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**Material Surface Chemistry Influences the Differentiation of Human Macrophages and their
Degradative and Inflammatory Response**

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**MATERIAL SURFACE CHEMISTRY INFLUENCES THE
DIFFERENTIATION OF HUMAN MACROPHAGES AND THEIR
DEGRADATIVE AND INFLAMMATORY RESPONSE**

by

DONNA LEE M. DINNES

A thesis submitted to the Faculty of Graduate and Postdoctoral Studies in partial
fulfillment of the requirement for the degree of

DOCTOR OF PHILOSOPHY

Department of Biochemistry, Microbiology and Immunology

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ABSTRACT

Numerous medical devices are comprised partially or completely of polyurethane (PU) materials, used as a result of their flexible, segmented structure, tensile strength and relative biocompatibility. The polycarbonate-based PU (PCNU), has been incorporated in the use of many devices as a result of their enhanced resistance to oxidative and hydrolytic degradation. Although relatively biocompatible, these materials still are sensitive to hydrolytic degradation and attachment of inflammatory cell types. Monocyte-derived macrophages (MDM) have been found as the primary cell type to adhere and remain at the cell-material interface of long-term implant devices. Although efforts are continually targeting the modification and design of improved PCNUs, cellular responses and MDM-mediated degradation of PCNU materials are not completely understood. The participation of inflammatory phospholipase A₂ (PLA₂) pathways were investigated for their participation in mechanisms leading to PCNU degradation by macrophages. Moreover, the influence of these materials on the stimulation of PLA₂ pathways and the hydrolysis product, arachidonic acid, was further explored. Fully differentiated human MDM have been typically used for subsequent studies evaluating PCNU degradation, however, studies here were the first to examine the morphology and function of monocytes differentiating along the macrophage lineage under the influence of altered material surface chemistry. Finally, the tools of proteomics were employed here for the first time in the investigation of material surface influence on both the differentiation of MDM and in assessments of degradative capacity of MDM adherent to PCNU. The results presented here in this collection of manuscripts has provided evidence that PCNU materials induce changes in expression and activation of PLA₂

enzymes, that in turn may have indirect participation in the mechanisms of degradation. In addition, the material surface upon which MDM differentiate, greatly influences not only cellular morphology but also function in terms of degradative capacity and cytoskeletal protein expression and rearrangement of actin-based structures. Proteomics techniques have identified proteins that may link contributors to the multi-factorial foreign body response to PCNU materials *in vitro*, involving PLA₂ and the structural proteins actin, vinculin and vimentin that would ultimately lead to the release of hydrolytic enzymes that contribute to material breakdown. The understanding of these cellular responses and mechanisms of material destruction is an essential base of knowledge required for efficient design of medical implant materials for their intended use.

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LIST OF ABBREVIATIONS

2-DE	2-dimensional electrophoresis
AA	arachidonic acid
AFM	atomic force microscopy
ANOVA	analysis of variance
ARIST	aristolochic acid
ATCC	American Type Culture Collection
BD	1,4-butanediol
BMSF	Bioanalytical Mass Spectrometry Facility
BPB	bromophenacyl bromide
CD	Cluster of Differentiation
CE	cholesterol esterase
CIHR	Canadian Institutes of Health Research
COX-1	cyclooxygenase-1
COX-2	cyclooxygenase-2
cPLA ₂	cytosolic phospholipase A ₂
CPM	counts per minute
CVT	cytosol-to-vacuole targeting
CXE	carboxyl esterase
Da	dalton
DMAC	dimethylacetamide
DPBS	Dulbecco's phosphate buffered saline
DTT	dithiothreitol

EDTA	ethylenediaminetetraacetic acid
F-actin	filamentous actin
FBGC	foreign body giant cell
FBR	foreign body response
FBS	fetal bovine serum
FTIR/ATR	Fourier transform infrared / attenuated total reflectance spectroscopy
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	granulocyte/macrophage-colony stimulating factor
HDA	hexamethylene diamine
HDI	1,6-hexane diisocyanate
HDI321	HDI:PCN:BD – 3:2:1
HDI431	HDI:PCN:BD – 4:3:1
IFN- γ	interferon-gamma
IL-4	interleukin-4
IL-13	interleukin-13
IPG	immobilized pH gradient
iPLA ₂	calcium-independent phospholipase A ₂
kDa	kiloDalton
kVh	kilovolt-hours
LPS	lipopolysaccharide
LSCM	laser scanning confocal microscopy
mA	milliAmps
MALDI-ToF	matrix assisted laser desorption ionisation – time of flight

MDA	methylene dianiline
MDI	4,4'-methylene bis-phenyl diisocyanate
MDI321	MDI:PCN:BD – 3:2:1
MDM	monocyte-derived macrophage
MS	mass spectrometry
MSE	monocyte-specific esterase
MudPIT	multi-dimensional protein identification technology
MW	molecular weight
NSAID	non-steroidal anti-inflammatory drugs
NSERC	Natural Sciences and Engineering Research Council
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCN	polycarbonate / poly (1,6-hexyl carbonate) diol
PCNU	polycarbonate-based polyurethane / polycarbonate-urethane
PESU	polyester-based polyurethane / polyester-urea urethane
PEUU	polyether-based polyurethane / polyether-urea urethane
<i>pI</i>	isoelectric point
PKC	protein kinase C
PLA ₂	phospholipase A ₂
PMA	phorbol 12-myristate 13-acetate
PMF	peptide mass fingerprinting
PMK	palmityl trifluoromethyl ketone
PMSF	phenyl methyl sulfonyl fluoride

PNB	<i>p</i> -nitrophenylbutyrate
PS	tissue culture grade polystyrene
PU	polyurethane
QUIN	quinacrine
RGD	Arg-Gly-Asp
RPMI	Roswell Park Memorial Institute
RR	radiolabel release
SDS	sodium dodecyl sulfate
SEM	scanning electron microscopy
sPLA ₂	secretory phospholipase A ₂
TBST	Tris-buffered saline Tween-20
TCA	trichloroacetic acid
TCPS	tissue culture grade polystyrene
TLR	Toll-like receptor

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1.0 INTRODUCTION

The human body is constantly challenged with environmental contaminants, infectious organisms and tissue injury. Fortunately, there exists a finely tuned immune system to immediately defend the delicate internal environment from such challenges. The innate immune system, either independently or in concert with the adaptive immune system, functions to rescue the body from these threats. More specifically, the innate immune system is comprised of inflammatory cells that are immediately “armed and ready” to recognize and destroy foreign antigens and send off distress signals to recruit help in order to achieve the goal of removing or inactivating the invading insult. The innate immune system is pre-programmed for several challenges the body may come into contact. Of particular interest, and central to this thesis, is to investigate how the innate immune system orchestrates a foreign body response to polyurethane (PU) implant materials, which is currently not well understood.

Medically implanted devices provide a wide range of therapeutic applications including long-term implanted pacemakers and drug-eluting stents to scaffolds that are used in tissue regeneration applications. In several of these devices, specific components, or the entire apparatus, are composed of flexible, diverse PU materials. The wide range of applications that PU can be utilized for is attributed to their flexible segmented structure, tensile strength, relative biocompatibility and the ability to drastically change the physical and chemical characteristics of an individual PU by slight changes in the chemistry of their components. Classically, these materials have been used in applications that require long-term implantation. However, concerns regarding biocompatibility and biostability

issues launched the biomaterials field into investigating the mechanisms of PU biodegradation. Recently, the need to understand these processes was broadened in order to design materials that were not only intended for long-term use but for drug delivery systems and regenerative medicine techniques as well.

Several studies, both *in vivo* and *in vitro*, in the past few decades have led to a better understanding of what determines the sensitivity of a particular PU to degradation by oxidative chemicals and hydrolytic enzymes [reviewed in Santerre et al., 2005]. Blood proteins and immune cells such as neutrophils and monocytes that adhere to and coat the surfaces of PU materials have been shown to participate in material degradation. The monocyte-derived macrophage (MDM) has been accepted as the primary cell type which contributes to PU-mediated biodegradation [Anderson et al., 1993; Labow et al., 2001a]. As a result of this, considerable efforts are being taken to elucidate the mechanisms through which these cells recognize and respond to PU materials. The central focus of our research group is to examine the participation of the human MDM, and simultaneously, the intracellular and secretory processes that result from the foreign body response to these implanted materials. Recent work has demonstrated that hydrolytic enzymes, such as monocyte-specific esterase (MSE) and cholesterol esterase (CE) are released from PU-adherent MDM [Matheson et al., 2002; 2004] that can degrade model PU materials [Labow et al., 2001b; 2002a]. Furthermore, the highly oxidative compounds HOCl and H₂O₂ that are released from neutrophils and activated macrophages respectively [Beutler, 2004] have also been shown to contribute to PU degradation in cell free systems [Christenson et al., 2004; Labow et al., 2002b; McBane

et al., 2005] and MDM models of biodegradation [McBane et al., 2006]. Despite the knowledge gained of the degradative capacity of MDM and their biological activities released into the extracellular space, the mechanism(s) of the initiation of the inflammatory and intracellular pathways that occurs during MDM-adherence to PU surfaces has yet to be determined.

Although evidence has been presented for the involvement of hydrolytic enzymes such as MSE and CE in the process of MDM-mediated PU degradation, little is known regarding the entire complement of proteins that may orchestrate the foreign body response of MDM to PU. Previous *in vitro* studies have utilized chemical inhibitor screening assays to determine potential pathways that may lead to cell-mediated degradation. These studies with neutrophils revealed a role for serine proteases and esterases (MSE and CE as mentioned above), protein kinase C (PKC) and also phospholipase A₂ (PLA₂) enzymes [Labow et al., 2001c]. Experiments outlined in the following publications contained in this thesis further examine the role that PLA₂ enzymes may have in the pathways leading to the release of degradative substances in addition to their participation in the inflammatory response induced by these model PU materials via macrophages.

To continue to individually select candidate proteins and assess their potential role in PU recognition and degradation would be a tremendous task. Increased popularity and improvement of proteomics techniques allows rapid, relatively simple and reproducible methods to analyze complex protein mixtures such as whole cell preparations. This provides a method through which we can detect how changes in the cellular

microenvironment can influence entire protein profiles of a cell population. Proteomic evaluation in the biomaterials field has largely been limited to characterization of proteins that adsorb to the material surface [Oleschuk et al., 2000; Griesser et al., 2004; Kim et al., 2005]. With the assistance of visiting research scholarships to the University of New South Wales (Sydney, AUS) and working in collaboration with the Bioanalytical Mass Spectrometry Facility, an expressional proteomics investigation of the intracellular and secreted MDM proteins that mount the response to PU surfaces was initiated.

In addition to an assessment of inflammation and the foreign body response and how these mechanisms ultimately contribute to MDM-mediated PU biodegradation, the influence of PU materials on monocyte to macrophage differentiation was also addressed in this thesis. Although monocyte to macrophage differentiation is relatively well characterized in “typical” *in vitro* cell culture conditions [Dougherty and McBride, 1989], monocyte differentiation during adherence to a PU surface has been largely uninvestigated. This thesis will address the concept of monocyte to macrophage differentiation on PU materials and the drastic effect this has on features such as morphology, function and monocyte maturation that could influence subsequent degradative capacity and inflammatory response towards these materials. In summary, the collection of manuscripts presented in this thesis will address the following hypothesis and objectives:

1.1 HYPOTHESIS

Material surface chemistry (specifically polycarbonate-urethanes) influences the inflammatory, degradative and differentiation capacity of MDM.

1.2 GLOBAL OBJECTIVE

The overall objective is to investigate the human MDM response to polycarbonate-urethane (PCNU) materials focusing on the PLA₂ inflammatory pathway, macrophage differentiation capacity and changes in MDM proteome profile that may contribute to inflammation and the degradative potential of these cells during the foreign body response.

1.2.1 SPECIFIC OBJECTIVES

- 1) To investigate the role of phospholipase A₂ enzymes in MDM-mediated biodegradation and PCNU-mediated inflammation.
- 2) To determine the effect of PCNU surfaces on the differentiation of human monocytes to macrophages in terms of morphology and function.
- 3) To utilize proteomics techniques to establish the effect of a PCNU surface on the MDM proteome profile.

1.3 BACKGROUND

The following sections will provide an overall review of the literature that pertains to the concepts presented in the collection of manuscripts contained in this thesis. Although each manuscript includes an independent introduction and presentation of background material, the following sections allow for a more in depth exploration of the concepts that are relevant to and important for understanding macrophage-mediated responses to PU materials. The first section will examine the innate immune system and inflammation and how this component of immunity is both essential to and central in the foreign body response. Next, in order to fully appreciate the specific foreign body response to material surfaces that was investigated in this thesis, a comprehensive review of PUs in general is presented. The evolution of biomaterials research that led to the use of PCNU materials rather than other long-term implant materials will be discussed. Furthermore, mechanisms that have been elucidated thus far regarding both enzyme- and cell-mediated PU degradation will be reviewed. In addition, the consideration of the potentially toxic biodegradation products that may result from PCNU biodegradation will be presented.

Proteomics techniques were used for the first time in order to investigate cell-material interactions and characterize proteins that may be involved in the MDM response to PCNU surfaces. Therefore, relevant background information is presented in order to understand the expressional proteomics approaches that were taken to investigate this new area of biomaterials research. Proteomics applications hold great promise in the biomaterials field as a method to identify multiple proteins from complex mixtures, such as whole MDM cell lysates, in a 'shot gun' approach that has the potential to expose

pathways and proteins that have not yet been considered in the foreign body response to PCNU materials.

1.3.1 THE INNATE IMMUNE SYSTEM AND INFLAMMATION

The defense mechanisms of the human body can be broadly categorized into innate and adaptive immunity. Although these contrasting immune systems are usually separated, there are cellular components of innate and adaptive immunity that overlap. In fact, some would say that it is difficult to determine where the line between innate and adaptive immunity lies. Expert innate and adaptive immunologists would perhaps even argue as to which branch of immunity is “more important”; the innate immune system that comprises cellular components that immediately respond to foreign insults, or the adaptive immune system that follows, which has the ability to “tailor” responses to the specific antigens and retain lasting memory for future insults. The bottom line is that the innate immune system is the first line of defense in a foreign body response. One of the initial barriers to external foreign bodies or organisms is the skin. Once this line of defense has been crossed, the innate immune system consists of a host of cells that are pre-programmed to distinguish these non-self antigens from self. If this primary battery of immune cells is overcome, it is at this point that the proteins and cells that comprise the innate system will recruit the components of the adaptive immune system and their ensuing response.

1.3.1.1 Cells of the innate immune response

Within the innate immune system, there are three types of phagocytic or inflammatory cells that construct the initial line of defense; macrophages, dendritic cells and

granulocytes [Janeway et al., 2001]. Inflammation is often considered synonymous with innate immunity since the responses characterized by each event are quite intertwined. The highly inflammatory macrophage and dendritic cell arise from the same monocyte precursor. However, MDMs are involved in the immediate response and direct phagocytic removal of foreign antigens, whereas a dendritic cell is considered the classic professional antigen presenting cell of adaptive immunity. For these purposes, of these two cell types only the MDM will be considered further. Granulocytes are named such since they harbour their destructive capacity in specialized granules contained within their cytoplasm. The most abundant granulocyte in blood is the neutrophil, which is a relatively short-lived leukocyte that is usually the initial cell to respond in infection and inflammation. In the case of an implanted material, the neutrophil is the first cell to arrive at the blood-material interface in the acute phase of the response [Anderson, 1993]. Once this cell responds to the material and recognizes this surface as foreign, it has the ability to release chemokines to recruit monocytes which will differentiate into longer living MDMs. Although MDMs may contribute chronically to the foreign body response to material surfaces [Marchant et al., 1983], neutrophils, along with the array of reactive oxygen species and hydrolytic enzymes released from these cells, have been shown to play a part in the initial events that lead to biomaterial degradation (see Section 1.3.3). Both MDMs and neutrophils, upon initiation of phagocytosis, will produce an assortment of oxidative and hydrolytic compounds designed to destroy internalized pathogens and degrade foreign antigens. However, in the case of an implanted biomaterial, the surface is much too large for the cell to engulf. MDMs, however, will alter their morphology and function in order to chronically respond to this non-phagocytosable foreign body.

1.3.1.2 Macrophages, foreign body giant cells and inflammation

Macrophage-mediated phagocytosis

Once a monocyte encounters a foreign body such as a large, flat material surface, it will adhere, differentiate and attempt to spread over the surface of the material. As a result of the inability of the macrophage to engulf this foreign body, it may release the degradative contents in an attempt to breakdown this material. Studies have shown that macrophages can form a tight seal with the substrate they adhere to which could facilitate the concentration and targeting of biological substances at the cell-material interface [Wright and Silverstein, 1984]. The features described in this study are suggestive of the formation of a phagocytic cup. These cell surface specializations are formed as a membrane extension that can encircle a foreign particle during early phagocytosis [Buccione et al., 2004]. In the case of a macrophage encountering a foreign object larger than what it can engulf, such as a biomaterial surface, a process through which macrophage phagocytic contents may be released has been described in a process called “frustrated phagocytosis” [Cannon and Swanson, 1992]. Other atypical secretion pathways that have been identified are cytosol-to-vacuole targeting (CVT) as well as ectocytosis. CVT has primarily been studied in budding yeast cells however could represent a potential mechanism of release of oxidative and degradative contents of macrophages [Kim and Klionsky, 2000; Shaw et al., 2001]. Ectocytosis has been described as a process by which vesicles are released with right-side-out membranes via membrane blebbing [Stein and Luzio, 1991; Gasser et al., 2003]. Proteins as varied as actin, various receptors (β 1-integrin, Fc γ RIII, Mac-1, L-selectin), galectin-3, matrix metalloproteinases and complement proteins have been detected on ectosomes [Lee et al.,

1993; Mehul and Hughes, 1997; Gasser et al., 2003]. The specific mechanism responsible for the release of the compounds that cause breakdown of the material surface is still unknown. However, it is possible that each of the above mechanisms are contributing candidates, releasing oxidative and hydrolytic agents that lead to PU degradation.

Foreign-body giant cell formation

Upon activation, monocytes and macrophages have the ability to fuse and form large multinucleated cells called foreign body giant cells (FBGC)s. These large phagocytic cells, typical of chronic inflammation, can contain >20 nuclei [Anderson, 2000; Möst et al., 1997]. Increased FBGC formation has been demonstrated in macrophages adherent to certain biomaterial surfaces and coincided with decreased apoptosis in this cell population [Collier and Anderson, 2002; Collier et al., 2004]. These findings suggest a protective mechanism in FBGC formation, perhaps contributing to the long-term survival of these cells on implant materials.

In order for such a great number of macrophages to fuse into large FBGCs, there must be significant rearrangement of cytoskeleton and cell membranes. In order to facilitate fusion, the environment to which macrophages are infiltrating, such as the surface of a biomaterial, must have a high enough density of cells in order for cell communication and membrane fusion to occur. Studies have demonstrated that cell fusion can be initiated between freshly isolated monocytes and also between differentiated MDM and newly introduced monocytes [Most et al., 1997]. This demonstrates that new cells may

continue to be recruited to an implant site, fusing with the FBGCs already established there, contributing to the further destructive capacity of cells at the material interface. However, there is evidence that a small percentage of local macrophages can proliferate [Gordon and Taylor, 2005] contributing to macrophage numbers and therefore cells that can increase the FBGC population.

Fusion of differentiating macrophages into FBGCs *in vitro* can be induced by human serum [Dougherty and McBride, 1989], several different cytokines such as IL-4, IL-13 [McNally and Anderson, 1995; DeFife et al., 1997] and IFN- γ [Nagasawa et al., 1987; Möst et al., 1990; Fais et al., 1994], as well as phorbol esters such as phorbol myristate acetate (PMA) [Hassan et al., 1989] (suggesting an involvement of PKC), vitamin E [McNally and Anderson, 2003] (suggesting a role for diacylglycerol) and even antibodies [Lazarus et al., 1990; Orentas et al., 1992; Tabata et al., 1994]. Receptors such as macrophage mannose receptors [McNally et al., 1996; Dadsetan et al., 2004], and also β 1 and β 2 integrins [McNally and Anderson, 2002] have been demonstrated as a requirement for fusion between macrophages. Once fusion of MDM has been initiated, considerable changes in cell membrane structure must occur. In order to facilitate significant plasma membrane changes, there must be reorganization of the plasma phospholipid content of the fusing MDM. Supporting this hypothesis, calcium-independent PLA₂, known to be involved in membrane reorganization [Murakami et al., 1998], has been suggested to be important in FBGC formation in biomaterial adherent cells [McNally and Anderson, 2005]. Not surprisingly, the actin cytoskeleton is likely to become completely altered when cells must fuse their cytoskeletal contents. The

requirement for actin depolymerization and polymerization would require the action of several actin-binding and modifying proteins [Revenu et al., 2004]. DeFife *et al* demonstrated, through the use of actin polymerization inhibitors latrunculin A [Coue et al., 1987] and cytochalasin D [Wodnicka et al., 1992], that disruption of actin microfilaments compromises MDM fusion to FBGCs and demonstrates the importance of cytoskeletal rearrangements in proper FBGC formation [DeFife et al., 1999]. Although the complete mechanisms of FBGC formation as well as their functions have not been elucidated, the processes leading to their formation are multi-factorial and their functions varied.

Macrophage-mediated inflammation

During the foreign body response to a biomaterial, it is inevitable that inflammatory signaling pathways will be initiated in MDMs and FBGCs. Although the release of inflammatory mediators and cytokines provide a normal defense mechanism to the body, they may under certain circumstances become destructive to the host tissue and implanted medical device and result in damaging the very cells that were intended to be repaired, leading to the ultimate failure of the device. The challenge to understanding the mechanisms of biodegradation is to be able to tailor the macrophage response for the desired end result.

One of the most potent inflammatory pathways is the PLA₂ pathway. The family of PLA₂ enzymes consists of a group of enzymes that hydrolyze the *sn*-2 ester bond of membrane glycerophospholipids to liberate arachidonic acid (AA) and lysophospholipid

as products. AA is a highly potent mediator of inflammation and at the centre of an enormous pharmaceutical market of non-steroidal anti-inflammatory drugs (NSAID). The focus of these pharmaceutical targets, such as aspirin and ibuprofen, lies in the fact that AA is metabolized by either cyclooxygenase-1 or -2 (COX-1 and COX-2) enzymes to eventually lead to a myriad of potentially inflammatory prostaglandins. Prostaglandins can act on a variety of cells and play a role in modulating effects such as vascular constriction and dilation (vascular smooth muscle), pain (central nervous system) and platelet aggregation. There are 3 broad categories of PLA₂ enzymes that are capable of hydrolyzing these membrane phospholipids to release AA: secretory (sPLA₂), cytosolic (cPLA₂) and calcium-independent (iPLA₂). PLA₂ enzymes and their proposed roles in macrophage-mediated PU degradation are further discussed in Manuscript #1 and #2.

So why are PLA₂ enzymes important in the foreign body response to PU? The first 2 manuscripts in this thesis describe experiments which further investigate a potential role for PLA₂ enzymes in the foreign body response to PU materials. What led to these investigations was a study by Labow *et al* using PU adherent neutrophils to assess several intracellular pathways with the potential to contribute to PU degradation *in vitro* [Labow et al., 2001c]. Using chemical inhibitors, these studies demonstrated that inhibitors of PLA₂ enzymes could decrease neutrophil-mediated PU degradation. Since MDM caused significantly more PU degradation than neutrophils [Labow et al., 2001a], the role of PLA₂ in MDM-mediated degradation was investigated and found to be involved [Matheson et al., 2002]. Not only are PLA₂ enzymes central to inflammation, in addition, some of these enzymes possess signaling functions which potentiates a role for these

enzymes in triggering degradative pathways as well as eliciting a strong inflammatory response. Furthermore, as a result of the damaging effects of inflammatory mediators originating from AA, if these enzymes are activated, they could also lead to damage of new tissue attempting to form around the surgical site of the medical device. Studies in Manuscript #1 and #2 focus on PLA₂ enzymes and explore the potential association of PLA₂ enzymes and PCNU degradation, the ability of PCNU surfaces to activate PLA₂ enzymes, determine which categories of PLA₂ enzymes may be responsible for the effects seen and determine if PCNU materials have the ability to alter intracellular macrophage PLA₂ expression as part of the foreign body response. In order to further appreciate the response initiated in these inflammatory cells, the following sections will present a review of the materials studied in this thesis in order to gain a better understanding of the material surfaces these cells encounter.

1.3.2 POLYURETHANE MATERIALS

As a result of advances in biomedical engineering and polymer chemistry, conditions such as congenital heart valve defects, degenerating electrical conduction systems of the heart and occluded blood vessels can be repaired or corrected by the use of implanted medical devices. One of the most common materials used in the manufacture of such medical devices for the past few decades has been PU elastomers [reviewed in Gunatillake et al., 2003; Santerre et al., 2005]. Their favorable properties of flexibility and tensile strength, as well as relative stability and blood compatibility, make them an attractive material for such diverse applications as indwelling catheters, pacemaker lead insulation, artificial valves, blood oxygenating tubing and surgical patches, just to name a

few. PU-based devices have been more commonly used in long-term implantation where biostability is of utmost importance. Despite this, it has been pointed out that degradation of PU materials is not surprising because of the structure of these materials [Santerre et al., 2005]. Certain categories of PU materials still maintain relative biostability *in vivo* and so have maintained popularity in the market. However, the publicly addressed studies of degradation of PU coating of breast implants [Slade and Peterson, 1982; Benoit, 1993], launched intense investigations into the mechanisms of material degradation. As a result of our increased understanding of PU biodegradation, these materials are now also being employed in applications where controlled breakdown of the material is required.

1.3.2.1 Polyurethane structure and biostability

In order to understand the mechanisms of degradation of PUs, it is necessary to understand the components that make up the final structure of these materials. PU materials contain three basic components: a diisocyanate, a polydiol and a chain extender, to form the final segmented PU chain (Figure 1-1). The composition of each individual component and the stoichiometric ratio of these components together in a synthesized PU elastomer can drastically change the physical, chemical and degradative properties of the final material [Santerre et al., 1994; Tang et al., 2001a; b; Matheson et al., 2004]. The polydiol of a PU can be polyester-, polyether- or polycarbonate-based, although traditional PU materials were polyester or polyether based. In terms of susceptibility to degradation, polyester-based PUs (PESU) are sensitive to hydrolytic degradation [Stokes

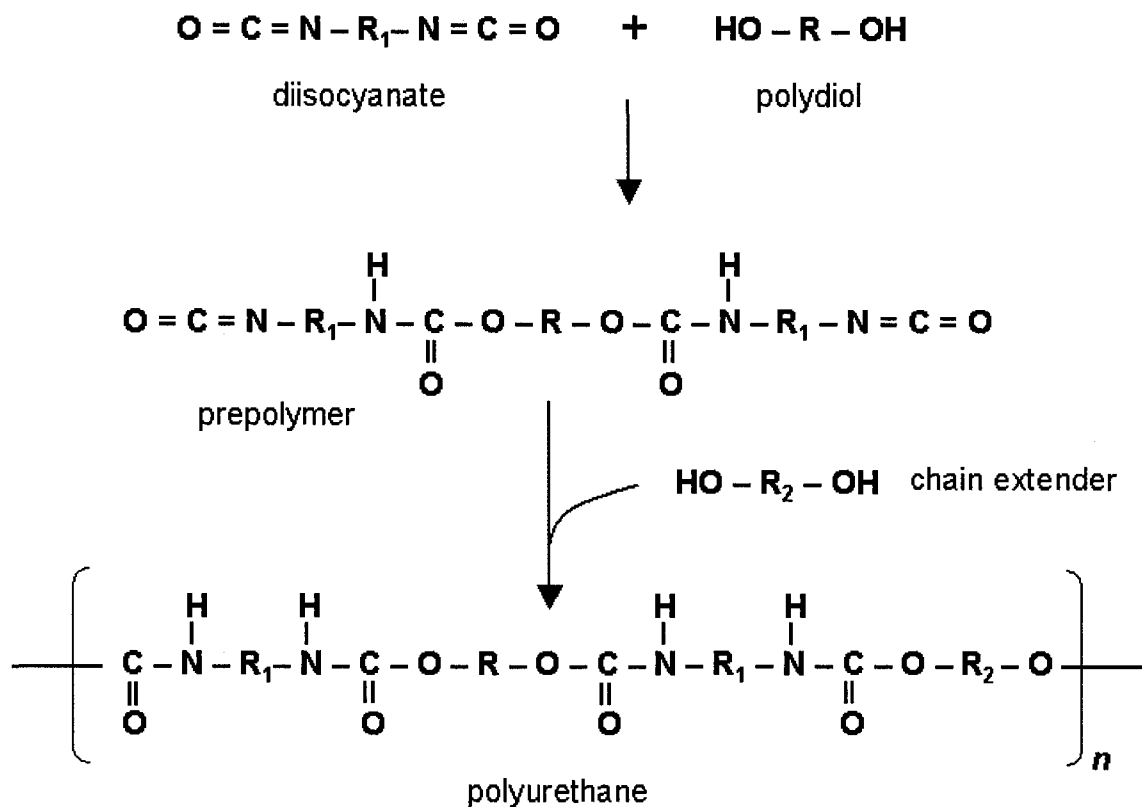


Figure 1-1 Polyurethane synthesis. Synthesis of the final polyurethane chain is a two-step process. The starting components, diisocyanate and polydiol (R usually = polyester, polyether or polycarbonate), are reacted together to form a prepolymer. This prepolymer is then reacted in equal molar ratios with a chain extender until the desired polymer chain length for the final polyurethane is achieved. [Adapted from Gunatillake et al., 2003]

and McVennes, 1995], whereas polyether-based PU (PEUU) have shown to be more sensitive to oxidative degradation [Stokes, 1988]. In attempts to overcome these degradative activities seen, PU began to be synthesized with polycarbonate (PCN) polydiol segments. These PCNU materials, although still far from completely stable, were found to be more resistant to hydrolytic and oxidative degradation than PESU and PEUU respectively [Tanzi et al., 1997; Pinchuk, 1994]. As a result of this apparent

increased biostability of PCNU materials over PESU and PEUU elastomers, PCNU materials seemed more attractive for long-term implantation.

1.3.2.2 Polycarbonate-based polyurethanes

Throughout the manuscripts contained in this thesis, only PCNU materials were utilized and therefore will be the type of PU material referred to from this point on. The PCNU materials employed in the following studies contained either an aromatic 4,4'-methylene bis-phenyl diisocyanate (MDI) or aliphatic 1,6-hexane diisocyanate (HDI) combined with the polydiol poly(1,6-hexyl carbonate)diol and 1,4-butanediol (BD) as the chain extender (Figure 1-2). These PCNUs were synthesized with various stoichiometric ratios of the starting components in order to study the effects of differences in diisocyanate chemistry (MDI:PCN:BD in a ratio 3:2:1 versus HDI:PCN:BD 3:2:1) or differences in the ratio of starting components (HDI:PCN:BD in a ratio of 4:3:1 versus 3:2:1). An example of the final polymer chain structure for HDI:PCN:BD in a ratio of 4:3:1 is shown in Figure 1-3.

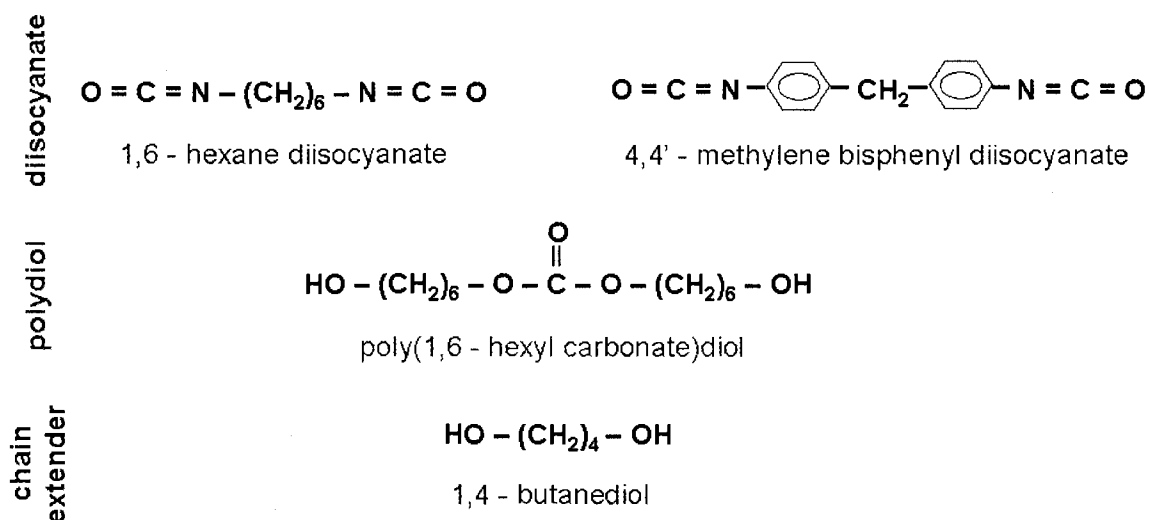


Figure 1-2 Polycarbonate-urethane starting components.

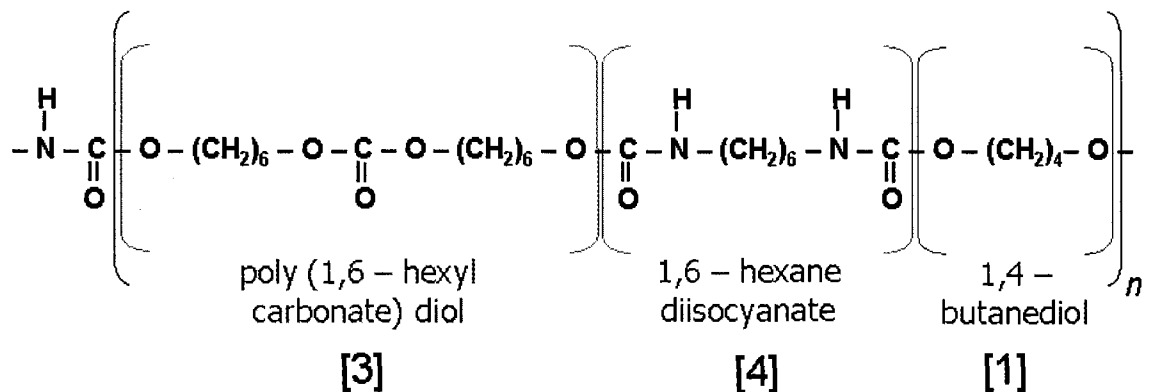


Figure 1-3 Polycarbonate-urethane chain structure.

The aim of studies by Labow and Santerre is to understand the cellular responses, particularly of human macrophages, to PCNUs in order to design new materials that are either sensitive or resistant to degradation depending on their intended application. Studies thus far have demonstrated that the model PCNU materials above, with their slight chemical differences, have varying sensitivities to enzymatic degradation, and also elicit quite different responses from MDM in terms of morphology and function. Although it is quite apparent that there are chemical differences in these three model PCNU materials, they also exhibit different surface topography that can influence protein and cell adherence as well as cellular responses.

1.3.2.3 PCNU Morphology

The PCN component of a PCNU is referred to as the “soft” segment. The soft segment, where no H bonds can form, imparts flexibility to the PCNU. The diisocyanate component (HDI or MDI) in combination with the chain extender (BD) constitutes the

“hard” segment, and gives strength to the polymer due to the degree of H bond formation. Upon synthesis of PCNU materials, the two-phase separation that occurs generates regions enriched with either soft or hard segments. This results in an aggregation of hard segment domains that are contained within a soft-segment matrix termed a “two-phase microdomain” structure [Lamba et al., 1998]. The segregation of phases is largely due to the insolubility of each phase in the other, and the high degree of hydrogen bonding between hard segments, therefore resulting in rearrangement into a semi-crystalline 3D structure [Gunatillake et al., 2003]. Therefore, characteristics such as the hard to soft segment ratio, as well as chain length (extensible by the BD moiety) will contribute to the degree of phase “mixing”, texture and surface morphology of the individual PCNU. The soft segment possesses mobility allowing the polar and non-polar groups to rearrange allowing the lowest interfacial free energy possible [Santerre et al., 2005]. The polar hard segments will migrate to a cell-material interface since this is a polar environment. If these materials are present in a vacuum environment, the non-polar soft segments will be present at the air-material interface. A diagrammatic representation is given below (Figure 1-4) of the microphase separation that occurs between hard and soft segments of PCNU. The differences in surface topography, illustrated by atomic force microscopy images (Figure 1-5), for the PCNUs used in the following studies are shown. By altering the diisocyanate component of the synthesized PCNU, gross differences in surface topography are evident (Figure 1-5 A) and B) versus C)). These physical differences will undoubtedly influence protein adsorption and cell adhesion altering the foreign body response and material degradation.

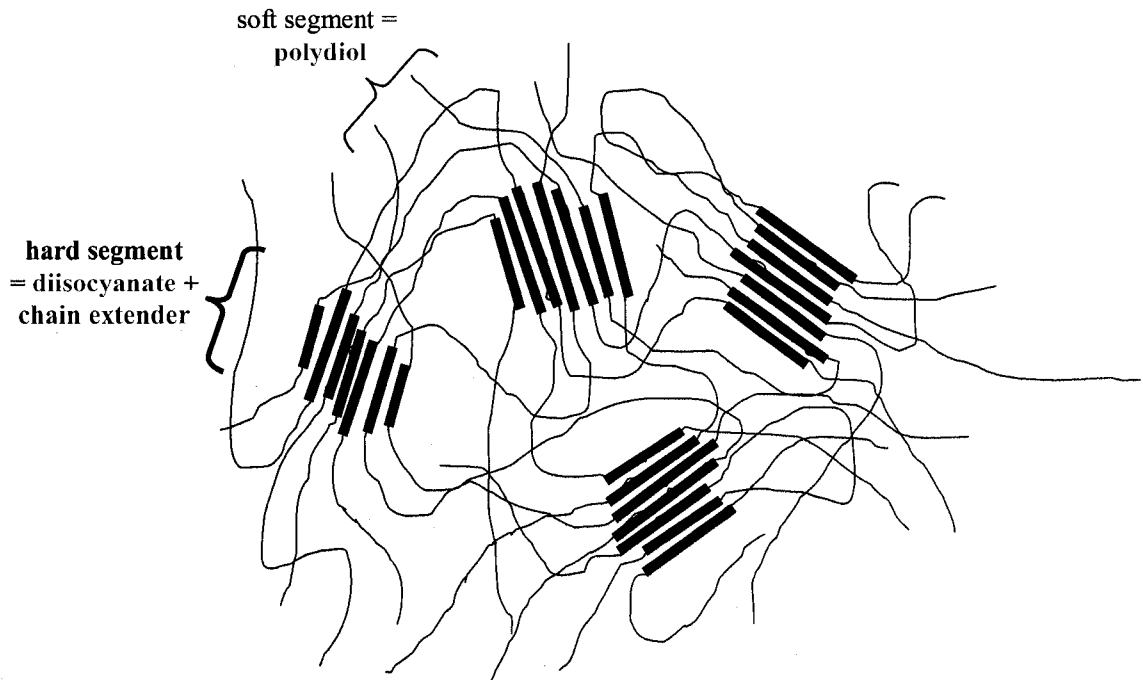


Figure 1-4 PCNU microphase separation. Upon synthesis of PCNU chains, the hard segments and soft segments will form a 3-dimensional structure. The hard and soft segments will phase separate due to insolubility of each component within the other and inter- and intra-molecular hydrogen bonding that occurs within the hard segments. [Adapted from Gunatillake et al., 2003].

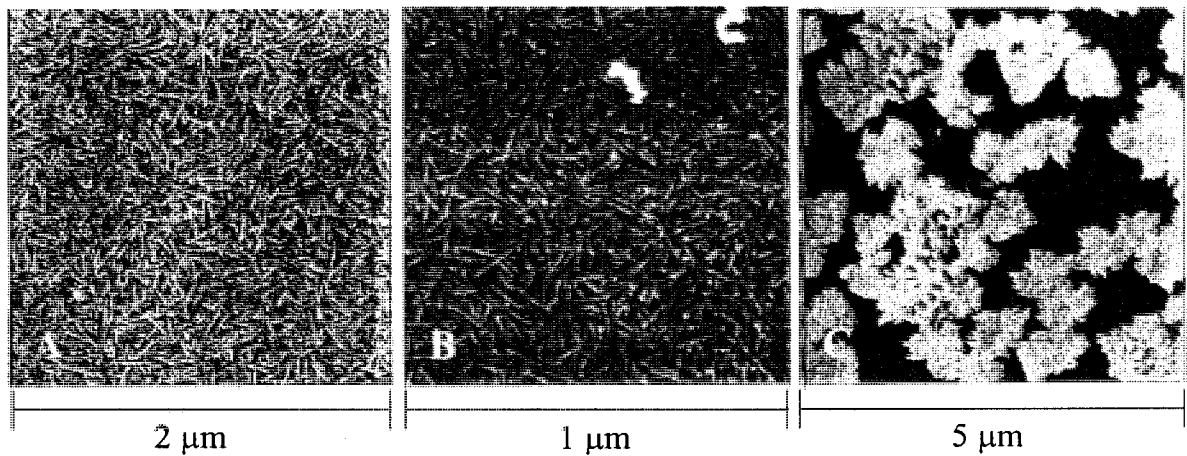


Figure 1-5 Atomic force microscopy images of PCNU surface topography. Atomic force microscopy images were taken of the surface topography of each of the PCNUs A) HDI431, B) HDI321, C) MDI321. [Adapted from Tang et al., 2002 © Brill Academic Publishers – reprinted with permission from Koninklijke Brill NV].

1.3.3 PCNU BIODEGRADATION

1.3.3.1 Enzyme-mediated PCNU biodegradation

Earlier studies that focused efforts on understanding degradation of PU (particularly PEUU) held oxidative pathways responsible for breakdown and cracking of implant materials seen, a process known as environmental stress cracking [Stokes et al., 1995]. It is widely accepted that oxidation of PU surfaces does occur, at first from neutrophils during the acute response but mostly from macrophages during the chronic response. In addition, hydrolytic degradation has been shown to be a significant contributor to biodegradation [Anderson, 1988; Santerre et al., 1993]. However, the PCNU class of PUs was developed in order to overcome or reduce the susceptibility to oxidative degradation experienced with PEUU. Although PCNU materials seem to be more resistant to oxidation, they have still been shown to undergo oxidative degradation [McBane et al., 2005], and still remain very susceptible to hydrolysis [Labow et al., 2002b; 2001b; 2002a].

Several studies by Santerre and Labow have focused efforts on evaluating the capacity of hydrolytic enzymes to degrade PESU, PEUU and PCNU [Tang et al., 2001a; b; 2002; 2003a; Labow et al., 1999; 2002a; b]. In order to measure this degradation in a simple and sensitive manner, they developed a method by which a ^{14}C radiolabel is incorporated into the PU base structure [Santerre et al., 1994]. In this way, upon cleavage of bonds in the PU and release of these products into culture media or enzyme buffer, the ^{14}C radiolabel can be easily quantified by scintillation counting. This method has proven sensitive to both enzyme [Santerre et al., 1994] and cell mediated degradation [Labow et

al., 2001a; b]. Upon assessment of several candidate enzymes, it was determined that the hydrolytic enzymes with the greatest capacity to degrade PU materials were esterases. More specifically, commercial preparations of CE and carboxyl esterase (CXE) were found to have high degradative activity towards PU materials [Labow et al., 1994]. Although a PU chain is not the natural substrate for these enzymes, a PCNU has bonds that could be susceptible to esterolytic activity. Figure 1-6 depicts the carbonate and urethane linkages that have been shown susceptible to enzymatic cleavage [Tang et al., 2003b].

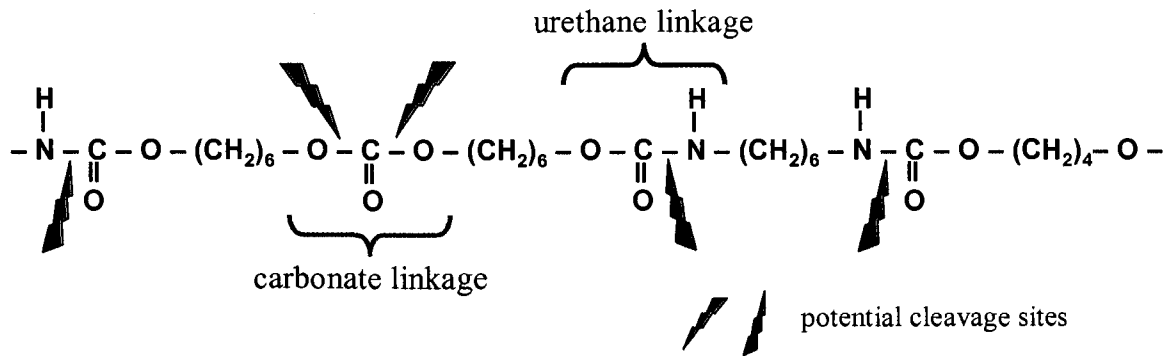


Figure 1-6 Sites on PCNU chain that are susceptible to esterolytic cleavage. Segmented PCNU chains contain urethane and carbonate linkages that are sensitive to cleavage by esterolytic enzymes such as CE and CXE.

1.3.3.2 Cell-mediated PCNU biodegradation

The original studies that assessed the contributions of immune cells to biomaterial degradation were initiated by Anderson and colleagues [Marchant et al., 1984]. Anderson's group was able to establish that MDMs were the primary cells that were likely participating in the biodegradative processes seen in implanted hydrogels [Marchant et al., 1983]. The most abundant cell types that were found present on explanted long-term implant materials were also MDM and FBGCs demonstrating that

these cells can remain at the material interface for the lifetime of the device [Kao et al., 1995]. Although these studies suggested an involvement of MDM in PU degradation processes, comprehensive studies of the mechanisms that led to MDM-mediated biodegradation were initiated by Santerre and Labow.

Both *in vitro* neutrophil and MDM model systems were investigated for their destructive capacity towards a model PESU [Labow et al., 2001a]. In this study, MDM were found to have 5 - 10 times more degradative capacity than neutrophils (measured by esterase activity and ^{14}C radiolabel release from ^{14}C -PESU). Although neutrophils may be the initial cell to arrive at the cell-material interface and possess oxidative and hydrolytic properties, it is most likely MDM that are responsible for degradation of implanted PU. Perhaps the most convincing evidence of the destructive capacity of these cells arose from a study which demonstrated the “pitting” that occurs in model PCNU materials by the action of adherent MDM (Figure 1-7).

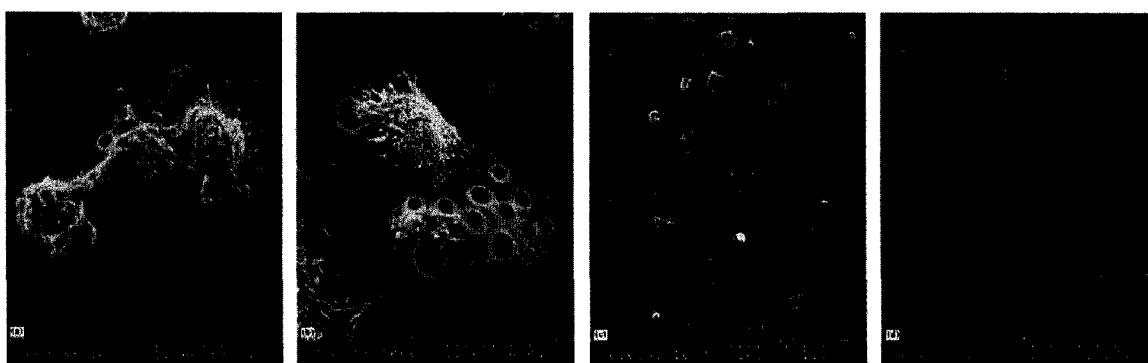


Figure 1-7 SEM photomicrographs of MDM on PCNU. Monocytes were cultured to MDM on tissue culture polystyrene, trypsinized and re-seeded onto PCNU and cultured for an additional 28 days. MDM were fixed (a) at 1 day (x1500); (b) at 28 days (x1500); (c) at 28 days (x200); (d) a media only control was fixed at 1 day (x2000). [Labow et al., 2001b © Wiley-Liss, Inc - reprinted with permission of John Wiley & Sons, Inc]

Based on this initial study showing the destructive capacity of these cells, a MDM cell system has been used to investigate the pathways and processes that lead to the degradation of model PCNU materials.

As discussed in section 1.3.1.2, macrophages fuse to form FBGCs, a hallmark of chronic inflammation [Anderson, 2000]. It is clear that inflammation is a central part of the foreign body response to implanted PCNU materials. Studies were conducted that assessed the influence of PCNU surfaces on fully differentiated MDM morphology and function [Matheson et al., 2004]. These studies demonstrated that MDM displayed the greatest amount of multinucleation as well as *de novo* protein synthesis on the most degradable PCNU, HDI431. In addition to the fact that commercial CE and CXE degraded PU materials, this study has provided evidence that these enzymes (CE and MSE (the MDM homologue of CXE)) were synthesized and secreted by MDM that were cultured on PCNU materials [Matheson et al., 2004]. Moreover, CE and MSE were present in increased amounts on a more degradable PCNU (HDI431 versus MDI321). Furthermore, MSE was found to be synthesized *de novo* and secreted in MDM in response to the more degradable PCNU, HDI431. These studies on the hydrolytic degradation of PCNU materials have provided evidence that esterases such as CE and CXE contribute to degradation of PCNU surfaces and that MDM, shown to remain at the biomaterial site, synthesize these enzymes differentially in response to the surface with which they are in contact. Although strong evidence has implicated CE and MSE secreted from activated MDM as prime candidates for the biodegradation seen with

PCNU materials, the mechanisms of how the secretion of these enzymes is triggered as well as the mechanism of the secretion from MDM has yet to be elucidated.

Two cell signaling pathways have been identified to contribute to PCNU degradation. Activation of PKC and PLA₂ has been implicated in the overall mechanism that leads to PU degradation [Labow et al., 2001c]. In an important study looking at the relationship of oxidative and hydrolytic degradation mediated by MDM, a clear role for PKC activation was evident in the overall degradative mechanisms of PCNU materials [McBane et al., 2005]. PKC activation, which results in the release of reactive oxygen species, caused varied effects on the hydrolytic degradation of model PCNU materials. Depending on the chemistry of the model PCNU, PKC activation had a protective mechanism against further hydrolytic attack. Furthermore, pre-treatment of PCNU materials with H₂O₂, the predominant reactive oxygen species from MDM, either exacerbated or reduced subsequent hydrolytic degradation. This study demonstrated that there is an interplay between oxidative and hydrolytic degradation and the sensitivity of PCNU materials to this combined attack is highly dependent on the chemical structure of the PCNU material. The role of PLA₂ enzymes in the foreign body response and PCNU degradation is discussed in detail below in Manuscripts #1 and #2. Although the overall picture of exactly how MDM elicit biodegradation of PCNUs has yet to be completed, it is well accepted that these materials are susceptible to degradation. An important aspect of cell-mediated biodegradation yet to be considered is the products generated by hydrolysis. The by-products of this degradation should be characterized as they may have a potential toxic effect on the recipient of the implanted material.

1.3.3.3 PCNU biodegradation products

It is apparent that PCNU materials are susceptible to degradation, whether by enzymatic cleavage alone or the overall cellular attack. Therefore, it is essential when considering PCNU degradation to also consider the cleavage products that are formed. With the concern of methylene dianiline (MDA), a potential cleavage product of MDI-based PCNUs, as a potential carcinogen [McQueen and Williams, 1990], and interest in the sites of enzymatic cleavage in PCNU, studies were initiated by Tang *et al* to assess the CE-mediated products of various PCNU materials [Tang et al., 2003b]. Although these studies characterized enzyme-mediated and not cell-mediated degradation products, CE is a candidate enzyme in PU degradation (section 1.3.3.1 and 1.3.3.2) and these studies have given us an indication of the potential products that could be liberated from PCNU materials implanted *in vivo*. This study demonstrated that although MDI321 is the most stable of the 3 model PCNU materials, it was the only one to produce its diamine derivative MDA. The HDI-based PCNU materials did not produce hexamethylene diamine (HDA) although it was hypothetically possible to be a cleavage product by CE. Furthermore, BD was found to be a cleavage product from these model PCNUs. Since MDA and BD have been determined to be biodegradation products of these model PCNUs, a study was initiated to assess the effects of these products on further MDM-mediated PCNU degradation (Manuscript #3) and intracellular MSE expression (Appendix V). An important follow-up to these studies will be to assess the products that are generated as a result of MDM-mediated biodegradation. Although this is a necessary step, these studies will be complicated in attempts to isolate PCNU products from secreted proteins and cellular debris.

1.3.4 HOW DO MACROPHAGES RECOGNIZE MATERIAL SURFACES?

With a better appreciation of MDMs and FBGCs that respond to and degrade these model PCNU materials, described in section 1.3.2.2, how is it that these cells recognize such a foreign surface? It is highly unlikely that a monocyte will “recognize” and attach *directly* to the PCNU surface itself. In addition, it is not likely that the bare surface of the material will even be exposed to these cells upon their arrival. Prior to the arrival of neutrophils and monocytes, the material surface will be in contact with blood as a result of the surgical procedure of implantation, and additional proteins as a result of altered vascular permeability which allows plasma proteins to infiltrate the material site. As a result of this, the initial effect in the foreign body response is a coating of the biomaterial with a protein layer. When blood is in contact with a foreign material surface, the initial protein adsorption can occur in a matter of seconds [Nygren, 1997; Eskin et al., 2004]. Subsequently, the cells that arrive at the interface will encounter these proteins that have adsorbed onto the surface. Even in *in vitro* cell culture conditions, culture medium containing serum will deposit protein onto the material surface before cells have a chance to attach. It is then the combination of material surface chemistry, profile of adsorbed proteins and conformation of adsorbed proteins that will influence the attachment of monocytes and MDM [Jenney and Anderson, 2000]. A recent study examining the adsorption of fibrinogen and attachment of platelets to model PU materials demonstrated that it was not the amount of fibrinogen that influenced platelet adsorption and activation, but likely the pattern of protein adsorption and conformation of the protein that became adsorbed that influenced cell behaviour [Massa et al., 2005].

The basis of the innate immune system is to recognize self from non-self. The monocytes and MDM of the innate immune system have evolved receptors that recognize conserved and integral structural components of bacteria [Han and Ulevitch, 2005]. These are usually germline encoded receptors that bind to specific patterns on the surface of invading microbes. Probably the most well known of these receptors is the family of Toll-like receptors (TLR). For example, TLR4 is known to bind the ligand lipopolysaccharide (LPS) from gram-negative bacteria. It is the recognition of this ligand as “non-self” by the TLR4 receptor that initiate signaling cascades eventually leading to uptake, removal and/or neutralization of these pathogens. In addition, macrophages express other pattern-recognition receptors such as mannose receptors and scavenger receptors that will recognize certain patterns of oligosaccharides and lipids respectively on foreign microbes [Aderem and Smith, 2004]. Material surfaces certainly do not possess these molecular patterns that will be recognized by evolutionarily conserved receptors. However, the proteins that are adsorbed to the material in a specific manner could be recognized. It has been suggested that the proteins perhaps “translate” the surface of the material for the cell. It is believed that the protein interaction occurs through variations in hydrogen bonding and hydrophobic interactions between protein and material surface [Gregonis and Andrade, 1985]. Several proteins have been identified that adsorb to various material surfaces; albumin, fibrinogen, fibronectin, vitronectin, IgG, transferrin and α_2 -macroglobulin are a few examples [Oleschuk et al., 2000; Kim et al., 2005]. Many of these proteins contain a similar sequence of Arg-Gly-Asp (RGD) which is one of the best known cell-binding motifs and can be recognized by integrin receptors (Kao et al., 2001). Integrins, such as CD11b/CD18 or β 1- and β 2-

integrin, may facilitate the primary interaction between a macrophage and the proteins coated on a material surface. When antibodies to integrin receptors were introduced into an osteoclast cell system, decreased cell attachment was observed, suggesting the involvement of these heterodimeric receptors in the interaction between the protein coated surface and the cell [Grzesik and Robey, 1994]. Once integrin receptor engagement has been initiated, clustering of integrin receptors may begin along with the binding to cytoskeletal proteins for the formation of cell adhesion complexes [Clark and Brugge, 1995].

Once a monocyte has bound to the material interface, it must attach more firmly and spread itself over the surface which requires cytoskeleton and intermediate filament components of the cell. In order to facilitate cell attachment to the material surface, cell adhesion complexes must be formed between the cell and substrate. One such adhesion structure that has gained much attention recently is the podosome (reviewed in [Linder and Aepfelbacher, 2003]). Macrophages are one of the few cell types that are known to spontaneously form podosomes [Linder and Aepfelbacher, 2003; Buccione et al., 2004]. Podosomes are distinct from focal adhesion complexes; however possess some similar characteristics [Linder and Aepfelbacher, 2003]. Podosomes consist of punctate actin-rich structures on the ventral surface of the cell in contact with the extracellular matrix or material surface that are surrounded by a ring of proteins such as vinculin and talin [Linder and Aepfelbacher, 2003]. These structures may provide a means of intimate contact with the material surface and a mechanism of release of degradative cell contents to intimate sites between the MDM and material interface.

1.3.5 MONOCYTE TO MACROPHAGE DIFFERENTIATION

Following the initial acute neutrophil response to PU materials, cytokine release from these cells will recruit monocytes to the implant site that will be the source of MDMs and FBGCs at the cell-material interface. As previously mentioned, the MDM has been characterized as the primary cell type found adherent to implanted biomaterials [Anderson et al., 1993]. Despite this, it is remarkable that few studies have assessed the effect that these surfaces have upon differentiating MDM that will eventually mount a response to the surface. Monocytes are one of two types of mononuclear leukocytes, the other being lymphocytes. Lymphocytes are cells typical of the adaptive immune system, whereas monocytes are classical innate immune cells providing an initial defense mechanism. Monocytes are non-proliferative cells originating from the bone marrow that normally remain in blood circulation until required for inflammatory responses. In addition to their recruitment to sites of inflammation, monocytes are required in several tissues and will differentiate into resident macrophages, induced by influential cues in the microenvironment. Resident macrophages are present at specific anatomical sites in tissue that is *not* inflamed and can be activated in situations of infection or when encountering other foreign antigens.

As monocytes alter their differentiation state, they may possess varied expression of surface molecules called CD markers (Cluster of Differentiation). CD markers are often unique to certain cell types allowing one cell type to be distinguished from another. Using these markers is frequently a means for identification of specific cell types. When considering monocytes and differentiated macrophages, there are several CD markers

expressed on these cells. These CD markers will demonstrate increased or decreased expression depending on the differentiation state of the cell. Some of the markers that are well known for characterizing the differentiation state of monocytes and macrophages include CD11b, CD14, CD68 and CD71. CD14 and CD11b are two markers known to decrease in expression as monocytes mature into macrophages [Andreesen et al., 1990; Jacob et al., 2002]. On the other hand, cells differentiating along the monocyte to macrophage lineage demonstrate increased expression of CD68 [Ramprasad et al., 1996; Boynton et al., 2000] and CD71 [Jacob et al., 2002] antigen allowing the determination of the degree of maturation of this cell lineage. The use of these markers along with other morphological and functional characteristics may assist in determining the differentiation state of MDM. Differentiated MDM are known to have characteristics of increased size and spreading, increased metabolic activity as well as higher capacity for secretion [Ross and Auger, 2002]. An additional specific functional characteristic of maturing MDM is increased esterase activity [Labow et al., 1998]. As previously mentioned (Section 1.3.3.2), esterase protein expression and enzyme activity has been demonstrated to be an indicator of degradative capacity of MDM towards PU materials, therefore suggesting that mature MDM may possess greater degradative capacity relative to undifferentiated monocytes.

Studies were conducted in order to determine how PCNU material surfaces influence monocytes undergoing differentiation towards MDMs. The first study (Manuscript #3) investigated MDM differentiated on various PCNU surfaces compared to tissue culture grade polystyrene (TCPS)(control, non-degradable surface) considering protein

expression and subsequent degradative activity towards PCNU materials *after* their differentiation period. As a result of the findings from these studies, monocytes differentiating to MDM were then characterized *during* their course of differentiation on PCNU material surfaces to determine if the material surface alters the maturation and/or inflammatory state of these cells that could then contribute to their altered degradative capacity (Manuscript #4). These studies have shown that the material environment that monocytes first encounter as they differentiate into MDM will have an impact on the foreign body response of the mature MDM.

1.3.6 PROTEOMICS

The foreign body response of MDM to biomaterial surfaces and the influence this microenvironment has on these cells is undoubtedly multi-factorial. Although the numerous studies outlined above have indicated certain proteins and pathways (i.e. CE, MSE, PLA₂ and PKC) that participate in this response, continually selecting individual proteins and pathways for further investigation to piece together the entire process would be a phenomenal task. Applying proteomics techniques would allow a large “fishing expedition” to identify multiple proteins that have altered expression in MDM in response to a changed microenvironment such as interaction with PCNU materials.

The Human Genome Project that has successfully completed the sequence of the human genome certainly accelerated the study of ‘genomics’ and gene function. However, studying the human genome will not give us a complete picture of ultimate gene function. In order to achieve this, we must study the gene products, or proteins that carry out the

ultimate function of the genes that encoded them. The term *proteomics* arose from a combination of *proteins* and *genomics*, with the term *proteome* first defined in the mid 1990's [Williams et al., 1997]. Proteomics is generally described as the identification and study of all the proteins in a cell, tissue or organism. However, more recently, it has been suggested [Lawrence et al., 2005] that this definition be expanded as “the effort to establish the identities, quantities, structures, and biochemical and cellular functions of all proteins in an organism, organ or organelle, and how these properties vary in space, time, or physiological state” [Kenyon et al., 2002]. In a way, it is just not ‘good enough’ to identify the protein profile of a specific cell or organism, we must also look into characteristics such as structure and function to truly understand the *proteome*.

In general, the study of proteomics can be divided into expressional and functional proteomics. Expressional proteomics simply being the identification of the proteins expressed in the cell type, whereas functional proteomics encompasses studies of such characteristics as structure and post-translation modifications that will contribute to how a protein may function. However, in an initial effort to study the proteins of a new cell type or new microenvironment influence on a cell type, the proteins expressed or altered in expression must first be identified.

The ‘gold standard’ of proteomic analysis utilizes 2-dimensional electrophoresis (2-DE) as a method of protein separation [Ong and Pandey, 2001]. This method harnesses the characteristics of isoelectric point (pI) and MW of a protein in order to separate multiple proteins in two dimensions. Methods for isoelectric focusing (separation of proteins by

pI) have seen remarkable improvement mainly as a result of commercialized premade immobilized pH gradient (IPG) strips. Previous methods for 2-DE required the operator to cast their own pH gradient gels utilizing capillary tube gel casters that were cumbersome and non-reproducible. Several manufacturers now provide precasted IPG strips of various pH ranges (i.e. broad 3 – 10 or narrow and specific 5.5 – 6.7) that have greatly increased the quality of protein separation by *pI* and much higher reproducibility between gels. The next step in this 2-dimensional process is separation of proteins by MW. Not much has changed in techniques of SDS-PAGE for protein separation by MW, however, equipment has improved somewhat to allow the operator to run multiple gels in tandem so that samples are run under identical conditions and therefore increasing confidence and reproducibility of the 2-DE gels. Once proteins within a sample have been successfully separated by 2-DE, these proteins must be identified. Often 2-DE is combined with matrix assisted laser desorption ionisation – time of flight mass spectrometry (MALDI-ToF MS) to identify these proteins. Proteins spots from a 2-DE gel can be excised from the gel and subjected to tryptic digestion before MALDI-ToF MS analysis. MALDI-ToF MS will result in the generation of a peptide mass fingerprint (PMF) for each protein spot analyzed. From this PMF, online databases (i.e. Mascot) can be searched to match this PMF with known protein tryptic digest fingerprints to generate protein identifications. The combination of these proteomic techniques (2-DE and MALDI-ToF MS) constitutes the most common combination of proteomics methods for identifying multiple proteins in a sample.

There are still several drawbacks to the 2-DE and MALDI-ToF MS approach. One of the greatest drawbacks is that it is time consuming. If the entire protein complement of the sample analyzed must be identified and not just a few spots of interest, this approach can be quite tedious in order to excise and attempt to identify each protein spot. In addition, MALDI-ToF MS does not have very high sensitivity, and therefore proves difficult to use for identification of low abundance proteins. The 2-DE step is also only able to separate proteins to a certain degree. If there are proteins of highly similar MW and *pI*, these proteins will be difficult to separate unless using specific pH gradients and percentage of acrylamide gels to 'tweeze' out the proteins in a particular range of *pI* and MW. Another highly used method in proteomics that can overcome these drawbacks is 2D liquid chromatography combined with tandem MS (2D-LC MS/MS), also referred to as MudPIT (**M**ultidimensional **P**rotein **I**dentification **T**echnology). This provides a gel-free approach to 'shot-gun' proteomics. In addition to overcoming some of the difficulties outlined above from 2-DE + MALDI-ToF, MudPIT can analyze quite complex protein mixtures relatively easily. In addition, MudPIT allows the identification of very high and low MW proteins that will be missed on a 2-DE gel, as well as very high and low *pI* proteins that never resolve properly on a typical IPG strip. In addition, due to the fact that a mixture of proteins must be resolubilized and rehydrated in an IPG strip, it is possible that not all proteins will completely resolubilize e.g. membrane proteins that are highly hydrophobic. The MudPIT approach can overcome these hurdles and result in a greater representation of the entire proteome profile of the desired sample.

As discussed in more detail in Manuscript #5, few efforts have been taken to harness the field of proteomics and utilize these methods in the field of biomaterials. Thus far, most studies carried out have been used to identify the profile of proteins that adsorb to various material surfaces [Oleschuk et al., 2000; Griesser et al., 2004; Kim et al., 2005]. A recent review has highlighted the potential of proteomics techniques in studying cell-material interactions [Gallagher et al., 2006]. Very few studies have explored the proteome of any cell in contact with a biomaterial. Manuscript #5 presented here, to our knowledge, is the first to take a comparative proteomics approach to MDM in contact with any material (in this case PCNU) relative to the normal TCPS environment of typical cell culture.

1.4 SUMMARY

It is imperative that we gain a better understanding of the foreign body response to biomaterials and particularly the behaviour of macrophages at the cell-material interface. Only recently is considerable effort being made to unravel the mechanisms that occur within these inflammatory cells in response to the materials used in a variety of medical devices. It is interesting that it is well known that there is a “strong correlation between the inflammatory response and the biocompatible nature of implanted materials” [Rice et al., 1998], however very few studies have assessed cell signaling pathways and inflammation that occur as a result of these interactions except for extensive studies on FBGC formation [Anderson, 1988; Collier et al., 2002; 2004; Dadsetan et al., 2004; Jenney and Anderson, 2000; Kao et al., 1995; 2001; Matheson et al., 2004].

The following studies began to further assess an inflammatory pathway, PLA₂ that contributes to the foreign body response to PCNUs which indirectly influences degradation of these materials. In addition, the first study of MDM-PCNU interactions using proteomics is outlined as a potential tool to explore the proteome for proteins and pathways that may participate in the overall MDM response. And finally, two studies were carried out, the first determining that the material surface upon which MDM are differentiated has an influence on their subsequent function, the second demonstrating that morphological and functional parameters were also influenced along the monocyte-to-macrophage differentiation pathway. The manuscripts describing the studies of which this thesis is composed have given us a better knowledge of the inflammatory response of MDM to PCNU materials, as well as a better appreciation that the differentiation of monocytes upon arrival at the material interface will indeed influence their subsequent capacity to function as inflammatory macrophages and destructive cells to material surfaces. In addition, the tools of proteomics have been shown to be quite powerful and should be used more extensively to isolate specific proteins that may either directly participate in material degradation or activate signaling pathways that initiate an appropriate foreign body response to implanted PCNU materials.

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2.0 MANUSCRIPT #1

Phospholipase A₂ pathway association with macrophage-mediated polycarbonate-urethane biodegradation

STATEMENT OF AUTHOR CONTRIBUTIONS

Donna Lee M. Dinnes was the primary author and sole contributor to the experimental data for this manuscript. **Dr. J. Paul Santerre** is a co-investigator of Dr. Rosalind S. Labow, and principal investigator on the CIHR operating grant that funded this research. In addition, he has contributed to the editing and suggestions in the preparation of this manuscript. Dr. Santerre's Polymer Chemistry laboratory at University of Toronto synthesized the model PCNU materials used for the following studies. **Dr. Rosalind S. Labow** is the supervisor of Donna Lee M. Dinnes and contributed to experimental design and editing / suggestions for this manuscript.

SUMMARY

The following manuscript entitled "Phospholipase A₂ pathway association with macrophage-mediated polycarbonate-urethane biodegradation" has recently been published in *Biomaterials* [(2005) 26:3881-3889]. Based on preliminary data with neutrophils on PESU, it was determined that chemical inhibitors of PLA₂ enzymes inhibited material degradation (measured by the use of ¹⁴C-PESU) [Labow RS et al. *J Cell Physiol* (2001) 186:95-103]. As a result of these findings, the following studies were explored to further characterize the specific PLA₂ enzymes that could potentially have a role in the mechanisms leading to macrophage-mediated degradation.

Since PLA₂ enzymes have been implicated in biodegradation, when activated by the material surface, there may be a two-fold detrimental effect. Not only would PLA₂ enzymes contribute to the breakdown of implanted materials, in addition, activation of these enzymes would trigger an inflammatory response via the highly pro-inflammatory mediator AA that would be generated. Hence, not only would material breakdown and potential device failure *in vivo* ensue, the surrounding local tissue could potentially be damaged as a result of the inflammatory response that is initiated. Therefore, to assess the capacity of PCNU materials to activate the inflammatory PLA₂ pathway, ³H-AA release assays were incorporated as an assay of PLA₂ enzyme activation. Finally, a potential mechanism of PLA₂ influence on the release of degradative enzymes from macrophages was proposed from the results of these studies.

The experiments in this manuscript were carried out with the U937 cell line. The promonocytic U937 cell line has been well characterized by Matheson *et al* as a model system for human MDM-mediated biodegradation [Matheson LA et al. *J Biomed Mater Res* (2002) 61:505-13]. Upon differentiation with phorbol esters, this cell line takes on macrophage-like characteristics [Hass R et al. *Eur J Cell Biol* (1989) 48:282-293]. Using this system, it was possible to expedite the experiments and eliminate donor macrophage variation. However, some of the following experiments were confirmed in MDM to validate the use of U937 cells for these studies. Validation experiments with primary human MDM, and additional methods employed in these studies not included in the following publication are included in Appendix I and II.

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Phospholipase A₂ pathway association with macrophage-mediated polycarbonate-urethane biodegradation

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Key words: biodegradation, polyurethane, phospholipase A₂, U937 cell line, inflammation

ABSTRACT

Activation of the phospholipase A₂ (PLA₂) pathway is a key cell signaling event in the inflammatory response. The PLA₂ family consists of a group of enzymes that hydrolyze membrane phospholipids resulting in the liberation of arachidonic acid (AA), a precursor to pro-inflammatory molecules. Given the well documented activating role of biomaterials in the inflammatory response to medical implants, the present study investigated the link between PLA₂ and polycarbonate-based polyurethane (PCNU) biodegradation, and the effect that material surface had on PLA₂ activation in the U937 cell line. PCNUs were synthesized with poly(1,6-hexyl 1,2-ethyl carbonate)diol, 1,4-butanediol and one of two diisocyanates (hexane 1,6-diisocyanate or 4,4'-methylene bis-phenyl diisocyanate) in varying stoichiometries and incubated with adherent U937 cells. PLA₂ inhibiting agents resulted in significantly decreased PCNU biodegradation ($p < 0.05$). Moreover, when activation of PLA₂ was assessed (³H-AA release), significantly more ³H-AA was released from PCNU-adherent U937 cells than polystyrene-adherent U937 cells ($p < 0.05$) which was significantly decreased in the presence of PLA₂ inhibitors. The pattern of inhibition of U937 cell-mediated biodegradation and ³H-AA release that was modulated by PCNU surface differences, suggests a role for secretory PLA₂ along with cytosolic PLA₂. Understanding PCNU activation of intracellular pathways, such as PLA₂, will allow the design of materials optimized for their intended use.

INTRODUCTION

The interaction of cells with a foreign material surface has the potential to initiate several signaling pathways. One of the primary cell signaling events in inflammation is activation of the phospholipase A₂ (PLA₂) pathway. PLA₂ hydrolyzes the *sn*-2 ester bond of cell membrane glycerophospholipids releasing arachidonic acid (AA) as one of its products (1). There are three general categories of PLA₂ enzymes; secretory (sPLA₂), cytosolic (cPLA₂) and Ca⁺⁺-independent (iPLA₂) (1, 2). Following activation of any or all of these PLA₂s, the AA released (or its metabolites) may indirectly lead to the biodegradation of material surfaces by triggering secretion of degradative enzymes (3, 4) from adherent phagocytic cells such as neutrophils or monocyte-derived macrophages (MDM).

Although neutrophils are the first phagocytic cells to respond to an implanted medical device, MDM are the most abundant phagocytic cells at the time of explantation (5). It has been shown that an MDM cell system had 5-10 times degradative potential towards a ¹⁴C-labeled polyester-urea urethane (PEUU) when compared to a neutrophil cell system (6). Polycarbonate-based polyurethanes (PCNU)s, although originally introduced because of their increased resistance to oxidation (7), have been shown to be degraded by an *in vitro* MDM cell system (8). With more recent attention given to biodegradable drug delivery systems and tissue regeneration (9), the chemistry of polyurethanes (PU) has been further altered to undergo programmed degradation. This raises the need to further understand mechanisms of interaction between the polymers and cells that will be breaking them down.

Labow *et al.* have shown in previous studies that esterases are candidate enzymes for the degradation of PCNU (10). Commercial sources of both cholesterol esterase (CE) (11-13) and carboxyl esterase (CXE) (10), when incubated on PCNU films, have been shown to cause degradation demonstrating the destructive role of these enzymes in material degradation. Using antibodies to CE and CXE which cross react with the MDM forms of these enzymes (CE and monocyte-specific esterase (MSE) respectively), immunoblot analysis confirmed the presence of these esterases in both cell lysates and conditioned media (14). Scanning electron micrographs have depicted the “pitting” of PCNU surfaces that occurs only directly under adherent MDM, demonstrating that hydrolytic enzymes that are released from the activated MDM may be a significant contributor to the breakdown of the material which occurs immediately in contact with the cell (8). How the material surface of these devices activates these cells to secrete degradative enzymes, as well as how and where these enzymes are released from the cell has yet to be elucidated. However, it is possible that intracellular pathways, such as the PLA₂ pathway, may have a role in this process.

A previous study by Labow *et al.* demonstrated that PLA₂ enzyme activity does not degrade PU (15). However, neutrophil (16), U937 and MDM cell systems (17), when cultured on ¹⁴C-PU, show a significant decrease in degradation when the PLA₂ inhibitors aristolochic acid (ARIST) or quinacrine (QUIN) were included in the media. Moreover, these inhibitors also inhibited the release of ³H-AA (a measure of PLA₂ activation) from neutrophils adherent to a standard non-biodegradable tissue culture grade polystyrene surface (PS) and PEUU suggesting that activation of PLA₂ pathways were involved in

material degradation. In addition, it appeared that material surface may play a role since significantly more $^3\text{H-AA}$ was released from neutrophils adherent to PEUU than for the same cells adherent to PS (16).

In the present study, the effect of PCNU surfaces on the activation of PLA_2 pathways was studied using PCNU synthesized with either 1,6-hexane diisocyanate (HDI) or 4,4'-methylene bis-phenyl diisocyanate (MDI) and a polycarbonate (PCN) poly(1,6-hexyl 1,2-ethyl carbonate)diol soft segment and 1,4-butanediol (BD) chain extender in different stoichiometric ratios (11, 12). A ^{14}C radiolabel was incorporated into their structures in order to measure degradation by radiolabel release. Using the U937 cell system, PLA_2 inhibitors with different specificities were assessed for their effects on $^{14}\text{C-PCNU}$ biodegradation and PCNU initiated $^3\text{H-AA}$ release as a measure of PLA_2 activation. Using this approach, it was possible to establish an association between biodegradation, material activation and PLA_2 .

MATERIALS AND METHODS

Unless otherwise specified all reagents were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada).

Preparation of material surfaces

Model PCNUs used in this study were synthesized with a PCN soft segment, BD chain extender and either HDI or MDI as the diisocyanate as described in detail previously (11, 12). A ^{14}C radiolabel was incorporated into the PCNU structure as a method to assess

biodegradation (11, 12). Three PCNUs were used for this study (Table 1); 1) a PCNU with a stoichiometric ratio of 4:3:1 (HDI:PCN:BD) with the radiolabel (^{14}C) in the HDI moiety (referred to as ^{14}C -HDI431), 2) a PCNU with a stoichiometric ratio of 3:2:1 with the radiolabel in the BD moiety (referred to as ^{14}C -HDI321B), 3) a PCNU with a stoichiometric ratio of 3:2:1 with the radiolabel in the BD moiety (referred to as ^{14}C -MDI321B). These PCNUs were also synthesized without a ^{14}C -radiolabel for ^3H -AA release assays, and are referred to as HDI431, HDI321 and MDI321 respectively.

The PCNUs were dissolved overnight in a 10% dimethylacetamide solution (w/v), centrifuged and filtered (0.45 μm Teflon filter; Chromatographic Specialties, Toronto, ON, Canada), then coated as a thin layer (100 μL) onto 15 mm diameter glass cover slips (Fisher Scientific, Ottawa, ON, Canada) under sterile conditions in a laminar flow hood, as previously described (6). The slips were dried overnight at 50°C, purged for 24 hours and then dried under vacuum for 72 hours. Prior to using the slips for an experiment, they were hydrated by incubating with Dulbecco's phosphate buffered saline at 37°C and 5% CO_2 with 100% humidity overnight. This process equilibrates the polymer slips with an aqueous environment and removes trace solvent that may possibly interfere with the experiment.

U937 cells

In the current study, U937 cells were employed as a model for MDM-mediated PCNU biodegradation (17). Cells were maintained as a promonocytic cell suspension and fed every second day with RPMI medium containing 10% fetal bovine serum, 1 mM sodium

Table 1 - Polycarbonate-based polyurethanes used in this study

Reagent	Stoichiometry	Acronym
*HDI/PCN/BD	4:3:1	¹⁴ C-HDI431
HDI/PCN/*BD	3:2:1	¹⁴ C-HDI321B
MDI/PCN/*BD	3:2:1	¹⁴ C-MDI321B
HDI/PCN/BD	4:3:1	HDI431
HDI/PCN/BD	3:2:1	HDI321
MDI/PCN/BD	3:2:1	MDI321

*indicates the moiety that is radiolabeled in the polymer structure

pyruvate (Gibco Invitrogen Corp., Burlington, ON, Canada) with 100 units/mL penicillin and 0.1 mg/mL streptomycin (Gibco Invitrogen Corp.), as described in detail previously (17). Cells were differentiated by seeding 1×10^6 cells/mL suspension onto PS 12-well plates with phorbol 12-myristate 13-acetate (PMA) (1×10^{-7} M) for 72 hours. The cells were disrupted by gentle pipetting, resuspended at a concentration of 1×10^6 cells/mL and reseeded onto the required material surface for the experiment. Control wells, which contained no cells but included media, inhibitor and/or vehicle for each material surface and experimental condition, were also assessed. These controls established if there was any effect of inhibitor or vehicle on the material surface which was not due to the cells.

Effect of PLA₂ inhibitors on PCNU degradation

A 1 mL aliquot of a 1×10^6 cells/mL differentiated U937 cell suspension was reseeded into 24-well plates containing ¹⁴C-HDI431, ¹⁴C-HDI321B or ¹⁴C-MDI321B coated slips. The cells were allowed to adhere for 1 hour, incubating at 37°C and 5% CO₂ with 100% humidity. One plate was then analyzed for 'T=0' DNA (the cells that had adhered at 1 hour), the media from a second plate was removed and replenished with media with and without PLA₂ inhibitors. These cells were then incubated for 48 hours, after which, conditioned medium from each well was removed and radiolabel release measured by adding 600 µL of this medium in 10 mL of scintillation fluid (Formula 989; Packard Instrument Co. Inc., Meriden, CT, USA) and measuring ¹⁴C in a scintillation counter (LKB Wallac 1219 Rackbeta, Gaithersberg, MD, USA). Each well was then analyzed for DNA to relate radiolabel release to the number of adherent cells at 48 hours as described below.

A second time point for determining the effect of PLA₂ inhibitors on PCNU degradation was also assessed during differentiation. At 48 hours of differentiation, PLA₂ inhibitors were added to the U937 cell cultures. Each inhibitor remained present for the duration of the experiment. PLA₂ inhibitors ARIST (sPLA₂) (16), bromophenacyl bromide (BPB)(sPLA₂) (2), 12-epi-scleradiol (sPLA₂) (18), manoolide (sPLA₂) (2), palmityl trifluoromethyl ketone (PMK)(cPLA₂ and iPLA₂) (19), QUIN (sPLA₂ and cPLA₂) (20) and bromoenol lactone (iPLA₂) (21) were assessed at concentrations previously established (see Table 2). Appropriate vehicle controls were assessed with the cells to ensure the effect seen was an inhibitor and not a vehicle effect. Solubility in some cases was the limiting factor as well as cytotoxic effects of increased vehicle.

³H-arachidonic acid release

At 48 hours of U937 cell differentiation, 0.10 μ Ci/mL of ³H-AA was added to each well of differentiating U937 cells (22). Cells were then allowed to differentiate for the final 24 hours. At the end of the differentiation period, it was determined that ~50% of the ³H-AA added was incorporated (data not shown). The cells were then resuspended and washed twice in media to remove any unincorporated ³H-AA. Cells were then resuspended at a concentration of 1×10^6 cells/mL in media containing calcium ionophore (A23187) [1 μ M] and reseeded into PS 24-well plates with and without HDI431 slips. A23187, a stimulator of AA release, was included in the culture medium for ³H-AA release experiments since previous results demonstrated that activation by PMA alone could not stimulate U937 cells to release the maximum amount of ³H-AA (22). Four separate plates were seeded that were incubated for 10, 20, 30 or 60 minutes

Table 2 – PLA₂ inhibitors used to assess effects on biodegradation (radiolabel release) and PLA₂ activation (³H-AA release) by PCNU-adherent U937 cells

Inhibitor	Concentration	Specificity	Vehicle
Aristolochic Acid (ARIST)	200 μM	sPLA ₂	Dimethylsulfoxide
Bromo-enol Lactone	500 nM	iPLA ₂	Dimethylsulfoxide
Bromophenacyl Bromide (BPB)	25 μM	sPLA ₂	Acetone
12-epi-scleradiol	54 μM	sPLA ₂	Dimethylsulfoxide
Manoalide	100 μg/mL	sPLA ₂	Dimethylsulfoxide
Palmityl trifluoromethyl ketone (PMK)	4 μM	iPLA ₂ , cPLA ₂	Dimethylsulfoxide
Quinacrine (QUIN)	1 mM	sPLA ₂ , cPLA ₂	Culture Medium

respectively. At each time point, the conditioned media were collected and centrifuged to pellet any non-adherent cells and the supernatant was counted by scintillation counting for $^3\text{H-AA}$ release from the cells into the media. The cells that remained adherent to the material surface were then analyzed for DNA to relate $^3\text{H-AA}$ release to the number of cells adherent to the different surfaces at that time point.

$^3\text{H-arachidonic acid release with PLA}_2$ inhibitors

The PLA_2 inhibitors that inhibited radiolabel release were also used to determine the effects on $^3\text{H-AA}$ release. Three different time points for adding the PLA_2 inhibitors were assessed, 1) 48 hours into differentiation, 2) at the time of reseeding the cells onto the material surfaces and 3) at 5 minutes after reseeding. The time points used for the addition of PLA_2 inhibitors were investigated as a result of the $^3\text{H-AA}$ release pattern observed from experiments in the previous section. A 500 μL loading of a $^3\text{H-AA}$ treated 2×10^6 cells/mL suspension were seeded into each well. After 5 minutes, an additional 500 μL of medium with or without the respective inhibitor was added to each well. The cells were then allowed to incubate for another 5 minutes for a total of 10 minutes before measuring releasates for $^3\text{H-AA}$ release as outlined above.

DNA analysis

In order to measure DNA, as described in detail previously (6, 14), 240 μL 0.05% Triton-X 100 was added to each well. Hoechst dye (number 33258; Fisher Scientific) was diluted with TRIS buffer (0.01 M Tris, 0.001 M EDTA, 0.2 M NaCl) pH 7.4 immediately prior to analysis. Cell lysates collected for DNA (10 μL) were added to the dye (100 μL)

in 96 well microtiter plates (Microfluor 2 Black;VWR, Mississauga, ON, Canada). The lysates were compared to a DNA standard (Calf Thymus DNA) and each well to be analyzed also contained the same amount of 0.05% Triton-X 100 as in the lysate samples. The samples were analyzed using a fluorescence microplate reader (POLARstar Galaxy, BMG Labtechnologies, Durham, NC, USA). All data were related to 10 µg DNA which was representative of the number of cells that remained adherent to the material surface.

Statistical Analysis

All data was analyzed by a 2-way ANOVA, except for the effect of material surface alone on ³H-AA release which was analyzed by a 1-way ANOVA. The p-value for significance (0.05) was adjusted for multiple comparisons. All analyses were performed using SAS[®] version 8.2 for Windows.

RESULTS

Effect of PLA₂ inhibitor on PCNU degradation

Initial PLA₂ inhibitor studies were performed using ¹⁴C-HDI431, to determine which PLA₂ inhibitors would inhibit radiolabel release (biodegradation). ¹⁴C-HDI431 has demonstrated to be more sensitive to biodegradation than ¹⁴C-HDI321 and ¹⁴C-MDI321 and was therefore selected for initial assessments of PLA₂ inhibitors. Those inhibitors that significantly influenced radiolabel release were further investigated using all three material surfaces. At the concentrations listed previously in Table 2, ARIST, BPB, PMK, and QUIN significantly inhibited radiolabel release on ¹⁴C-HDI431 in comparison to media alone in adherent U937 cells. The inhibitors manoalide, 12-epi-scleradiol and

bromo-enol lactone failed to inhibit radiolabel release at the concentrations listed in Table 2 (data not shown).

The four PLA₂ inhibitors that inhibited biodegradation on ¹⁴C-HDI431 (ARIST, BPB, QUIN, PMK) were then assessed on ¹⁴C-HDI321B and ¹⁴C-MDI321B (Figure 1). Each of the above inhibitors significantly decreased radiolabel release on all three surfaces relative to U937 cells in media only. Furthermore, significant differences were seen in the amount of biodegradation, caused by U937 cells incubated with ARIST and BPB, between the three surfaces. With the sPLA₂ inhibitor ARIST, radiolabel release from both ¹⁴C-HDI321B and ¹⁴C-MDI321B adherent U937 cells was lowered significantly more than on ¹⁴C-HDI431. Also, with the inhibitor BPB, a significantly greater reduction in radiolabel release was seen with ¹⁴C-MDI321B adherent U937 cells than with either ¹⁴C-HDI431 or ¹⁴C-HDI321B.

When PLA₂ inhibitors were added during U937 cell differentiation, a different pattern of inhibition of ¹⁴C-PCNU radiolabel release over 48 hours was seen (Figure 2). ARIST showed the greatest inhibition, resulting in only 30-55% of the original radiolabel release depending on the material surface. Significant inhibition of radiolabel release was seen on each material surface with significant differences in the degree of inhibition by ARIST between ¹⁴C-HDI321B and ¹⁴C-MDI321B adherent cells.

PMK did not significantly inhibit radiolabel release on any material surface when added during U937 cell differentiation. Both BPB and QUIN appeared to damage the cells

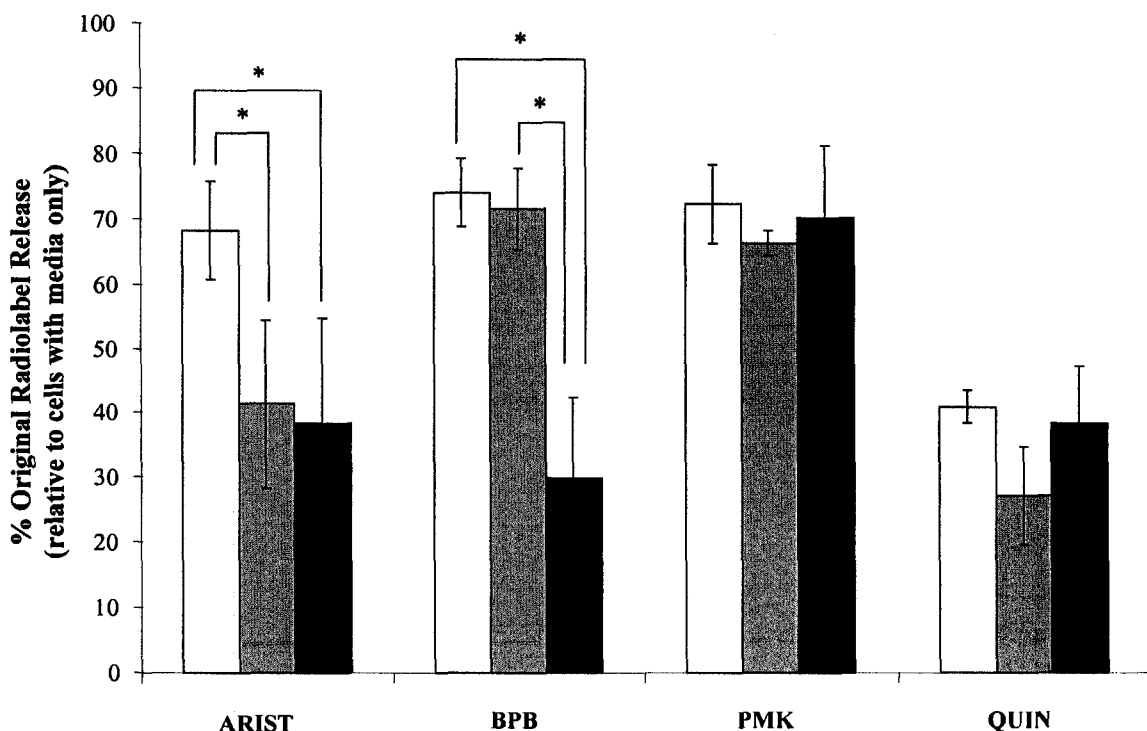


Figure 1 - Effect of PLA₂ inhibitors on radiolabel release from ¹⁴C-PCNU-adherent U937 cells. Differentiated U937 cells were cultured on ¹⁴C-PCNU coated slips, ¹⁴C-HDI431 (open bars), ¹⁴C-HDI321B (shaded bars) and ¹⁴C-MDI321B (closed bars) for 48 hr. PLA₂ inhibitors aristolochic acid (ARIST), bromophenacyl bromide (BPB), palmityl trifluoromethyl ketone (PMK) and quinacrine (QUIN), were added to the wells 1 hr after adding the cells to the ¹⁴C-PCNU slips. Change in radiolabel release is expressed as % original radiolabel release of cells in media without any inhibitors. Each inhibitor significantly decreased radiolabel release compared to cells with media only. * indicates significant material differences for a specific inhibitor.

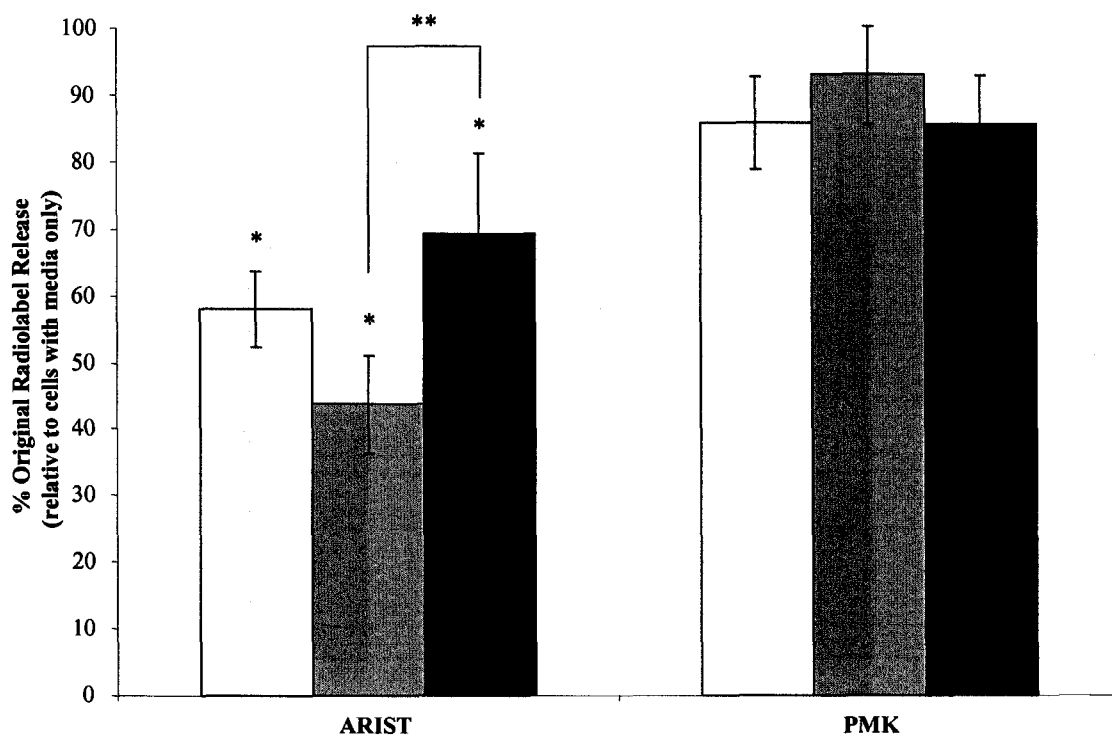


Figure 2 - Effect of PLA₂ inhibitor addition during U937 cell differentiation on radiolabel release from ¹⁴C-PCNU-adherent U937 cells. PLA₂ inhibitors aristolochic acid (ARIST) or palmityl trifluoromethyl ketone (PMK) were added at 48 hours of U937 cell differentiation and remained present throughout the experiment. Differentiated U937 cells were cultured on ¹⁴C-PCNU coated slips, ¹⁴C-HDI431 (open bars), ¹⁴C-HDI321B (shaded bars) and ¹⁴C-MDI321B (closed bars) for 48 hr. * indicates a significant decrease in radiolabel release, ** indicates a significant material difference. Change in radiolabel release is expressed as a % original radiolabel release compared to cells with media only.

when added during the differentiation period. Cells became undifferentiated, and lifted from the differentiation surface, leaving an inadequate number of cells to reseed onto the PCNU surfaces at the end of the differentiation period.

³H-arachidonic acid release assays

In order to determine if PMA, used to differentiate U937 cells, was sufficient for stimulating maximal release of ³H-AA from U937 cells, ³H-AA release studies were carried out with and without A23187 (stimulator of AA release). Figure 3 shows the significant increase in ³H-AA release when A23187 was added to U937 cells adherent to PS. ³H-AA release was significantly greater at 10 minutes as compared to any other time point. Hence, the activation of PLA₂ was assessed at 10 minutes on the material surfaces (Figure 4). Significantly more ³H-AA release was measured from U937 cells on the 3 PCNU surfaces when compared to PS. However, no significant differences in ³H-AA release was detected between the PCNUs.

Effect of PLA₂ inhibitors on ³H-arachidonic acid release

Since 10 minutes post-reseeding showed the greatest amount of ³H-AA release from PS-adherent U937 cells, experiments assessing the effect of PLA₂ inhibitors on ³H-AA release were also performed at 10 minutes. Three time points of addition for PLA₂ inhibitors were assessed to determine when the inhibitor needed to be present in order to achieve inhibition of ³H-AA release. As mentioned previously, these time points for PLA₂ inhibitor addition were, 48 hours into differentiation, at the time of reseeding, and at 5 minutes post-reseeding.

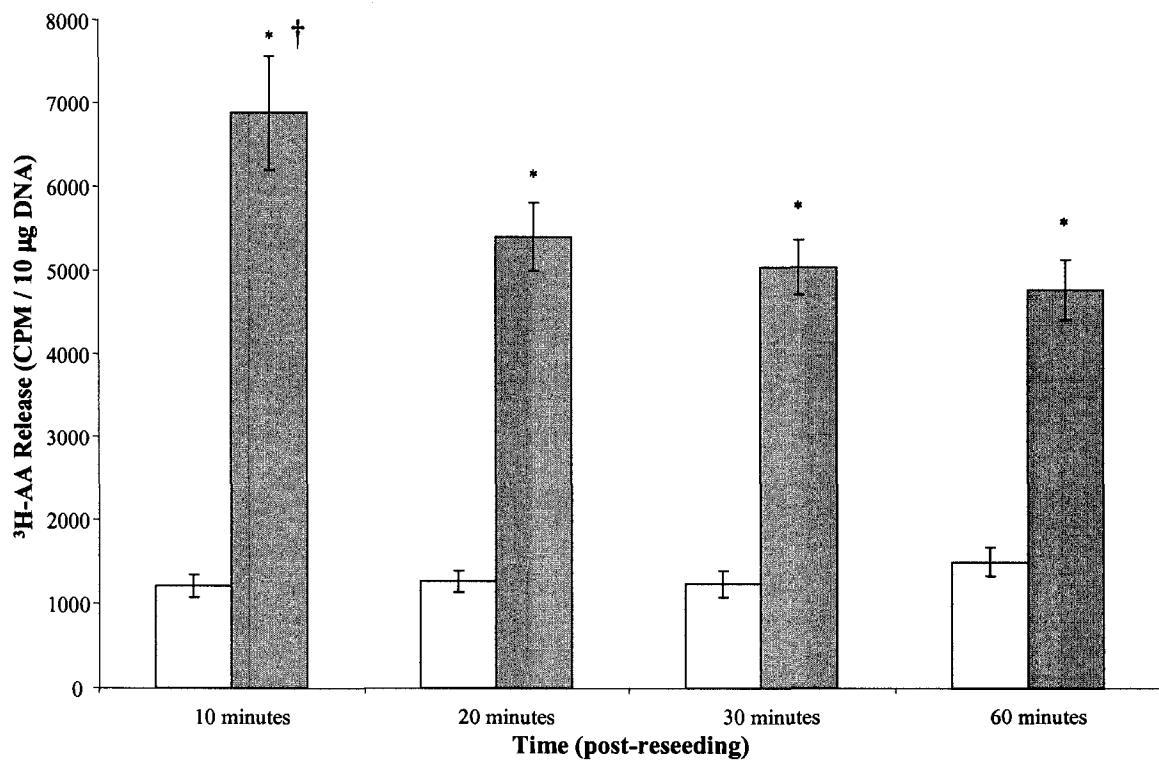


Figure 3 - Effect of calcium ionophore (A23187) on ³H-AA release from PS-adherent U937 cells. Differentiated, ³H-AA labeled U937 cells were cultured with PMA alone (open bars) or media with PMA and A23187 (shaded bars) on PS for 10, 20, 30 or 60 minutes. ³H-AA release from cells into the media was measured and normalized to 10 µg DNA. * indicates a significant increase in ³H-AA release, † indicates a significant increase in ³H-AA release between time points.

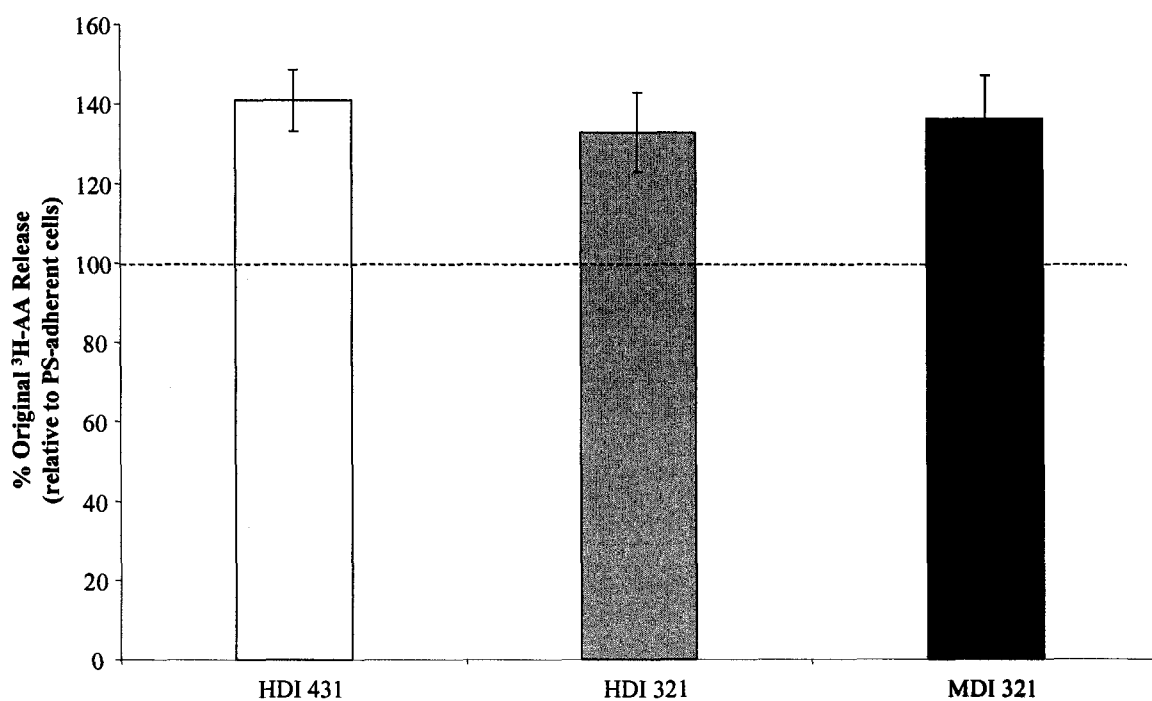
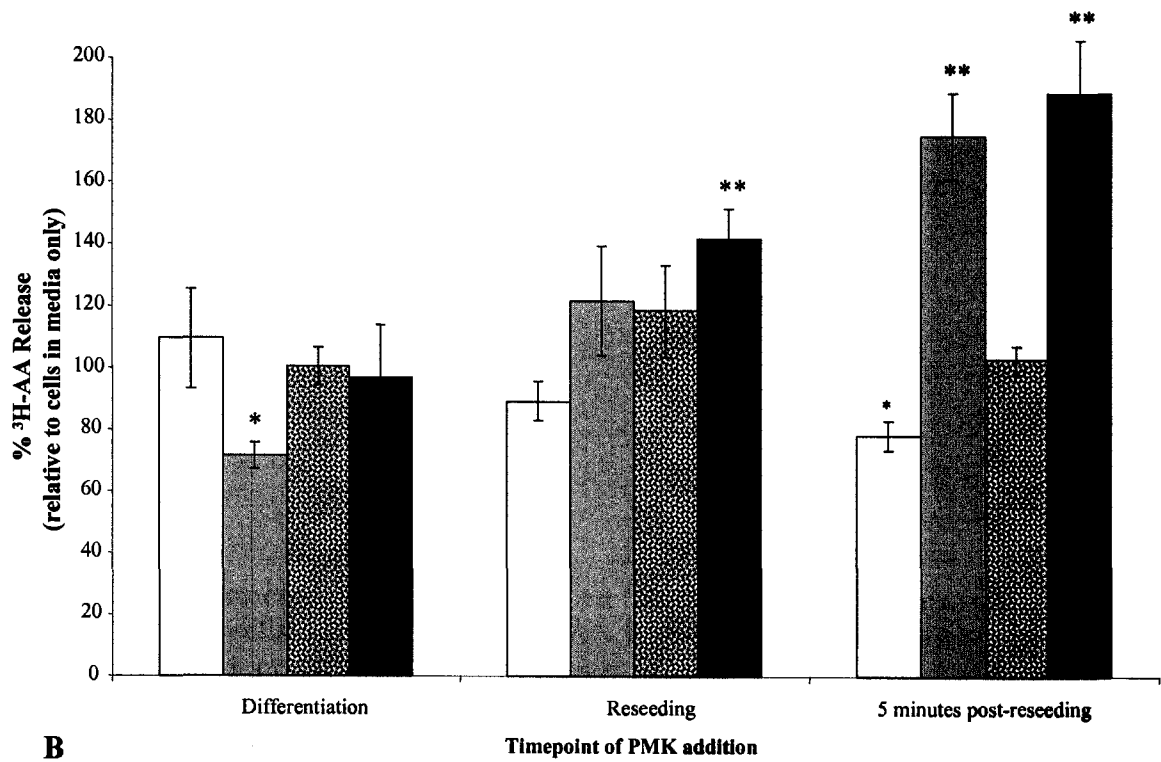
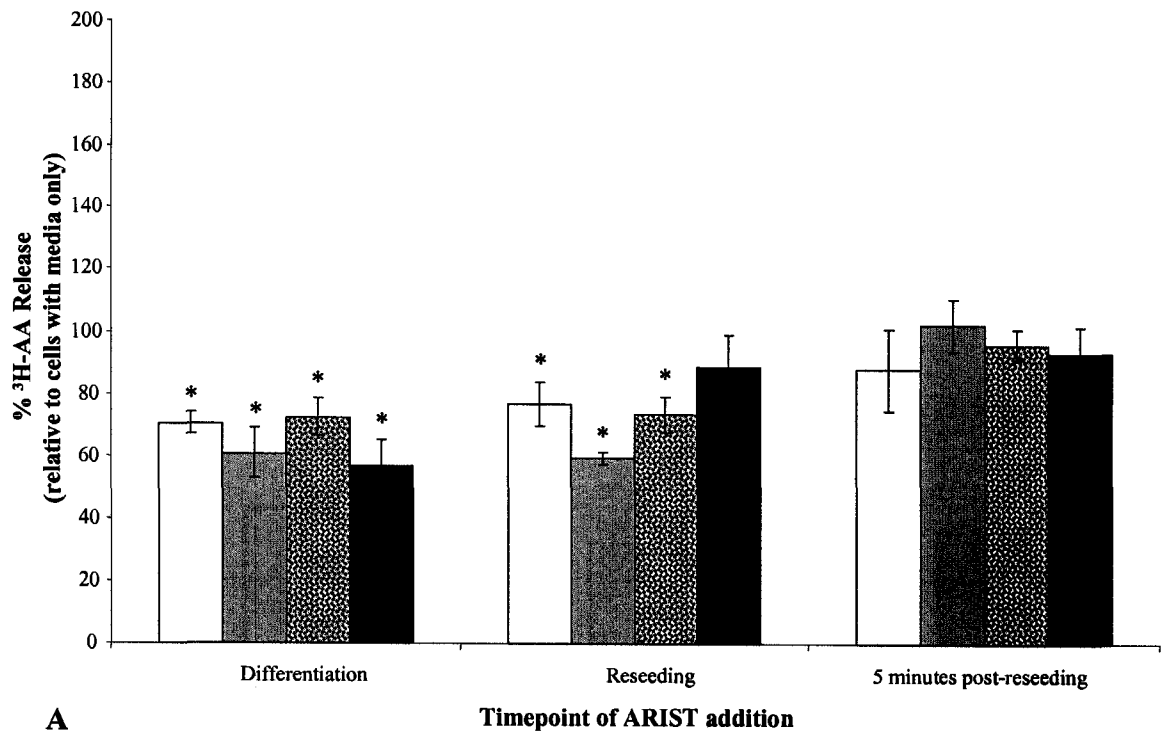


Figure 4 - Effect of material chemistry on ³H-AA release (PLA₂ activation) from adherent U937 cells. Differentiated, ³H-AA labeled U937 cells were cultured on PS, HDI431, HDI321 or MDI321 for 10 minutes. ³H-AA release from the cells into the media was measured and normalized to 10 μg DNA. Results are expressed as % original ³H-AA release relative to cells adherent to PS. Each PCNU significantly increased ³H-AA release from adherent U937 cells as compared to cells adherent to PS (dotted line).

ARIST, the PLA₂ inhibitor which decreased ¹⁴C-PCNU radiolabel release the most, showed the greatest inhibitory effect on ³H-AA release with the least harm to the cells (Figure 5A) on all material surfaces (~40% reduction) when ARIST was added during cell differentiation. Inhibition of ³H-AA release was seen on PS, HDI431 and HDI321 when ARIST was added at reseeding and significant material differences were seen between HDI431 and MDI321 at this time point (p=0.0197). When added at 5 minutes post cell reseeding, ARIST addition to culture medium did not result in significant inhibition of ³H-AA release.

PMK had minimal inhibitory effect on ³H-AA release overall (Figure 5B). When added at differentiation, PMK inhibited ³H-AA release in HDI431 adherent cells. Significant material differences in ³H-AA release were seen between PS and HDI431 (p=0.0003). When added during U937 cell reseeding, PMK did not result in significant inhibition of ³H-AA release on any material surface. However, ³H-AA release was significantly stimulated in MDI321 adherent cells. This stimulation of ³H-AA release was significantly increased in comparison to the ³H-AA release seen in PS adherent cells cultured with PMK at reseeding (p<0.0001). When PMK was added to U937 cells 5 minutes post-reseeding, significant inhibition of ³H-AA release was seen in PS adherent cells with significant stimulation from HDI431 and MDI321 adherent cells. Significant material differences in ³H-AA release were seen between all combinations of material surfaces except between HDI431 and MDI321 where the difference in stimulation of ³H-AA release was not significant. Since BPB and QUIN caused cell damage, the

Figure 5 - Effect of PLA₂ inhibitors and material chemistry on ³H-AA release (PLA₂ activation) from adherent U937 cells. Differentiated, ³H-AA labeled U937 cells were cultured on PS (open bars), HDI431 (shaded bars), HDI321 (dotted bars) or MDI321 (closed bars) for 10 minutes. PLA₂ inhibitors A) aristolochic acid (ARIST) or B) palmityl trifluoromethyl ketone (PMK) were added to the wells at 48 hours of differentiation, upon reseeding, or 5 minutes post-reseeding and was present in culture media for the remainder of the experiment. Cells were cultured on the PCNU surfaces for 10 min, ³H-AA release from the cells into the media was measured and related to 10 µg DNA. Results are expressed as % original ³H-AA release relative to cells with media alone. * indicates significant inhibition of ³H-AA release relative to no inhibitor condition, ** indicates significant stimulation of ³H-AA release relative to no inhibitor condition. A) Significant material differences in ³H-AA release were seen between HDI431 and MDI321 at reseeding. B) Significant material differences were seen between PS and HDI431 at differentiation, PS and MDI321 at reseeding, and at 5 minutes post-reseeding material differences were seen between all combinations of material surfaces except between HDI431 and MDI321.



significant inhibition seen with these inhibitors at all time points and on all material surfaces was difficult to interpret (data not shown) and will not be discussed further.

DISCUSSION

Since inflammation (in which the PLA₂ pathway is a major participant) is a part of the response to implanted medical devices, a better understanding of how materials affect the initiation of this response and subsequent induced biodegradation is important. The experimental approach of using inhibitors towards radiolabel release (i.e. material biodegradation) and AA release (i.e. activation of the PLA₂ pathway) was able to establish an association between specific PLA₂ pathway(s) and material degradation.

In this study with the U937 cell line, sPLA₂ appears to be the dominant PLA₂ pathway which is involved in PCNU biodegradation (as seen with ARIST-mediated inhibition results) and PLA₂ pathway stimulation (³H-AA release) by PCNUs. Although inhibitors of each group of PLA₂ decreased radiolabel release (Figure 1), ARIST (sPLA₂ inhibitor) had the greatest effect, both when added to cell culture during cell differentiation (Figure 2) and 1 hour post-reseeding (Figure 1). In ³H-AA release experiments with PLA₂ inhibitors, ARIST showed similar inhibition of ³H-AA release (~40% reduction)(Figure 5A) when added during differentiation as was seen in radiolabel release assays, providing the strongest link between biodegradation and activation of PLA₂. However, it is difficult to exclude other PLA₂ pathways (aside from the sPLA₂ pathway) as well as many other intracellular pathways since chemical inhibitors are rarely absolutely specific (2).

Although the cytotoxic effects seen upon addition of ARIST to cell culture during the final 24 hours of the differentiation period were not as severe as the effects seen with BPB and QUIN, cytotoxic side effects of using chemical inhibitors in the latter study is still a concern. It is possible that some of these chemical inhibitors are interfering with apoptosis or necrosis which would result in the altered cell viability. However, the results from PLA₂ inhibition by ARIST suggest that sPLA₂ does participate in the biological processes leading to macrophage-mediated PCNU biodegradation. It should be noted that PMK had some inhibitory effect on both radiolabel release (Figure 1) and ³H-AA release assays (Figure 5B), and its pleiotropic effects may cause inhibition of both iPLA₂ and cPLA₂ depending on the concentration used (21). The more specific iPLA₂ inhibitor, bromoenol lactone, showed no significant effect on radiolabel release when added 1 hour post-reseeding (data not shown), suggesting that iPLA₂ may not be involved in macrophage-mediated PCNU biodegradation.

The results from this study appear to be consistent with the proposed functions of each PLA₂ enzyme. sPLA₂ and cPLA₂ are suggested to be involved in membrane hydrolysis leading to AA release (23). Inhibition of sPLA₂ by ARIST did not result in 100% inhibition of radiolabel release or ³H-AA release. Since sPLA₂ and cPLA₂ have been shown to cross-talk (24), it is possible that cPLA₂ may compensate for the decreased activity of sPLA₂ and still result in a contribution (via AA release) to the release of activities that result in PCNU biodegradation. In this study, the inhibitor QUIN is used which is shown to inhibit both sPLA₂ and cPLA₂. In Figure 1, when QUIN was added to the experimental system at 1 hour post-reseeding, ¹⁴C- radiolabel release was decreased

by ~60-70% depending on the reseeding surface. This was the greatest inhibition by a PLA₂ inhibitor that was demonstrated on all 3 material surfaces similarly. iPLA₂, on the other hand, is suggested to contribute to membrane reorganization as opposed to phospholipid hydrolysis and AA release (25). These proposed functions of iPLA₂ coincide with the results herein. The iPLA₂ inhibitor PMK did not result in significant inhibition of radiolabel release when added to cell culture during differentiation, and varying effects were demonstrated in combined ³H-AA release/PLA₂ inhibitor studies with minimal inhibitory effects.

The effect of ARIST on both degradation and ³H-AA release showed sensitivity to the material surface depending on the time of addition of this inhibitor. Although there was no significant difference between the effect of ARIST on HDI321 and MDI321 degradation when ARIST was added 1 hr post-reseeding (Figure 1), there was a significant difference between these two surfaces when ARIST was added during differentiation (Figure 2). In another study (Labow *et al.*, *Biomaterials*, under review), degradation of PCNUs was affected by the surface on which the monocytes were differentiated. Although no inhibitor was involved, the difference in hard segment chemistry MDI321 surface compared to HDI321, was shown to promote differences in both the degree of PCNU degradation and the amount of esterase protein and activity subsequently measured.

While results have shown that esterases, CE and MSE, cause PCNU degradation and that the PLA₂ pathways are involved in the processes leading to this degradation, the direct

link between the two is yet unknown. The location of these esterases in macrophages has not yet been determined (26, 27). However, it has been demonstrated that these esterases are secreted from MDM (14). It is possible that the PLA₂ pathway, via PLA₂ itself or AA liberated by PLA₂, may have a role in esterase or other degradative enzyme secretion through pathways such as those considered in Figure 6. AA itself has been shown to be directly involved in lysosomal enzyme secretion (4). Both plasma membrane (28) and lysosomal (29) PLA₂ receptors have been reported, with studies showing the colocalization of certain groups of sPLA₂ with the lysosomal PLA₂ receptor (30). Group V sPLA₂ has been detected in human macrophages (23) and is a potential candidate as an endogenous ligand for the PLA₂ receptor (31). To date lysosomal hydrolases (i.e. acid phosphatase, hexosaminidase) have not been reported to be responsible for material degradation. Although CE and MSE are relatively neutral enzymes, it is possible that an acid cholesterol esterase originating from lysosomes may be secreted (therefore resulting in an acidic microenvironment beneath the cell) and participate in material degradation. Figure 6 represents a proposed schematic diagram which depicts potential mechanisms for PLA₂ and/or AA association with material degradation.

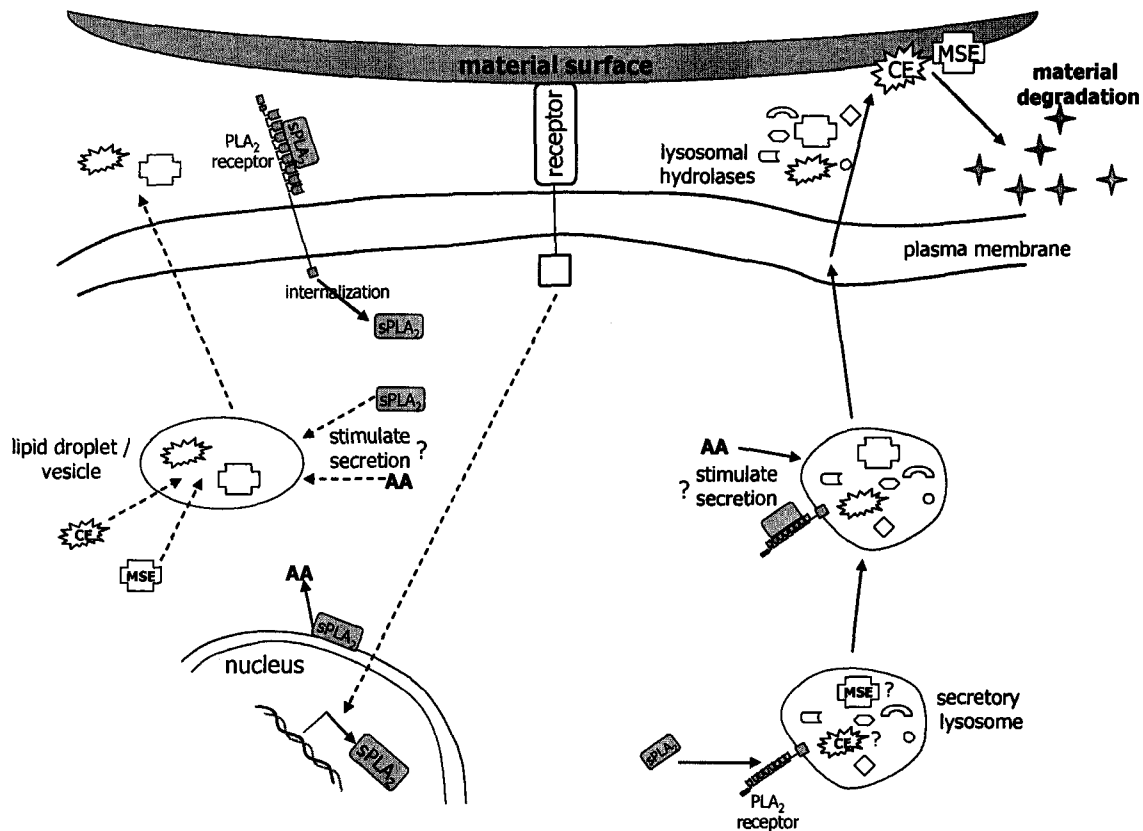


Figure 6 - Schematic diagram of hypothesized mechanism of sPLA₂ influence on material degradation. It is known that cholesterol esterase (CE) and monocyte-specific esterase (MSE) cause degradation of polyurethanes (10-13). The location of where these enzymes are released from macrophages, as well as how secretory PLA₂ (sPLA₂) may be involved in the release of degradative enzymes from macrophages is unknown. The mechanism for cell recognition of polyurethanes still remains to be elucidated, but it is possible that a surface pattern recognition receptor, such as a Toll-like receptor may be involved in macrophage recognition of polyurethanes (32). Upon material activation of a cell receptor, it is possible that this activation indirectly upregulates the synthesis of sPLA₂ or results in activation of sPLA₂ present in the cell. Both plasma membrane and lysosomal sPLA₂ receptors have been reported and certain groups of sPLA₂ have been shown to bind to these receptors (28-30). Upon binding to the plasma membrane receptor, sPLA₂ can be internalized, upon binding the lysosomal receptor, it is possible that sPLA₂ can stimulate the secretion of lysosomal enzymes (30). As well, arachidonic acid (AA) has also been shown to stimulate the release of lysosomal enzymes (4) and it may be possible that AA may be involved in the stimulation of release of CE and/or MSE from other cellular compartments or vesicles.

CONCLUSIONS

The current study has given insight into the members of the PLA₂ pathway that may participate in macrophage-mediated PCNU biodegradation. It has been shown herein that activation of sPLA₂ participates in biodegradation, but how this occurs remains to be elucidated. Exploring the mechanisms of biodegradation for PCNU-containing devices is important both for designing new materials for long-term implants that must resist degradation, as well as designing materials with controlled biodegradation for regenerative medicine applications and drug delivery systems. It is likely that a complex mechanism of cell-mediated biodegradation involves several intracellular pathways leading up to the release and/or secretion of destructive hydrolytic enzymes. Determining the role of specific PLA₂ enzymes in this key inflammatory pathway will be pivotal to understanding the foreign body response to PCNUs by human macrophages.

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3.0 MANUSCRIPT #2

Intracellular phospholipase A₂ expression and location in human macrophages: Influence of synthetic material surface chemistry

STATEMENT OF AUTHOR CONTRIBUTIONS

Donna Lee M. Dinnes was the primary author and sole contributor to the experimental data for this manuscript. **Dr. J. Paul Santerre** is a co-investigator of Dr. Rosalind S. Labow, and principal investigator on the CIHR operating grant that funded this research. In addition, he has contributed to the editing and suggestions in the preparation of this manuscript. Dr. Santerre's Polymer Chemistry laboratory at University of Toronto synthesized the model PCNU materials used for the following studies. **Dr. Rosalind S. Labow** is the supervisor of Donna Lee M. Dinnes and contributed to experimental design and editing / suggestions for this manuscript.

SUMMARY

This manuscript entitled "Intracellular phospholipase A₂ expression and location in human macrophages: Influence of synthetic material surface chemistry" has been submitted to *Journal of Cellular Physiology* (December 2006) and is currently under review (January 2007). The first manuscript in this thesis outlined how inhibitors of PLA₂ enzymes inhibited macrophage-mediated PCNU degradation. The results from this first manuscript suggested a potential role for sPLA₂ and cPLA₂ enzymes in the foreign body response to PCNU materials. Although chemical inhibitors of PLA₂ enzymes reduce PCNU mediated degradation, this does not indicate if or which PLA₂ enzymes may be induced as an inflammatory response.

The following manuscript further probes the involvement of the PLA₂ family of enzymes and their role in the macrophage response to PCNU undergoing degradation. The following work was also conducted in U937 cells differentiated to macrophage-like cells. However, further to the results in this manuscript, recent work during a short-term research visit to the University of New South Wales (Sydney, Australia) in collaboration with CBio BioPharmaceuticals Inc. (Brisbane, Australia), confirmed PCNU materials induce cPLA₂ protein expression in an additional macrophage cell line, the mouse RAW 264.7 macrophage cell line supporting the results presented here (Appendix IV).

ABSTRACT

Phospholipase A₂ (PLA₂) enzymes hydrolyze cell membrane glycerophospholipids liberating arachidonic acid (AA), a potent precursor to inflammatory eicosanoids. The presence of implanted polycarbonate-urethane (PCNU) materials, used in several medical applications, has the ability to influence the inflammatory response. This propogates the recruitment of monocytes, which differentiate into macrophages, to the cell-material interface with the ultimate goal of eliminating these materials. However, the inflammatory pathways that are activated upon macrophage attachment to PCNU surfaces are largely unknown. Previous studies suggested participation of PLA₂ pathways in material degradation with the use of chemical inhibitors, such as aristolochic acid (ARIST), however not accurately defining the specific PLA₂ enzymes involved. The current study aimed to establish specific groups of PLA₂ involved in the macrophage foreign body response to PCNU. ARIST was also assessed for specific effects on secretory PLA₂ (sPLA₂) protein expression and non-specific effects on key proteins, β-actin and monocyte-specific esterase, implicated in the macrophage attack on PCNU materials. Increased intracellular expression of cytosolic PLA₂ (cPLA₂), but not sPLA₂, was detected by immunoblot analysis, demonstrating a dual phase stimulation during the time course of increased cPLA₂ protein expression. Laser scanning confocal microscopy images indicated a change in location of cPLA₂ in macrophages adherent to PCNU surfaces compared to tissue culture polystyrene. This study has illustrated changes in macrophage cPLA₂ expression in response to cell-attachment to PCNU surfaces, demonstrating that the macrophage foreign body response to biomaterials induces a

potent inflammatory pathway, which may lead to tissue damage near the site of device implantation.

INTRODUCTION

One of the most important cellular signaling events initiated during an inflammatory response is the release of arachidonic acid (AA) from cell membranes by phospholipase A₂ (PLA₂) enzymes. The PLA₂ superfamily consists of a group of enzymes that hydrolyze the *sn*-2 ester bond of cell membrane glycerophospholipids to release AA and lysophospholipids as products [1]. To date, 14 groups of PLA₂ enzymes have been characterized with classification based on the substrate hydrolyzed from the *sn*-2 ester bond of phospholipids, amino acid sequence of the mature protein as well as sequence homology [2]. The three classical categories of PLA₂ are secretory (sPLA₂), cytosolic Ca²⁺-dependant (cPLA₂) and Ca²⁺-independent (iPLA₂) PLA₂ (reviewed in [2]). The sPLA₂ enzymes are low MW (13-18 kDa) proteins with an active site histidine. They have a requirement for mM Ca²⁺ and are secreted as indicated by the name. cPLA₂ enzymes are higher MW proteins (61-114 kDa) with a μM requirement for Ca²⁺, an active serine site and a high preference for AA in the *sn*-2 position of phospholipids. The final group, iPLA₂, was classified as such since it was the first PLA₂ cloned with no dependence for Ca²⁺. Both cPLA₂, as indicated by the name, and iPLA₂ are cytoplasmic enzymes. Since PLA₂ enzymes are pivotal to the inflammatory response, their role in human macrophages and the foreign body response to implanted biomaterials is important to define [3-5].

Materials used for the manufacture of implanted medical devices (such as pacemaker lead insulation, artificial heart valves and indwelling catheters) as well as tissue engineering and drug delivery applications, consist of a wide range of synthesized elastomeric

polymers depending on the properties desired for their intended use. Polyurethanes (PU) are one of the most common types of biomaterials in use today since they are relatively stable and biocompatible; however, these materials still maintain the ability to elicit immune responses as well as being shown to be susceptible to material degradation over time (reviewed in [6]). Whether biodegradation is an event that is desired or not (i.e. long-term implants versus short-term degradable materials for tissue engineering applications), the initiation of an immune or inflammatory response can result in negative and even harmful effects either at an implant site or the site of regenerating tissue.

The model polymers used in the current study represent an important family of PU, referred to as polycarbonate-based polyurethanes (PCNU). They were synthesized with either 1,6-hexane diisocyanate (HDI) or 4,4'-methylene bis-phenyl diisocyanate (MDI), a polycarbonate (PCN) [poly(1,6-hexyl carbonate)diol] soft segment, and 1,4-butanediol (BD) in varied stoichiometric ratios to examine sensitivity of PLA₂ responses to PCNU chemistry differences. PCNU materials were originally developed in order to overcome hydrolytic and oxidative degradation experienced with traditional polyester- and polyether-based PU materials [7, 8]. Although, some PCNUs are more stable to hydrolytic and oxidative degradation than polyester- and polyether-based PUs respectively [8-11], these materials remain susceptible to some biodegradation [12, 13]. Both neutrophils and monocyte-derived macrophages (MDM) are recruited to a material site as part of the foreign body response. Although both cell types participate in the foreign body response, the MDM contribution is much greater since neutrophils only survive a short time and MDM demonstrate 5-10 times greater degradative capacity

towards PU materials [3]. In addition, there is significant evidence that the MDM is also the greatest cellular contributor to *in vivo* PU degradation. These cells have been shown to be the most abundant cell type adherent to explanted long-term *in vivo* implant materials, as well as remaining at the cell-material interface for the lifetime of the device [14]. In addition, MDM have the capacity to fuse and form foreign body giant cells (FBGC), a hallmark of chronic inflammation [15]. Studies have shown that PCNU materials induced increases in FBGC formation in comparison to a control tissue culture polystyrene (TCPS) surface [16]. These findings provide supporting evidence that PCNU materials have the capacity to induce inflammatory responses. Despite this, very few specific inflammatory pathways have been characterized with the goal of advancing our understanding of the foreign body response to PU materials.

Previous studies have demonstrated that chemical inhibitors of various PLA₂ enzymes reduced macrophage-mediated PCNU degradation [5]. Inhibitors of sPLA₂, cPLA₂ and iPLA₂ enzymes each resulted in a reduction of macrophage-mediated PCNU degradation, with differences in inhibition elicited by only slight differences in PCNU chemistry. PLA₂ inhibitors caused a reduction in PCNU degradation demonstrating a potential contribution of PLA₂ enzymes in the material degradation pathways within macrophages. In addition, there was a difference in PLA₂ activation, as measured by ³H-AA release, between PCNU materials and TCPS, with significantly more ³H-AA being released from macrophages on PCNU [5]. Although cPLA₂ is primarily responsible for AA release (reviewed in [17]), both group IVA cPLA₂ and group V sPLA₂ have demonstrated the capacity to mobilize AA from phospholipid membranes of macrophages [18]. Since AA

release was increased from PCNU-adherent macrophages, group IVA cPLA₂ and group V sPLA₂ enzymes were investigated to determine if the increase in AA was correlated to changes in protein expression for either enzyme when macrophages interact with PCNUs.

The current study assessed the three model PCNU materials described above for their effect on PLA₂ expression in human macrophages and compared these effects to TCPS as the control surface. The effect of the sPLA₂ inhibitor, aristolochic acid (ARIST), on PLA₂ expression was compared to a cytoskeletal protein, β -actin, and an enzyme shown to be related to the degradation of PCNU, monocyte-specific esterase (MSE) [13, 16]. Changes in macrophage protein expression of both cPLA₂ and sPLA₂ induced by material chemistry were determined by immunoblotting at various time points of cell adherence to TCPS and the model PCNUs. In addition, changes in location of cPLA₂ on PCNU versus TCPS surfaces were investigated by immunofluorescence staining of U937 cells and imaging by laser scanning confocal microscopy.

MATERIALS AND METHODS

Unless otherwise specified, all reagents were purchased from Sigma-Aldrich Canada Ltd (Oakville, ON, CAN).

Preparation of material surfaces

The model PCNUs used in this study were synthesized with a PCN soft segment, BD chain extender and either HDI or MDI as the diisocyanate as described in detail previously [19]. Three PCNUs were used for this study (Table 1); 1) a PCNU containing

Table 1 - Polycarbonate-based polyurethanes used in this study

Reagent	Stoichiometry	Acronym
HDI/PCN/BD	4:3:1	HDI431
HDI/PCN/BD	3:2:1	HDI321
MDI/PCN/BD	3:2:1	MDI321

a linear aliphatic diisocyanate (HDI) with a stoichiometric ratio of 4:3:1 (HDI:PCN:BD - referred to as HDI431), 2) a HDI-containing PCNU with a stoichiometric ratio of 3:2:1 (HDI:PCN:BD - referred to as HDI321), and 3) a PCNU containing an aromatic diisocyanate (MDI) with a stoichiometric ratio of 3:2:1 (MDI:PCN:BD - referred to as MDI321). The first two materials vary in polar group content (HDI321 > HDI431) and are both highly degradable materials. For this study, TCPS was used as a control surface since this is the most commonly used material surface in cell culture.

The synthesized PCNUs were dissolved overnight in a 10% dimethylacetamide solution (w/v), centrifuged and filtered (0.45 μm Teflon filter; Chromatographic Specialties, Toronto, ON, CAN), then coated as a thin layer (100 μL) onto 15 mm diameter glass cover slips (Fisher Scientific, Ottawa, ON, CAN) under sterile conditions in a laminar flow hood, as previously described [3]. The slips were dried overnight at 50°C, purged for 24 hours and then dried under vacuum for 72 hours. Prior to using the PCNU slips or TCPS multiwell plates for an experiment, they were hydrated by incubating with phosphate buffered saline (PBS)(0.64 M KH_2PO_4 , 0.008 M NaCl, 0.2 M Na_2HPO_4) at 37°C and 5% CO_2 with 100% humidity overnight. This process equilibrates the polymer slips with an aqueous environment and removes trace solvent that may possibly interfere with the experiment.

U937 cell culture

U937 cells (American Type Culture Collection (ATCC™ catalogue # CRL – 1593.2)) were employed as a well characterized model for MDM-mediated PCNU biodegradation

[4]. U937 cells were maintained as a promonocytic cell suspension and fed every second day with RPMI 1640 medium containing 10% fetal bovine serum (Invitrogen Canada Inc., Burlington, ON, CAN), 1 mM sodium pyruvate (Invitrogen) with 100 units/mL penicillin and 0.1 mg/mL streptomycin (Invitrogen), as described in detail previously [4]. Only cells that were between passage 7 and 20 were used for differentiation into macrophage-like cells by culturing a 1×10^6 cells/mL suspension in TCPS 12-well plates with phorbol 12-myristate 13-acetate (PMA) (1×10^{-7} M) for 72 hours. At this time point cell division had ceased as determined by DNA analysis (see below), the cells were adherent and the parameters (maximal protein synthesis and esterase activity) that characterize a fully differentiated U937 cell for this *in vitro* model system occurred [4]. The cells were removed from the TCPS surface by gentle pipetting and were resuspended at a concentration of 1×10^6 or 2×10^6 cells/mL and reseeded onto the required material surface for the experiment.

Effect of ARIST on protein expression

Differentiated U937 cells were re-seeded (1 ml of a 2×10^6 cells/mL suspension) into 24-well plates alone (TCPS control) or into wells containing HDI431-, HDI321- or MDI321-coated glass slips. The cells were allowed to adhere for 1 hour after which the medium was replaced with or without the sPLA₂ inhibitor ARIST [200 μ M] [5, 20]. The cells were then incubated further for 48 hours after which the cells were lysed for DNA (see below) and prepared for immunoblot analysis.

DNA determination

Whole cell lysates were prepared and analyzed for DNA as described in detail previously [3, 16]. Cold Triton-X 100 buffer (180 μ L) (0.05% Triton-X 100, 10 mM EDTA in PBS) was added to each well and the cells were lysed for 1 hour on ice. Hoechst dye (number 33258; Amersham Biosciences., Baie d'Urfe, QC, CAN) was diluted with TRIS buffer (0.01 M Tris, 0.001M EDTA, 0.2 M NaCl, pH 7.4) immediately prior to analysis. Cell lysates collected for DNA (10 μ L) were added to the dye (100 μ L) in 96 well microtiter plates (Microfluor 2 Black; VWR, Mississauga, ON, CAN). The lysates were compared to a DNA standard (Calf Thymus DNA) and each standard well to be analyzed also contained the same amount of 0.05% Triton-X 100 buffer as in the lysate samples. The samples were analyzed using a fluorescence microplate reader (POLARstar Galaxy, BMG Labtechnologies, Durham, NC, USA) with an excitation and emission wavelength of 360 and 460 nm respectively.

Immunoblotting

Proteins from whole cell lysate samples were separated by SDS-PAGE using a Protean II Cell System (Bio-Rad Laboratories Ltd., Mississauga, ON, CAN) with 5% stacking and 10% (cPLA₂), 12% (β -actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), MSE) or 15% (sPLA₂) separating gel. U937 cells stimulated with PMA are non-dividing, terminally differentiated cells, therefore samples for electrophoresis were loaded into each well based on the amount of DNA in each sample. 250 ng of DNA was loaded into each well except for gels that were immunoblotted for cPLA₂ and sPLA₂, which required 1000 ng of DNA to be loaded per well (as a result of the low abundance of these

proteins). GAPDH was used as an additional control to ensure equal loading of protein in each well. Proteins from the resulting gels were transferred to nitrocellulose membranes (0.45 μ m, Bio-Rad) for 75 minutes at 130 V. The membranes were then blocked for 1 hour in Tris-buffered saline (TBST; 10 mM Tris, 0.1 M NaCl, 0.1% Tween-20, pH 7.5) containing either 5% non-fat dry skim milk or 5% bovine serum albumin. The membranes were then probed for 1 hour with rabbit anti-cPLA₂ (Research Diagnostics Inc, Concord, MA, USA), mouse anti-sPLA₂ (group V) (Cayman Chemical Co., Ann Arbor, MI, USA), rabbit anti-porcine esterase (Rockland Immunochemicals Inc., Gilbertville, PA, USA – reacts with MSE), mouse anti- β -actin or mouse anti-GAPDH (Chemicon International Inc., Temecula, CA, USA) and subsequently washed 8 times with TBST. The membranes were then incubated with either anti-rabbit IgG (Rockland) or anti-mouse IgG (Pierce Biotechnology Inc., Rockford, IL, USA) conjugated to horseradish peroxidase, for 1 hour followed by 8 washes with TBST. Protein bands were visualized using a chemiluminescence detection kit (Pierce). The intensity of the protein bands were quantified using Quantity One[®] software (Bio-Rad).

Immunofluorescence and laser scanning confocal microscopy

Differentiated U937 cells, as described above, were re-seeded at a concentration of 1×10^6 cells/mL into 24-well plates containing either HDI431-coated glass slips or TCPS cover slips (Sarstedt Inc., Montreal, QC, CAN) and allowed to adhere for 30 minutes or 2 hours. The adherent cells were washed 3 times with warmed PBS and fixed at room temperature with 4% paraformaldehyde (Fisher Scientific) in PBS for 10 minutes. The cells were then blocked for 1 hour with 10% rabbit serum (Cedarlane Laboratories Ltd.,

Hornby, ON, CAN) in PBS, followed by a 1 hour incubation with mouse anti-cPLA₂ (group IVA)(Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) in 5% rabbit serum in PBS. The slips were then washed 3 times in 5% rabbit serum in PBS followed by incubation for 45 minutes with rabbit anti-mouse Alexa Fluor[®] 488 (Invitrogen). The slips were finally washed 3 times in PBS and mounted onto microscope slides with 90% glycerol. The cells were visualized with an Olympus 100X oil immersion objective with a numerical aperture of 1.4, on an Olympus IX80 laser scanning confocal microscope operated by FV1000 software (version 1.4a). The Alexa Fluor[®] 488 fluorochrome was excited with the 488 nm line of the argon multi-line laser.

Statistical analysis

Statistical analyses were performed using a one-way ANOVA. The SAS[®] program (version 9.1) for windows was used and values were considered significant with a p-value of < 0.05.

RESULTS

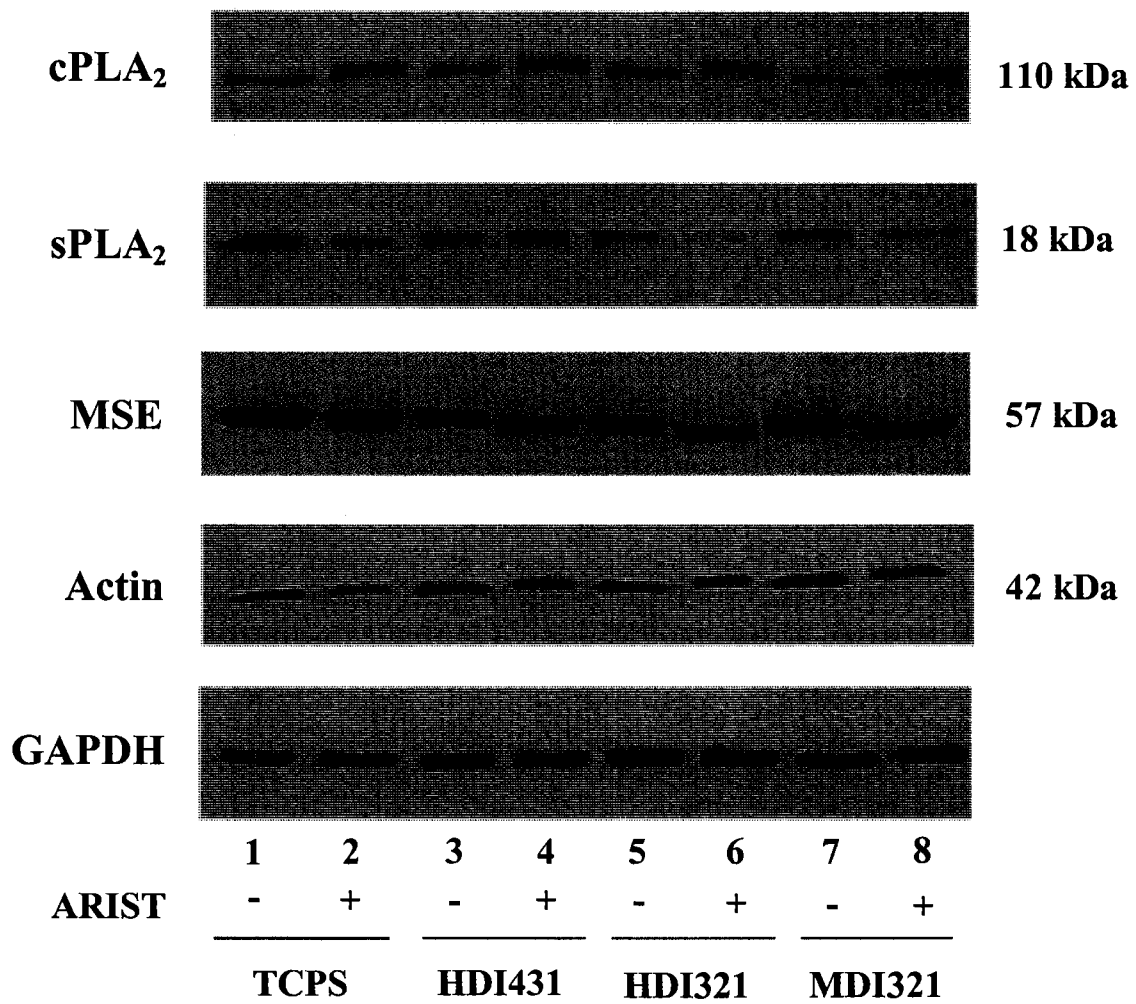
Specific inhibition of intracellular sPLA₂ protein expression by ARIST

Previous studies compared several chemical inhibitors of PLA₂ to assess their effect on U937 cell-mediated ¹⁴C-PCNU degradation and inhibition of PCNU-mediated U937 cell ³H-AA release [5]. The sPLA₂ inhibitor ARIST demonstrated the greatest reduction of both U937 cell-mediated ¹⁴C-PCNU degradation and PCNU-induced ³H-AA release by U937 cells. To assess the specificity of ARIST towards sPLA₂ versus cPLA₂, immunoblot analysis for intracellular cPLA₂ and sPLA₂ protein from lysates of cells

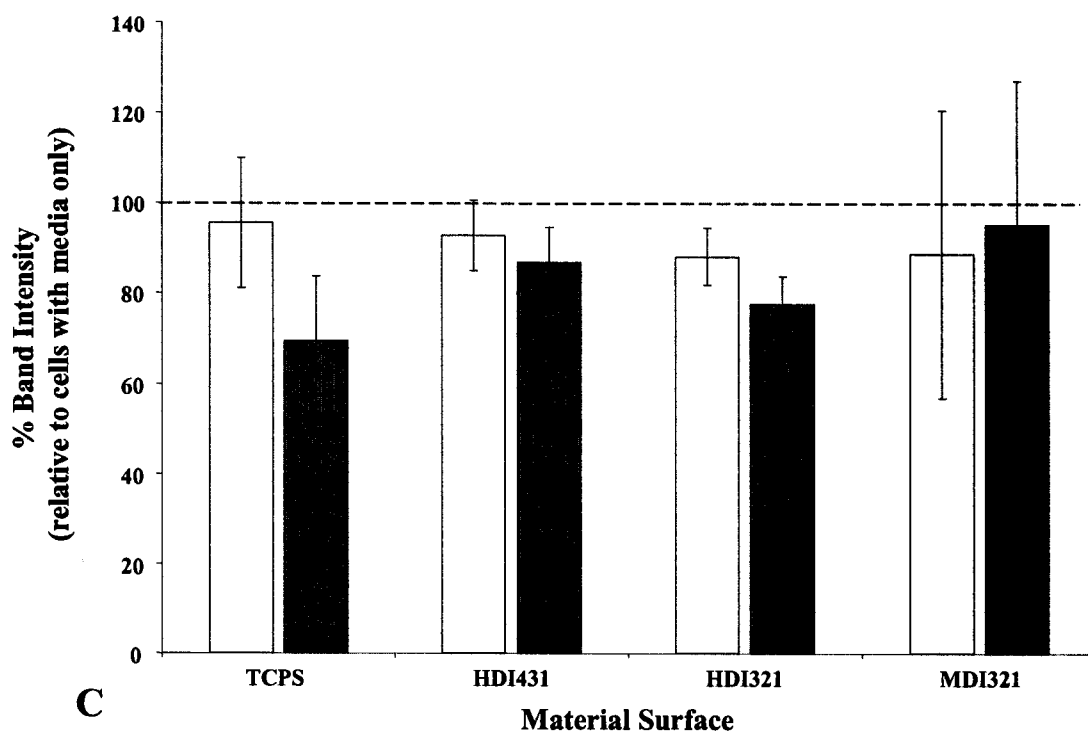
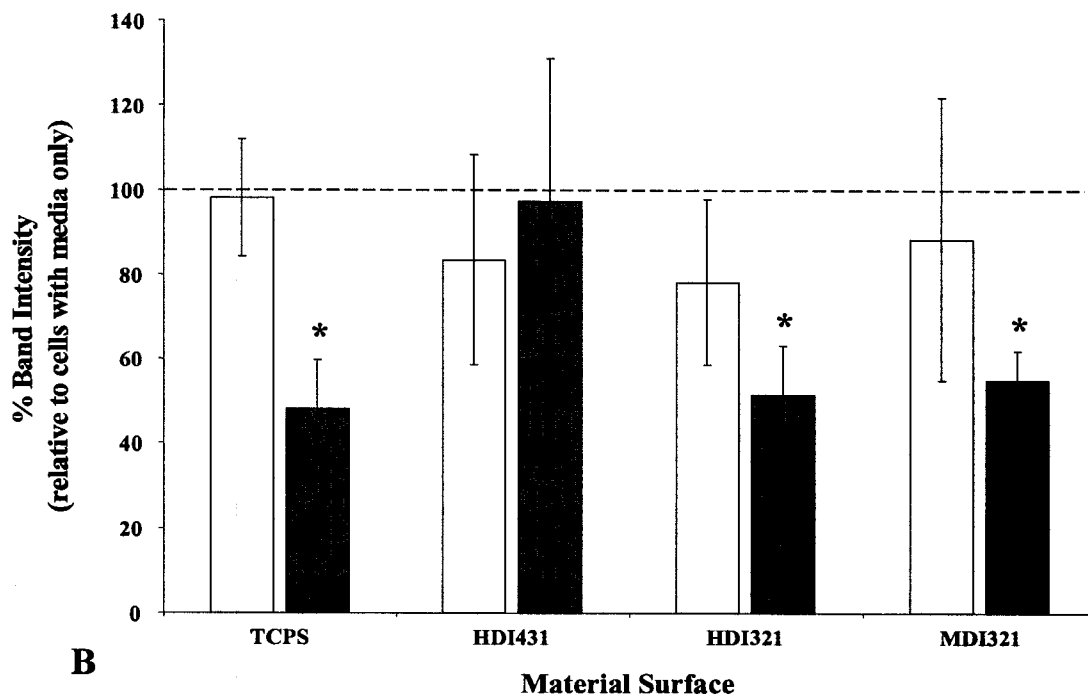
cultured with and without ARIST was performed to determine the effect of ARIST on intracellular protein expression. Figure 1A shows representative blots of cell lysates from U937 cells cultured on each material surface with and without ARIST for 48 hours. Lanes 1, 3, 5 and 7 of each blot represent U937 cell lysates from TCPS, HDI431, HDI321 and MDI321 respectively from cells cultured with media alone. Lanes 2, 4, 6, and 8 of each blot represent U937 cell lysates from TCPS, HDI431, HDI321 and MDI321 respectively from cells cultured with ARIST. The first blot demonstrates lysates probed with anti-cPLA₂ and the second blot probed with anti-sPLA₂. ARIST had no significant effect on cPLA₂ protein expression as indicated by quantification of blots from multiple experiments (Figure 1B). However, as seen in the second blot (Figure 1A), ARIST had a significant effect on sPLA₂ protein expression in U937 cells adherent to TCPS, HDI321 and MDI321 each demonstrating 50 – 60 % banding intensity of sPLA₂ protein relative to cells cultured with media alone (Figure 1B). ARIST had no significant effect on sPLA₂ protein expression in U937 cells adherent to HDI431.

In order to ensure that ARIST was not eliciting non-specific effects on key intracellular proteins, immunoblot analysis was performed on these U937 cell lysates to probe for β -actin and MSE. Immunoblot analysis determined that ARIST did not have significant effects on intracellular MSE protein as seen in Figure 1A (third blot) and quantified in Figure 1C. No significant effect on β -actin expression (fourth blot) was detected as outlined in the quantification of blots from multiple experiments (Figure 1C).

Figure 1 – PLA₂ inhibitor ARIST decreases U937 cell sPLA₂ expression. PMA-differentiated U937 cells were seeded onto TCPS (control) or PCNU surfaces for 1 hour. The culture medium was replaced with either fresh medium or medium containing ARIST (sPLA₂ inhibitor) as described in **Materials and Methods**. Cells were then incubated further for 48 hours before collection of cell lysates in preparation for immunoblotting. A) Representative blots of U937 cell lysates with and without ARIST. Blots were probed with anti-cPLA₂, anti-sPLA₂, anti-MSE or anti-β-actin. Cell lysates were loaded by equal amounts of DNA and blots were stripped and reprobed with anti-GAPDH as an additional loading control. Lane 1,2 – TCPS; 3,4 – HDI431; 5,6 – HDI321; 7,8 MDI321. (+) with PLA₂ inhibitor ARIST (200 μM), (-) no ARIST. B) and C) Quantification of immunoblots (from Figure 1A) from TCPS or PCNU adherent U937 cells. Protein bands were quantified using Quantity One. Data from repeated experiments (n = minimum of 3) were pooled and the data were statistically analyzed by a one-way ANOVA (*p<0.05). Relative amounts of cPLA₂ (open bars) or sPLA₂ (grey bars) (B) and MSE (open bars) or β-actin (grey bars) (C) on TCPS, HDI431, HDI321 and MDI321 are indicated in cells with ARIST compared to cells with media only (dotted line).



A



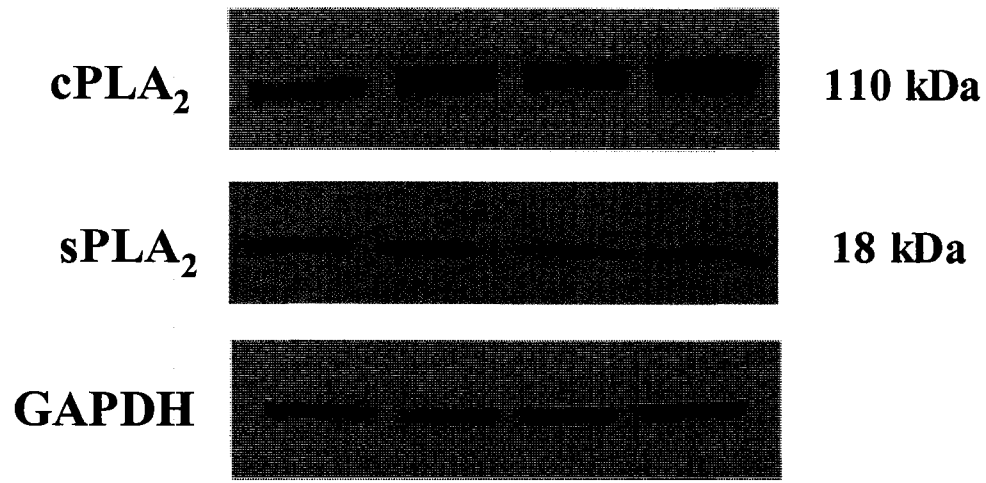
Effect of PCNU materials on intracellular cPLA₂ and sPLA₂ protein expression

Immunoblotting of lysates from U937 cells cultured for 2 or 48 hours on PCNU (HDI431, HDI321 and MDI321) and TCPS was performed to probe for group IVA cPLA₂ and group V sPLA₂. These time points were chosen based on established biodegradation assays [3-5, 12] in which cells were re-seeded onto the candidate surface for one hour (since the majority of cells have become adherent by approximately 1 hour post re-seeding). The culture medium was then replaced with or without the reagent to be tested and then incubated further for 48 hours for degradation analysis. Representative immunoblots show that at 2 hours post re-seeding to PCNU surfaces, cPLA₂ protein expression was increased relative to cells cultured on TCPS (Figure 2A) for all 3 PCNUs. 2 hours after U937 cell attachment to each PCNU surface, cPLA₂ protein was significantly increased by 3- to 3.5-fold on each PCNU relative to TCPS. By 48 hours post cell attachment, cPLA₂ protein amount, though increased on each PCNU relative to TCPS, was no longer significant (data not shown). Representative sPLA₂ blots (Figure 2A), verified by quantification from multiple experiments (Figure 2B), indicated that intracellular sPLA₂ protein expression was not significantly altered by the material surface to which U937 cells were adherent.

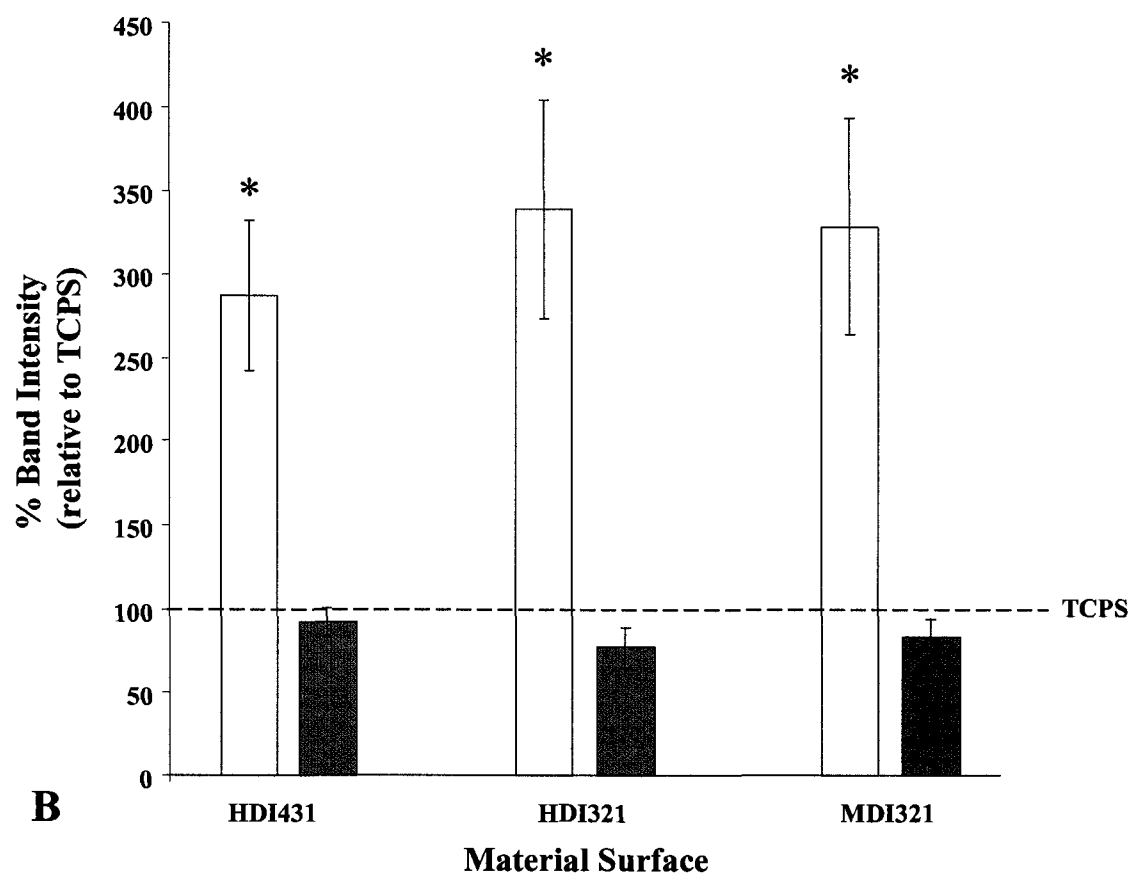
PCNU materials induce early changes in cPLA₂ protein expression

cPLA₂ protein expression was clearly altered by the material surface to which U937 cells were adherent (Figure 2A). Since there was such a large increase (~3-fold) in cPLA₂ protein expression at 2 hours post cell attachment, earlier time points were investigated to determine how early this response was initiated by cPLA₂ as a result of

Figure 2 – PCNU material surfaces induce increased intracellular U937 cell cPLA₂ expression. A) PMA-differentiated U937 cells were seeded onto TCPS (control), HDI431, HDI321, or MDI321 for 2 hours, as described in detail in **Materials and Methods**. Cell lysates were collected for immunoblotting and loaded by equal amounts of DNA. Resulting blots were probed with anti-cPLA₂ and anti-sPLA₂ antibodies, then stripped and reprobed with anti-GAPDH antibody as an additional loading control. B) Quantification of protein bands from immunoblots (from Figure 2A) of TCPS or PCNU adherent U937 cells. Protein bands were quantified using Quantity One, data from repeated experiments (n = minimum of 3) were pooled and data statistically analyzed by one-way ANOVA (*p<0.05). Relative amounts of cPLA₂ (open bars) or sPLA₂ (grey bars) are indicated for U937 cells adherent to TCPS, HDI431, HDI321 and MDI321.



A



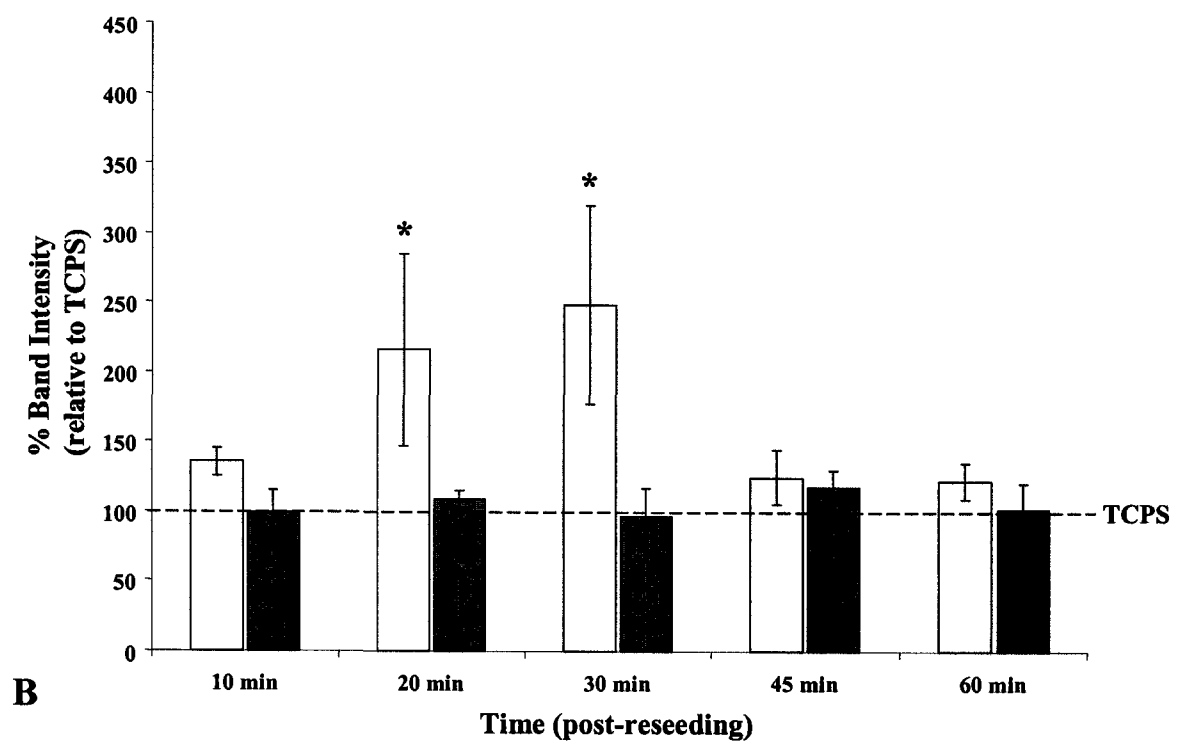
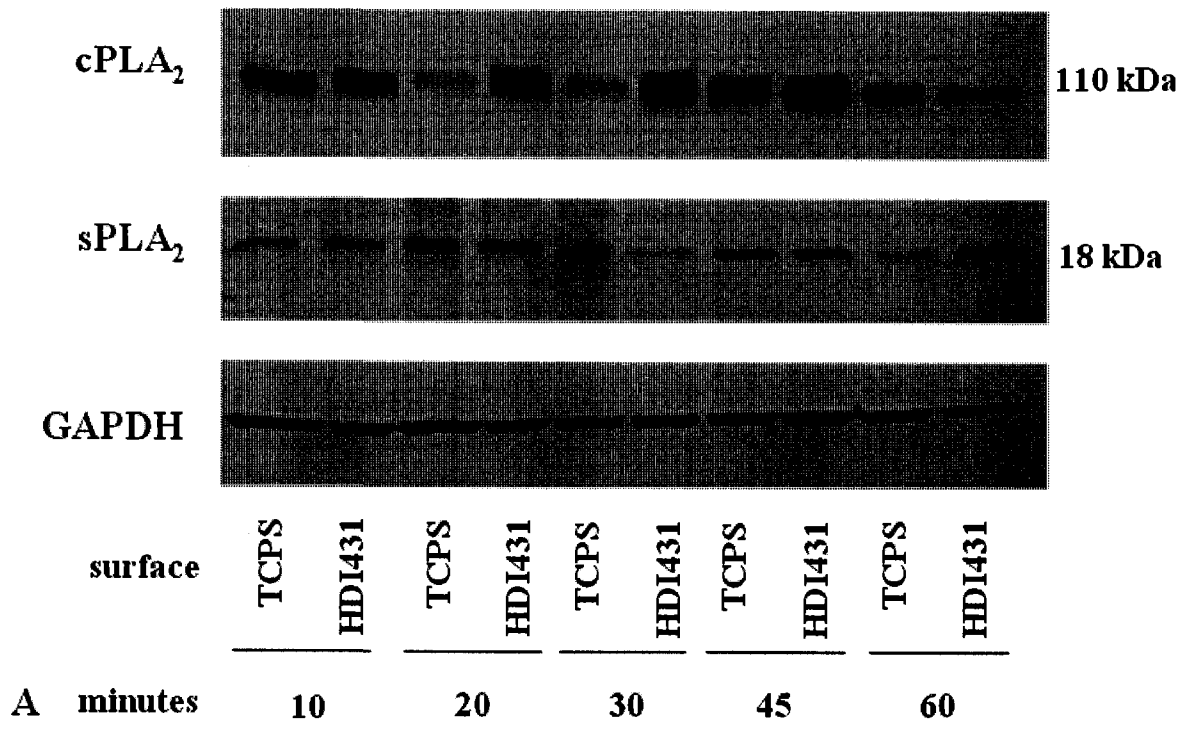
B

cell attachment to PCNU. At 2 hours, the altered cPLA₂ protein expression was comparable on each model PCNU material (Figure 2B), therefore further experiments exploring earlier time points of PLA₂ protein expression were carried out between only HDI431 and TCPS. Previous studies which demonstrated that PCNU materials induced increased ³H-AA release compared to TCPS illustrated that the greatest PLA₂ activation occurred within 10 minutes of U937 cell attachment (Figure 3 in [5]). Accordingly, a time course of protein expression between 10 and 60 minutes post cell attachment to HDI431 and TCPS was analyzed to assess the initial point at which PCNU had the ability to induce detectable changes in either cPLA₂ or sPLA₂ protein expression. Further to the results from 2 hours, sPLA₂ intracellular protein expression (Figure 3A) was not significantly altered in HDI431 adherent U937 cells compared to TCPS (Figure 3B). Elevated cPLA₂ protein expression on HDI431 relative to TCPS was detectable as early as 10 minutes post cell attachment (Figure 3A) with significantly increased cPLA₂ protein (~ 2 to 2.5 fold increase) at 20 and 30 minutes (Figure 3B). cPLA₂ protein expression returned to comparable expression levels seen in TCPS-adherent U937 cells by 45 minutes post cell attachment (Figure 3A and 3B).

cPLA₂ location was altered by U937 cell adherence to a PCNU surface

Considering the significant changes in cPLA₂ expression in response to material chemistry differences of the substrate on which U937 cells were cultured, cellular location of cPLA₂ (group IV) was assessed using laser scanning confocal microscopy to investigate material surface influences on cPLA₂ protein mobilization. U937 cells that were cultured for either 30 minutes or 2 hours on TCPS or HDI431 were

Figure 3 – PCNU material surfaces induce early changes in U937 cell cPLA₂ expression. PMA-differentiated U937 cells were cultured on TCPS or HDI431 for 10, 20, 30, 45 or 60 minutes. At the end of each time point, cell lysates were collected and prepared for immunoblotting as described in detail in **Materials and Methods**. A) Representative immunoblots of U937 cell lysates probed with anti-cPLA₂ or anti-sPLA₂ antibodies. Cell lysates were loaded using equal amounts of DNA. Blots were also stripped and reprobed with anti-GAPDH antibody as an additional loading control. B) Quantification of protein bands from Western Blots (from Figure 3A) from TCPS or HDI431 adherent U937 cells. Protein bands were quantified using Quantity One, data from repeated experiments (n = minimum of 3) were pooled and data statistically analyzed by ANOVA (*p<0.05). Relative amounts of cPLA₂ (open bars) or sPLA₂ (grey bars) indicated for U937 cells adherent to TCPS and HDI431 for a time course of 10 - 60 minutes.



immunofluorescently labeled with anti-group IVA cPLA₂ and visualized by laser scanning confocal microscopy. These time points, 30 minutes and 2 hours respectively, were chosen based on the fact that a peak of cPLA₂ expression was detected at 30 minutes (Figure 3B) and 2 hours (Figure 2B) on PCNU relative to TCPS. Fluorescent images illustrated a change in location of cPLA₂ in response to adherence to HDI431 compared to TCPS. At both 30 minutes and 2 hours, cPLA₂ was located diffusely throughout the cytoplasm in TCPS adherent cells (Figure 4A and 4C respectively). In contrast, U937 cells that were adherent to HDI431 for both 30 minutes and 2 hours exhibited a change in location of cPLA₂ towards the cell membrane (Figure 4B and 4D respectively) compared to U937 cells adherent to TCPS (Figure 4A and 4C).

DISCUSSION

The current study has been able to begin to establish the effect of PCNU surfaces on specific PLA₂ protein expression. Earlier studies showed that inhibitors of each category of PLA₂ enzymes induced a reduction in U937 cell-mediated degradation of PCNUs [5]. In addition, PCNU materials elicited an increase in AA release from U937 cells relative to a TCPS control, and this stimulated AA release was also primarily inhibited by the chemical inhibitors of sPLA₂ and cPLA₂ [5]. Group IVA cPLA₂ and Group V sPLA₂ were previously shown to be involved in AA release from macrophages with cPLA₂ being the main regulator [17, 18]. sPLA₂ may potentially enhance this cPLA₂-mediated AA mobilization [21], thereby generating an immediate and delayed AA mobilization response. In order to better define the specific groups of PLA₂ responsible for the early

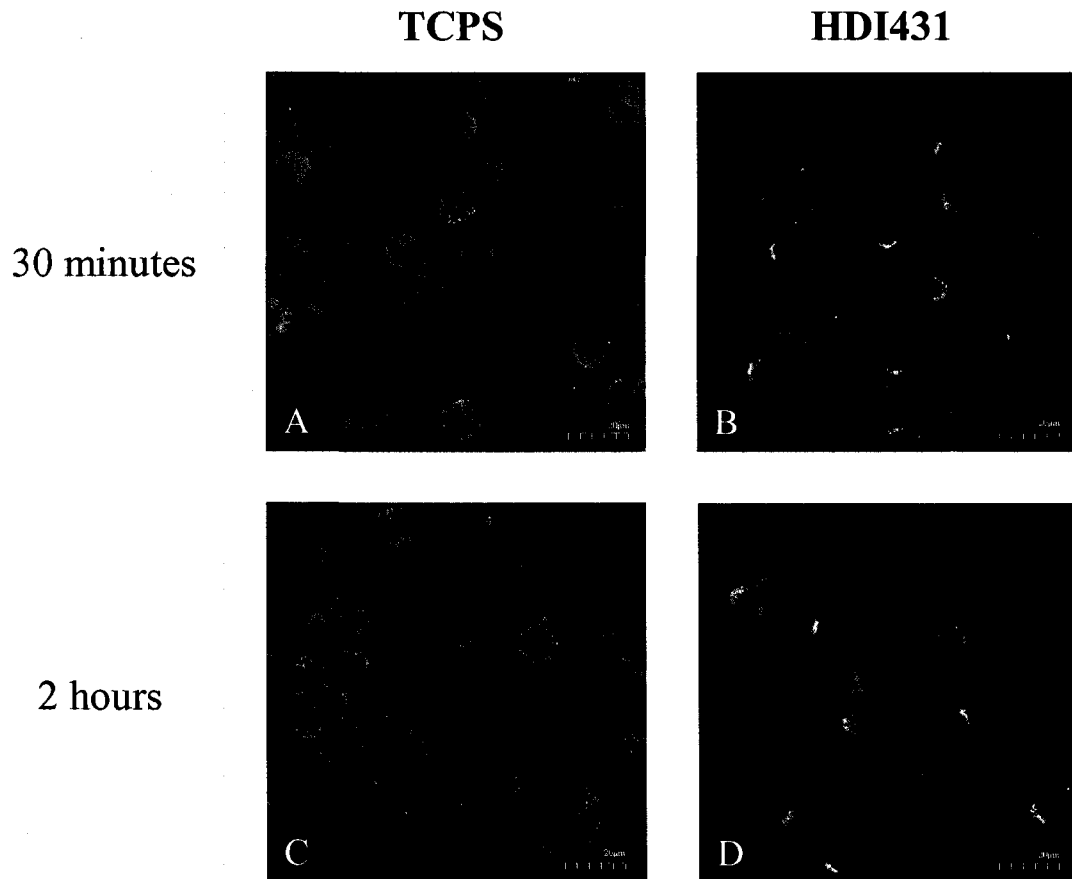


Figure 4 – Intracellular cPLA₂ expression patterns are influenced by material surface chemistry. PMA-differentiated U937 cells were seeded onto TCPS cover slips (control) or HDI431 coated glass cover slips for 30 min or 2 hours. As described in detail in **Materials and Methods**, cells were then fixed with 4% paraformaldehyde and labeled with mouse anti-group IVA cPLA₂ and rabbit anti-mouse Alexa Fluor[®] 488. Representative images were obtained using a laser scanning confocal microscope. (scale bar = 20 μm)

PCNU-mediated macrophage response, group IVA cPLA₂ and group V sPLA₂ were investigated for alterations in protein expression in response to cell-attachment to PCNU versus TCPS surfaces. iPLA₂ was not investigated since inhibitors of iPLA₂ elicited the least effect on ¹⁴C-PCNU degradation [5], in addition to the fact that iPLA₂ does not appear to contribute to AA release in U937 and other cells [22], most probably acting as a homeostatic enzyme regulating glycerophospholipid composition [23]. Several studies have illustrated that PU materials induce chronic inflammation [24-27], however, few studies have investigated specific signaling pathways that may contribute to PU-induced inflammation.

Specific inhibition of intracellular sPLA₂ protein expression by ARIST

Chemical inhibitors are often not as specific as claimed therefore experiments were carried out to determine if the inhibition of U937-cell mediated ¹⁴C-PCNU degradation elicited by ARIST [5] was a consequence of non-specific effects on protein expression. Particularly, expression of proteins implicated in macrophage-mediated degradation processes in addition to both sPLA₂ and cPLA₂ protein expression was evaluated. β -actin is a key cytoskeletal protein in many cell types but particularly motile cells such as macrophages [28]. Since β -actin is so central to cell shape and attachment, as well as cell spreading and adhesion [29, 30], protein expression of β -actin was assessed by immunoblotting to ensure no significant influences on β -actin protein expression were occurring in response to this inhibitor. Degradative substances can be released from macrophages into the extracellular space formed between the cells and the material surface that may contribute to extracellular matrix/biomaterial breakdown [31].

Candidate enzymes that have been well studied and implicated in macrophage-mediated PU degradation are esterases, specifically MSE [12, 13, 32]. MSE is an esterase that has demonstrated high degradative capacity toward PCNU materials, in addition to being newly synthesized and secreted from MDM in response to cell attachment to these surfaces [16]. Since this enzyme is central to macrophage-mediated degradation of PCNU, MSE protein expression was assessed in cells cultured with ARIST to ensure inhibition of degradation seen previously was not a result of effects on MSE protein expression.

Since both β -actin and MSE protein expression were not significantly altered in the presence of ARIST (Figure 1), it can be concluded that inhibition of ^{14}C -PCNU degradation elicited by U937 cells was not a result of non-specific effects on β -actin or intracellular MSE and was due to the effect of ARIST on PLA₂. Supporting this result was the previous study where the pattern of inhibition by ARIST of ^{14}C -PCNU degradation by U937 cells [5] was similar to the ARIST inhibition of sPLA₂ protein expression in HDI321 and MDI321 adherent cells (Figure 1B). The more prominent effect on both ^{14}C -PCNU degradation [5] and sPLA₂ protein expression (Figure 1A and 1B) was demonstrated on PCNU with a stoichiometric ratio of 3:2:1 of the starting components, regardless of the hard segment chemistry (HDI or MDI), in comparison to HDI431. This suggests the presence of a hard segment dependent response, possibly related to the strong polar chemistry contained within the hard segment, since polar groups have been shown to influence both degradation and adhesion [6].

The comparable results between ARIST influence on U937 cell-mediated ^{14}C -PCNU degradation and inhibition of sPLA₂ protein expression without non-specific effects on cPLA₂ expression indicate that ARIST inhibition of ^{14}C -PCNU degradation was indeed via sPLA₂ down-regulation. It is important to note that although ARIST inhibited U937 cell-mediated PCNU degradation, this was not due to the enzymatic activity of sPLA₂ on the material itself. No PCNU degradation, as measured by radiolabel release, occurred when sPLA₂ from bovine liver was incubated with ^{14}C -PCNUs (data not shown). It is unclear what the inhibitory effect of ARIST on sPLA₂ expression had on the macrophage response to the PCNU surfaces, since sPLA₂ expression was unchanged in U937 cells adherent to PCNUs (Figure 2). Although ARIST is generally classified as inhibiting sPLA₂, at the concentration used in this study [5, 20] it may be a more general inhibitor of PLA₂ activity. In a study where monocyte chemotaxis to MCP-1 was assessed in the presence of 100 μM ARIST, >80% inhibition was observed [33]. This process was then shown to involve cPLA₂ and iPLA₂ activity [33].

Effect of PCNU materials on intracellular cPLA₂ and sPLA₂ protein expression

As illustrated in Figure 2, each model PCNU stimulated an increase in cPLA₂ protein expression with no significant effect on intracellular sPLA₂ protein expression. In addition, sPLA₂ protein could not be detected in conditioned media by immunoblot analysis (data not shown). Although sPLA₂ is typically a protein that is secreted in response to stimulation, sPLA₂ has also been indicated to be internalized via a PLA₂ receptor shown to be located on the plasma membrane [34, 35], therefore potentially contributing again to the intracellular pool of sPLA₂ protein.

PCNU materials induce early changes in cPLA₂ protein expression

It is possible that the lack of induced expression of sPLA₂ protein observed at 2 hours (Figure 2) could have been due to an early burst of AA release occurring within minutes of cell attachment to the PCNU surface [5]. At 2 hours, there was no significant difference in the altered cPLA₂ protein expression in the lysates from cells on each model PCNU material (Figure 2B). Therefore further experiments exploring earlier time points of PLA₂ protein expression were only carried out for one PCNU (HDI431) and TCPS. HDI431 has been shown to be the most biodegradable model PCNU [13] in addition to inducing the greatest amount of macrophage fusion and multinucleation [16], therefore likely to be the model PCNU able to initiate the greatest inflammatory response. The significant induction of cPLA₂ protein expression that was detected at 30 minutes post cell attachment to HDI431 versus TCPS (Figure 3) that then diminishes again until 2 hours can be explained by an immediate and delayed AA release response that has been reported in response to cell activation [23]. This effect has been suggested to be mainly initiated by cPLA₂ and possibly sustained or enhanced by sPLA₂ [21]. Regardless, these results have clearly demonstrated a role for cPLA₂ in the inflammatory pathways induced in the foreign body response to PCNU materials.

cPLA₂ location was altered by U937 cell adherence to a PCNU surface

The role of cPLA₂, upon cell activation, is to hydrolyze membrane phospholipids to release AA as an initial precursor to several potent inflammatory mediators. cPLA₂ requires phosphorylation for activation in addition to possessing a requirement for μM levels of Ca^{2+} necessary for translocation to cell membranes [36]. Under resting

conditions cPLA₂ is typically constitutively expressed and located throughout the cytoplasm [37], as was indicated in TCPS adherent U937 cells (Figure 4A and 4C). Although not confirmed through co-localization to cytosolic compartments, upon adherence to PCNU (HDI431), cPLA₂ appeared to localize to the edges of the cytoplasm perhaps clustering towards the plasma membrane (Figure 4B and 4D). This would allow access to cell membrane phospholipids for AA release as has been extensively reported on [5, 38, 39]. Previous studies assessing various forms of cell activation have indicated localization of cPLA₂ to the plasma membrane [40, 41], nuclear envelope [37, 42], endoplasmic reticulum [37], in addition to the golgi apparatus [43].

CONCLUSIONS

The current study has provided evidence for the first time that signaling via cPLA₂ rather than sPLA₂, occurred when macrophages, cells pivotal to the innate immune response, adhere to PCNU surfaces. There was an increase in the intracellular expression of cPLA₂ that was time dependent along with a translocation of cPLA₂ that appeared to approach the plasma membrane where the hydrolysis of membrane phospholipids via PLA₂ enzymes would release AA, a potent inflammatory mediator of the innate immune response. Understanding the macrophage response at the cell-material interface is becoming increasingly important whether PCNU materials are used for long-term implants, drug delivery systems or regenerative medicine techniques. The initiation of an inflammatory response can result in negative and even harmful effects to the surrounding tissue and alter the desired outcome.

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4.0 MANUSCRIPT #3

The human macrophage response during differentiation and biodegradation on polycarbonate-based polyurethanes: Dependence on hard segment chemistry

STATEMENT OF AUTHOR CONTRIBUTIONS

Dr. Rosalind S. Labow is the primary author of this manuscript. **Danne Sa** performed biodegradation (Figure 1) and esterase activity (Figure 2) assays for cells differentiated on various PCNU surfaces. **Loren A. Matheson** performed immunoblotting for this manuscript (Figure 3). **Donna Lee M. Dinnes** performed the biodegradation and esterase activity assays with the biodegradation products (Figure 4). **Dr. J. Paul Santerre** is a co-investigator of Dr. Rosalind S. Labow, and principal investigator on the CIHR operating grant that funded this research. In addition, he has contributed to the editing and suggestions in the preparation of this manuscript. Dr. Santerre's Polymer Chemistry laboratory at University of Toronto synthesized the model PCNU materials used for the following studies.

SUMMARY

The following manuscript "The human macrophage response during differentiation and biodegradation on polycarbonate-based polyurethanes: Dependence on hard segment chemistry" was recently published in *Biomaterials* [(2005) 26:7357-7366]. This work is a result of the collective efforts from multiple members of the Labow research group. The rationale for this study comes from the fact that in an *in vivo* situation, monocytes that are recruited to the biomaterial interface will arrive at this surface, adhere and

differentiate into macrophages upon this surface. It is expected then that this surface will influence the phenotype of this macrophage. In the *in vitro* model used by Labow and Santerre, monocytes are differentiated into mature macrophages by adherence to TCPS, and subsequently trypsinized to be used for further experiments assessing the effect of these macrophages on model PCNU materials. The aim of the current study was to characterize the ensuing degradative capacity and esterase expression pattern of these macrophages that were differentiated on each of the model PCNU surfaces, as opposed to differentiation on TCPS only. In addition to these studies, the following manuscript contains data from experiments determining the effects of PCNU biodegradation products (see Section 1.3.3.3) on MDM-mediated biodegradation. Experiments not published detailing the effect of the PCNU biodegradation product MDA on MSE protein expression are outlined in Appendix V.

The human macrophage response during differentiation and biodegradation on polycarbonate-based polyurethanes: Dependence on hard segment chemistry

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Key words: macrophages, polyurethanes, enzymes, hard segment, biodegradation

ABSTRACT

Human monocytes, isolated from whole blood, were seeded onto tissue culture grade polystyrene (PS) and three polycarbonate-based polyurethanes (PCNUs) (synthesized with either 1,6-hexane diisocyanate (HDI) or 4,4'-methylene bis-phenyl diisocyanate (MDI), poly (1,6-hexyl 1,2-ethyl carbonate) diol (PCN) and 1,4-butanediol (BD) in different stoichiometric ratios (HDI:PCN:BD 4:3:1 or 3:2:1 and MDI:PCN:BD 3:2:1, (referred to as HDI431, HDI321 and MDI321 respectively). Following their differentiation to monocyte-derived macrophages (MDM) the cells were trypsinized and re-seeded onto each of the PCNUs synthesized with either ^{14}C -HDI or ^{14}C -BD and degradation was measured by radiolabel release (RR). When the differentiation surface was MDI321, there was more RR from ^{14}C -HDI431 than from any other surface ($p < 0.0001$) whereas the amount of esterase (identified by immunoblotting) as well as the esterase activity was the greatest in MDM differentiated on PS, re-seeded on ^{14}C -HDI431 ($p < 0.0001$). The effect of potential degradation products (methylene dianiline (MDA) and BD) from the PCNUs was carried out to determine possible links between products and substrate-induced activation of MDM. MDA was found to inhibit RR 60% from MDM seeded on ^{14}C -MDI321B ($p < 0.0001$), ~20% from ^{14}C -HDI431 ($p = 0.002$) and no effect from ^{14}C -HDI321B. MDA inhibited esterase activity 30% from MDM only on ^{14}C -MDI321B ($p = 0.003$), but no effect on esterase activity was observed for the other two polymers. BD had no inhibitory effect on RR from any PCNU, but did inhibit esterase activity in MDM on ^{14}C -HDI431 ($p = 0.025$). This study indicates that the degradation of a specific material is a multi-factorial process, dictated by its susceptibility to hydrolysis, the effect of specific products generated during this course of action, and perhaps not as

well appreciated, the material's inherent ability to influence enzyme synthesis and release.

INTRODUCTION

Both biocompatibility and biostability influence the ultimate fate of an implanted medical device. When monocytes arrive at the material surface, differentiation to monocyte-derived macrophages (MDM) is initiated due to the foreign body response. MDM are the most abundant white cell type found on explanted medical devices, secreting many hydrolytic enzymes as well as oxidants [1] which may lead to biodegradation. The effect of the material surface on the differentiation to MDM and their degree of activation (i.e. biocompatibility) [2], followed by the extent of degradation of the material (biostability) [3, 4] was assessed previously.

In previous work, monocytes, isolated from whole blood, were allowed to differentiate on three material surfaces, tissue culture grade polystyrene (PS), a polyester-urea-urethane (PESU) and a polyether-urea-urethane (PEUU) for up to 1 month. Significantly more esterase activity per cell was detected in the lysates after 1 week of differentiation on the PEUU surface when compared to PS and PESU. However, biodegradation of the polyurethanes (PU)s was not assessed in that study [2].

Earlier studies have shown that esterolytic activity is the most degradative towards a variety of PUs when compared to other hydrolytic activities [5, 6] and that PUs differed greatly in their biostability to commercially available cholesterol esterase (CE). PESUs [7] were much more degradable than PEUUs [8] as would be expected. Although polycarbonate-based PUs (PCNU)s were hydrolytically degraded by CE, they differed greatly in their relative biostability. When the stoichiometric ratios of hexane

diisocyanate (HDI): poly(1,6-hexyl 1,2-ethyl carbonate) diol (PCN):butanediol (BD) were varied, the amount of cumulative radiolabel release was greater for HDI:PCN:BD in the ratio 4:3:1 (^{14}C -HDI431) when compared to HDI:PCN:BD in the ratio of 3:2:1 (^{14}C -HDI321B), but this was observed beyond the first week of incubation with CE [9]. In addition, there was a very strong dependence on enzyme concentration required to achieve comparable degradation from polymers that differed in their hard segment chemistry, although the nature of the hydrolysable PCN segment remained the same [10]. The difference in degradability was even greater when the diisocyanate chemistry was varied as opposed to the hard segment size. When 4,4'-methylene bis-phenyl diisocyanate (MDI) was used instead of HDI, ^{14}C -MDI321B was 2.5-3 times less degradable by CE than ^{14}C -HDI321B, when assessed over the course of 8-10 weeks [11].

Esterases of different specificities have been identified in MDM. Li and Hui observed that in addition to lysosomal cholesterol esterase (CE) another secreted CE exists in MDM which is identical to pancreatic CE and as yet has no known function in MDM [12]. Another esterase in MDM is known as monocyte specific esterase (MSE). This esterase is also referred to as carboxyl esterase (CXE) depending on its tissue location. More commonly it is called non-specific esterase since it has no known natural substrate. It does however, have a distinctly different substrate specificity from CE, preferring short chain fatty acids and organometallic esters, with no activity towards cholesteryl oleate, the physiological substrate of CE. One hypothesis proposes that this enzyme acts as a detoxification mechanism for certain environmental agents [13]. However, this enzyme

is increased in certain pathological states and is absent in others suggesting that it plays a role in the inflammatory response.

In a recent study, it was shown that both CE and MSE were synthesized *de novo* in MDM on HDI431 by incorporation of ³⁵S-methionine, followed by immunoprecipitation with anti-CE and anti-CXE (reacts with MSE) antibodies [14]. In addition, the amount of each esterase in the conditioned media of MDM attached to the different material surfaces was determined by immunoblotting analysis following PAGE. Significantly more MSE was detected in the conditioned media of MDM reseeded on ¹⁴C-HDI431 than either PS or ¹⁴C-MDI321B. Moreover, there was much less esterase activity in the MDM attached to ¹⁴C-MDI321B along with less degradation of this PCNU [14]. These findings indicate that small changes in the biomaterial substrates dictate important changes in cellular responses, which direct surface biodegradation.

In this study, the response of monocytes and fully differentiated MDM to polyurethanes, which had different hard segment composition was assessed. In order to determine the effect of the polymer chemistry on both differentiation and activation after re-seeding, which is hypothesized to influence the extent of biodegradation, the monocytes were allowed to differentiate on PS and three different PCNU surfaces, followed by re-seeding onto any of the three PCNUs which had a radiolabel incorporated into their chemical structure. These PCNUs differed in their stoichiometry (HDI431 versus HDI321) as well as their diisocyanate chemistry (HDI321 versus MDI321). Both radiolabel release and esterase activity was measured. The amount of the esterases in the cell lysates was also

determined by immunoblotting analysis with antibodies raised against CE and CXE. In addition, since the potential MDM degradation products could differ from one substrate to another, based on the results obtained by degradation with CE [10], the effect of some of these products on radiolabel release and esterase activity was also assessed in order to determine how differences in biostability and biocompatibility of PCNUs may be related to polymer degradation product feedback loops or cytotoxicity of the degradation products.

MATERIALS AND METHODS

Materials

Unless otherwise specified all reagents were purchased from Sigma Chemical Company, St. Louis, MO.

Polymer synthesis

The diisocyanates used for the synthesis of the PCNUs (as described in detail previously) [9, 11] were HDI and MDI, (Aldrich, Milwaukee, WI, USA). The same chain extender and soft segment were used for both PCNUs and were BD (Aldrich, Milwaukee, WI, USA) and PCN (MW=1000) (Aldrich, Milwaukee, WI, USA). The stoichiometric ratios and acronyms for the PCNUs are given in Table 1 along with the specific radioactivities and the location of the ^{14}C radiolabel incorporated into the PU structure. Molecular weight values were in the range of $5\text{-}8 \times 10^4$ and similar to those previously reported [11].

Table 1

Reagent	Stoichiometry	Acronym	MW	CPM/100 mg
MDI/PCN/BD	3:2:1	^a ¹⁴ C-MDI321B	7.0 x 10 ⁴	0.73 x 10 ⁷
HDI/PCN/BD	3:2:1	^a ¹⁴ C-HDI321B	7.6 x 10 ⁴	0.79 x 10 ⁷
HDI/PCN/BD	4:3:1	^b ¹⁴ C-HDI431	7.8 x 10 ⁴	1.14 x 10 ⁷

Abbreviations: Diisocyanate: Methylene bis-*p*-phenyl diisocyanate (MDI), 1,6-Hexane diisocyanate (HDI); Macroglycoldiol soft segment: Poly (1,6-hexyl 1,2 ethyl carbonate) (PCN)(MW 1000); Diol chain extender: 1,4-butanediol (BD) (MW 90). For some experiments where ¹⁴C-labels were not required, the non-labeled analog was used and referred to without the ¹⁴C in the acronym. All radiolabel release data were normalized to the specific radioactivity of HDI431.

^a ¹⁴C-BD used in the synthesis

^b ¹⁴C-HDI used in the synthesis

The PCNUs were dissolved overnight in a solution of dimethylacetamide (DMAC) (Aldrich, Milwaukee, WI, USA) to create a 10% (w/v) solution. The polymer solutions were then passed through a 0.45 μm Teflon™ filter (Chromatographic Specialties, Toronto, ON) in order to remove any non-solubles that might be present. Round glass coverslips (15 or 22 mm diameter depending on the experiment (Fisher Scientific, Ottawa, ON)) were coated with the polymer solutions (100 or 200 μL) under sterile conditions in a laminar flow hood and dried overnight at 50°C, followed by purging at 50°C without vacuum for 24 hours and then under vacuum for 72 hours as described in detail previously [3]. Prior to experimentation the slips were placed in wells and hydrated with Dulbecco's Phosphate Buffered Saline (DPBS) for 24 hours. The radiolabel release data were normalized to HDI431 since the batchwise product of PCNUs yielded slightly different specific radioactivities (Table 1). When incubating the PCNUs with CE, it was previously found that the position of the radiolabel did not affect relative radiolabel release if these data were normalized [15].

Preparation of cell lysates

Isolation and culture of monocytes

Monocytes were isolated from the whole blood (120 mL per isolation) of healthy volunteers with the approval of the Research Ethics Committee of the University of Ottawa Institute (UOHI 91-102 protocol number) as described in detail previously [3]. Whole blood was collected in EDTA-containing Vacutainers™ (VWR) and was layered onto Histopaque 1077. The mononuclear cells (~20% monocytes) were then seeded at a concentration of 5×10^6 cells/mL on tissue culture grade polystyrene (PS) or HDI431,

HDI321 or MDI321 coated slips and allowed to differentiate with feeding every other day with 2.0 mL of media containing 10% fetal bovine serum (FBS) and 0.69 mM L-glutamine in RPMI. Monocyte differentiation was carried out on 12 well PS tissue culture plates or 22 mm diameter coated slips placed in the wells of a 12 well plate. After 2 weeks the mature MDM were gently trypsinized by treating the adherent cells with 0.25% trypsin-EDTA for 5 minutes and the reaction stopped by adding Hanks buffer containing 5% FBS. The trypsinized cells were collected and centrifuged at 450 x g with the entire process taking no longer than 15 minutes. Following this, the cells were resuspended (1×10^6 /mL in media containing 10% FBS) and re-seeded onto the ^{14}C -PCNU-coated slips (in this case 15 mm diameter) in 24 well tissue culture plates for 1 hour [3]. After this period, referred to as time = 0, fresh medium was added to the remaining wells, and the cells were incubated for 5 days which would accumulate enough esterase activity to potentially observe significant differences between surfaces. For each condition, representative wells for the experimental group were selected and the amount of adherent cells was determined by lysing the cells with a 0.05% Triton X-100-EDTA solution and determining the DNA content as described in detail below.

Radiolabel release and esterase activity in the cell systems

The cell supernatant (600 μL) was removed and counted for radioactivity on day 3 and day 5 of incubation and the cumulative counts calculated for the 5 days of incubation and normalized to the average DNA values from time = 0 and 5 days. The DNA content of the remaining adherent cells was determined on day 5 by lysing the cells with 0.05% Triton X-100 (240 μL). The lysate (50 μL) was assayed for esterase activity using *p*-

nitrophenylbutyrate (PNB) and DNA content as described in detail below. The latter were normalized to 10 µg DNA in the lysate.

Determination of DNA

The amount of DNA present in the 0.05% Triton X-100 lysate was determined by a modified method described in detail previously [2]. Briefly, Hoechst dye # 33258 (Amersham)(1 mg/mL) (10 µL) was diluted with TRIS buffer (0.01M Tris, 0.001 M EDTA, 0.2 M NaCl), pH 7.4 (10 mL) on the day of analysis. Cell lysate (15 µL) was added to the dye (100 µL) into a 96 well plate (Microfluor 2 Black; VWR, Mississauga, Ontario) and read against a DNA standard (100-300 ng DNA) (prepared with the same amount of Triton X-100 which was in the lysate sample) in a fluorescence microplate reader (POLARstar Galaxy, BMG Labtechnologies).

Gel Electrophoresis and immunoblotting analysis

Cell lysate protein samples from twelve conditions (four differentiation surfaces (PS, HDI431, HDI321, MDI321) in combination with the three re-seeding surfaces (¹⁴C-HDI431, ¹⁴C-HDI321B, ¹⁴C-MDI321B) were separated by SDS-PAGE using a Protean II cell (Bio-Rad Laboratories, Mississauga, ON) on a 12% polyacrylamide gel according to established methods [16]. Since MDMs are terminally differentiated cells, protein was loaded on the gel according to a standard amount of DNA. The separated proteins were transferred to a nitrocellulose membrane overnight at 30V. The membrane was then blocked for 1 hour in Tris-buffered saline (TBST: 10mM Tris, 0.1 M NaCl, and 0.1% Tween-20, pH 7.5) containing 5% non-fat dry milk or 5% gelatin (blocking buffer)

depending on the antibody. The nitrocellulose was incubated with either a polyclonal rabbit anti-porcine esterase (Rockland, Gilbertsville, PA) (referred to as anti-CXE, which cross-reacts with most mammalian forms of the enzyme) or a polyclonal rabbit anti-human CE serum (a gift from Dr. D. Hui, University of Cincinnati College of Medicine, Cincinnati, Ohio) (reacts with CE) for 1 hour and washed eight times with TBST. The membranes were then incubated with goat anti-rabbit IgG (Rockland, Gilbertsville, PA) conjugated to horseradish peroxidase for 1 hour and washed eight times with TBST. Protein bands were then visualized using an enhanced chemiluminescence detection kit (Pierce, Rockford, IL). The bands in the immunoblots were quantified with ImageQuant TL[®] (Amersham Biosciences).

Enzyme Assay for Esterase Activity

The cell lysates were assayed according to a modification of the procedure of Moore *et al* [17]. Sodium phosphate buffer (0.05 M), pH 7.0 (1.4 mL) and PNB solution (4 mM in acetonitrile, 50 μ L) were mixed with 50 μ L of the cell lysate prepared as described above. This solution was incubated at 37°C for 30 minutes and read in a spectrophotometer at 400.6 nm. One unit of activity was defined as the release of 1 nmol of *p*-nitrophenol ($\epsilon = 16,300 \text{ cm}^{-1}\text{M}^{-1}$) per minute at 37°C.

Determination of radiolabel release

After incubating the ¹⁴C-PCNU-coated slips for 24 hrs with the enzyme solutions or buffer only controls, 600 μ L of solution were collected from each of the wells and added to 10 mL of liquid scintillation cocktail (Formula-989, Canberra-Packard Instrument Co.

Inc., Meriden, CT) and counted in a liquid scintillation counter (LKB Wallac 1219 RackBeta, Gaithersburg, MD) to measure radiolabel release, as described in detail previously [3].

Effect of PCNU hydrolysis products

Some of the low molecular weight hydrolysis products, that could be purchased commercially and which were found to be generated by enzyme activity associated with cell-mediated biodegradation from HDI431, ^{14}C -HDI321B and ^{14}C -MDI321B were assessed for their effect on radiolabel release and esterase activity [18]. These were hexane diamine (HDA), methylene dianiline (MDA) and BD. Monocytes isolated as described above were differentiated for 14 days on PS, trypsinized and re-seeded onto ^{14}C -HDI431, ^{14}C -HDI321B and ^{14}C -MDI321B coated glass slips. HDA was dissolved in water, and MDA and BD were dissolved in ethanol at concentrations ranging from 25-250 $\mu\text{g}/\text{mL}$. The products were added to the MDM cell system following 1 hr of cell attachment to the material surface and incubated for 48 hrs. Although the amount of product to be added was empirical, an attempt was made to calculate theoretical amounts based on the amount of radiolabel release measured as a percent of the total polymer coated on the slip. For example, in the case of MDI321, 10 mg were coated on each glass coverslip. Based on the specific radioactivity of 0.34×10^7 CPM/100 mg, 340000 CPM would represent 100% hydrolysis. The MDM mediated radiolabel release from MDI321 only generated 1000 CPM ($\sim 0.3\%$ or 3000 μg). However, much less MDA was generated from the CE hydrolysis as was shown recently [18], so a concentration response was started at a much lower amount. The volume of ethanol required to solubilize MDA and BD was the limiting factor since a concentration of ethanol above

1% was cytotoxic. Radiolabel release and esterase activity were measured and normalized to 10 µg DNA in the adherent cells as described in detail above.

Statistical analysis

The effect of differentiation surface on re-seeding surface for both radiolabel release and esterase activity was analyzed by a 2-way ANOVA. The p-value for significance (< 0.05) was adjusted for multiple comparisons. Since there were 12 groups (Table 2), the p-value for significance for the analysis was 0.0045 ($0.05 \div n-1$ or 11). The data from the immunoblot analyses were compared for each differentiation surface separately using the raw data for the band intensity. For these analyses, the p-value was ($0.05 \div n-1$ or 2) or 0.025 using a 1-way ANOVA. In order to compare the effect of all differentiation surfaces, the band intensities were normalized to HDI431 as the re-seeding surface and then analyzed using a 2-way ANOVA ($n=11$, with a p-value = 0.0045). For the effect of products each PCNU was analyzed separately for 3 groups (media, MDA and BD), with a p-value for significance of 0.025. All analyses were performed using SAS[®] version 8.2.

RESULTS

Effect of the differentiating surfaces on biodegradation

The surface that the monocytes were differentiated on significantly affected the subsequent radiolabel release that was measured from the surface onto which the fully differentiated MDM were re-seeded (Figure 1). When analyzing all the data (i.e. the four differentiation surfaces as well as the 3 re-seeding surfaces) by a 2-way ANOVA there was a significant statistical interaction between differentiation surface and re-seeding

Table 2

Group Number	Differentiation surface	Reseeding surface
1	PS	¹⁴ C-HDI431
2	PS	¹⁴ C-HDI321B
3	PS	¹⁴ C-MDI321B
4	HDI431	¹⁴ C-HDI431
5	HDI431	¹⁴ C-HDI321B
6	HDI431	¹⁴ C-MDI321B
7	HDI321	¹⁴ C-HDI431
8	HDI321	¹⁴ C-HDI321B
9	HDI321	¹⁴ C-MDI321B
10	MDI321	¹⁴ C-HDI431
11	MDI321	¹⁴ C-HDI321B
12	MDI321	¹⁴ C-MDI321B

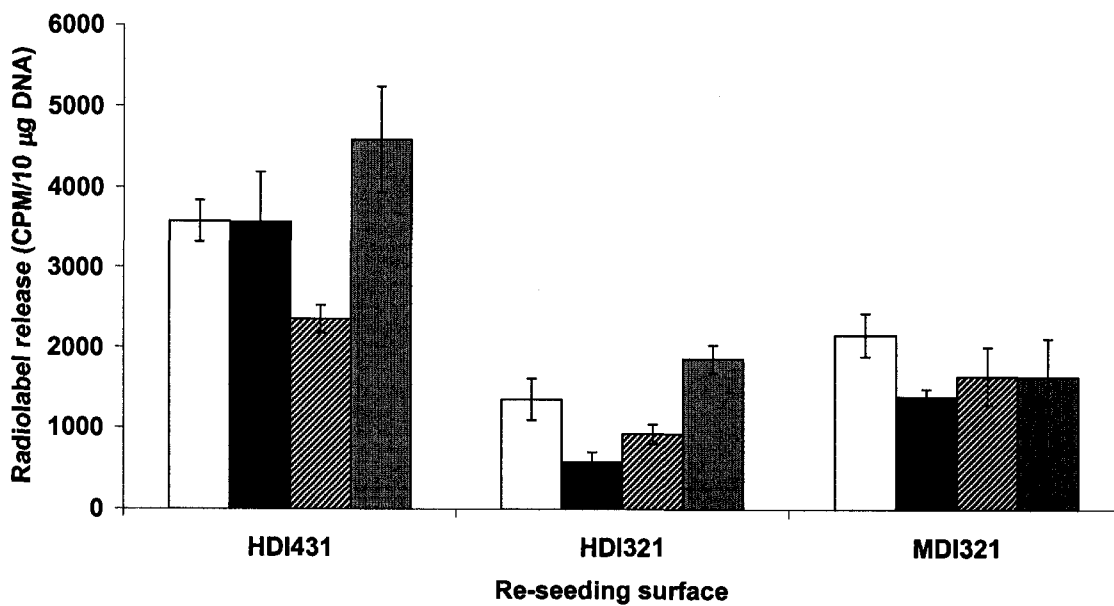


Figure 1 - Effect of differentiation surface on radiolabel release. Monocytes were differentiated on PS (white), HDI431 (black), HDI321 (diagonal), MDI321 (gray) for 14 days and re-seeded on radiolabeled ^{14}C -HDI431, ^{14}C -HDI321B and ^{14}C -MDI321B. Radiolabel release was measured 5 days later and plotted for each differentiation surface normalized to 10µg DNA as described in **Materials and Methods**.

surface ($p < 0.0001$). The most degradable surface (i.e. the reseeded surface) was ^{14}C -HDI431 (approximately 3-4 fold greater than most of the other surfaces) except when the MDM were differentiated on HDI321 and reseeded on HDI431 where differences with other substrates was not always significant.

Effect of the differentiating surfaces on esterases

When analyzing the effect of the differentiation surface on the esterase activity in the lysates from the MDM that had been reseeded onto the three PCNU surfaces, there was also an interaction between differentiation surface and re-seeding surface. This time it was PS as the differentiation surface, which elicited the most esterase activity from the MDM lysates that had been reseeded on the ^{14}C -HDI431 surface ($p < 0.0001$)(Figure 2).

In addition to assaying esterase activity, the amount of esterase protein was investigated for the conditions that were described in Figures 1 and 2. In order to compare all the differentiation-reseeding combinations (Table 2), it was necessary to normalize the amount of MSE and CE to one surface (See explanation in *Statistical Analysis* above).

Immunoblotting was carried out with the lysates of the MDM differentiated on the four surfaces (PS, HDI431, HDI321, MDI321), after a 5 day incubation period following re-seeding on the three surfaces (^{14}C -HDI431, ^{14}C -HDI321B, ^{14}C -MDI321B) (Figure 3), using anti-CXE (Figure 3A) and anti-CE (Figure 3B). In order to quantify the relative amount of MSE in the lysates following PAGE, all the bands visualized in each individual lane were added together and the banding intensity between lanes was

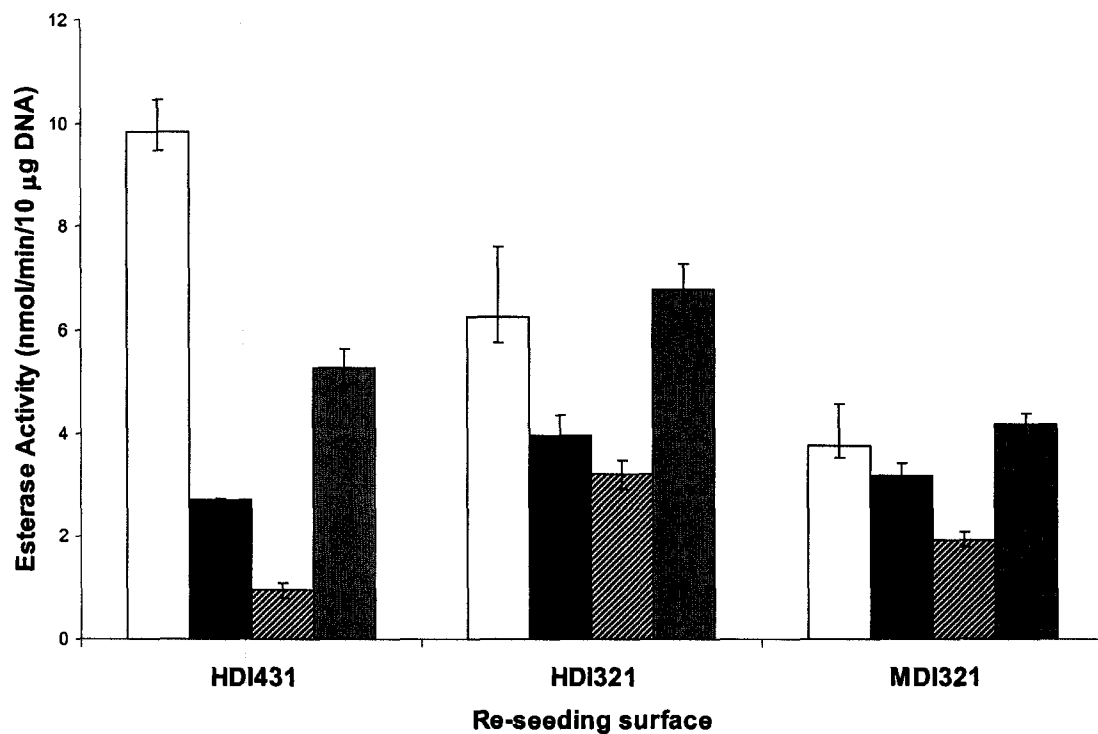
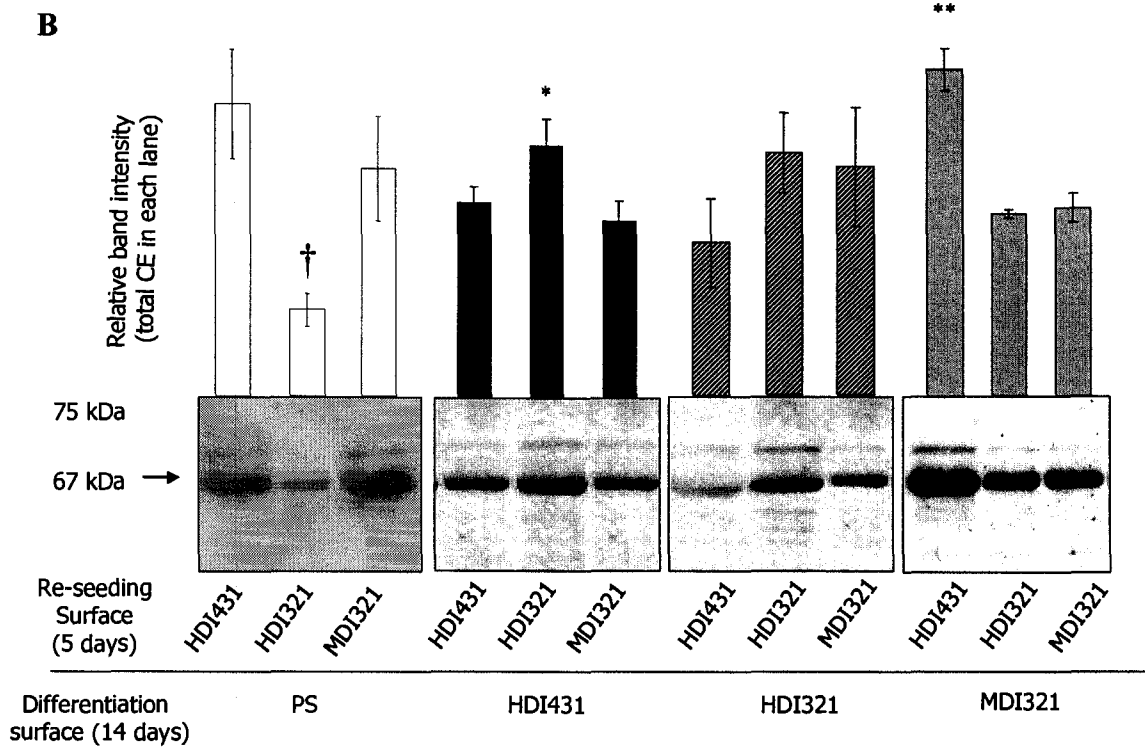
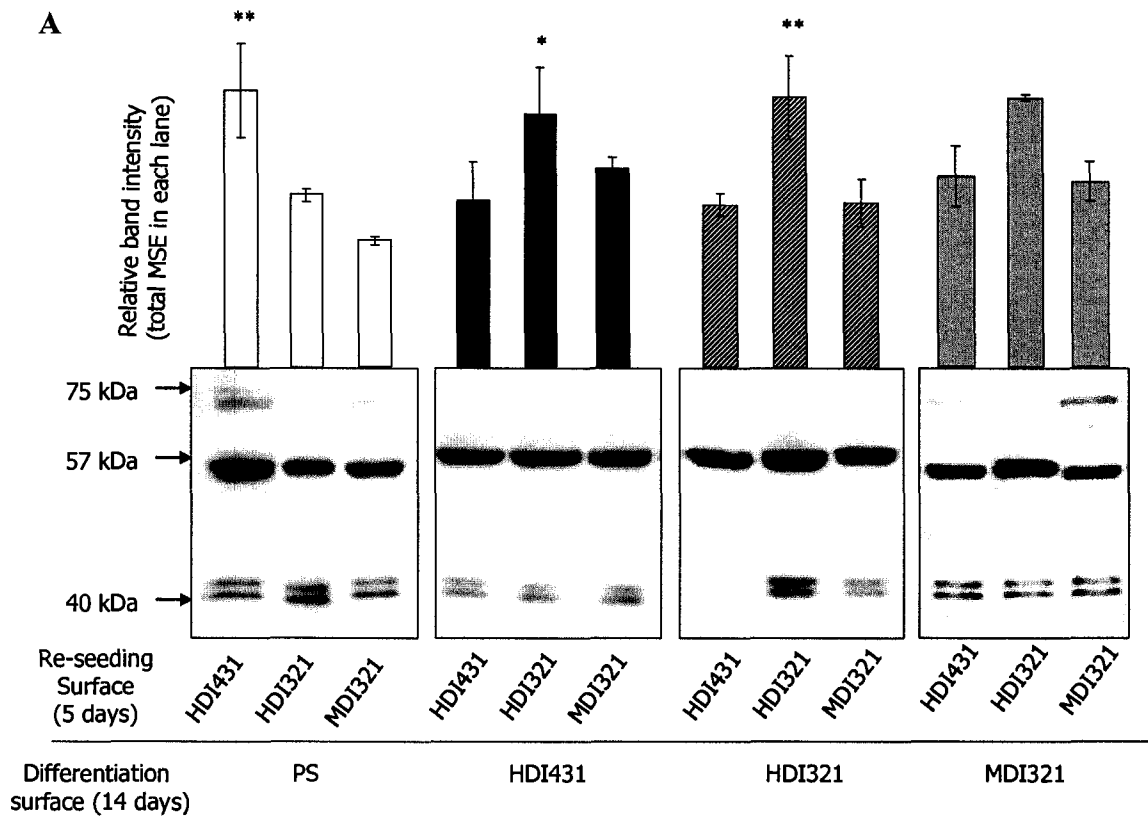


Figure 2 - Effect of differentiation surface on the esterase activity in MDM lysates. Monocytes were differentiated on PS (white), HDI431 (black), HDI321 (diagonal), MDI321 (gray) for 14 days and re-seeded on radiolabeled ^{14}C -HDI431, ^{14}C -HDI321B and ^{14}C -MDI321B. Esterase activity was measured 5 days later using *p*-nitrophenylbutyrate and plotted for each differentiation surface normalized to 10µg DNA as described in **Materials and Methods**.

Figure 3 - Effect of differentiation surface on the amount of monocyte-specific esterase and cholesterol esterase detected by immunoblotting analysis of MDM lysates. The amount of monocyte specific esterase (MSE) (A) and cholesterol esterase (CE) (B) in MDM lysates was determined by immunoblotting analysis using antibodies to CE and CXE following polyacrylamide gel electrophoresis (PAGE) of lysates prepared from cells differentiated on PS (white), HDI431 (black), HDI321 (diagonal), MDI321 (gray) for 14 days and re-seeded on ¹⁴C-HDI431, ¹⁴C-HDI321B and ¹⁴C-MDI321B for 5 days. * indicates significantly greater than 1 surface; ** indicates significantly greater than the other 2 surfaces; † indicates significantly lower than the other 2 surfaces.



compared. This was considered to be representative of the total amount of each esterase since MSE [19] and CE [20] exist in many isoforms. The highest level of MSE was detected when the monocytes were differentiated on PS and re-seeded on ^{14}C -HDI431 rather than on the other two reseeding surfaces. This was the same trend observed for the total esterase activity when cells were differentiated on PS (white bars on the three reseeded surfaces in Figure 2). Both MSE and total protein showed a positive correlation coefficient of 1.000 ($p < 0.0001$) for the latter condition. Likewise, the other three differentiation surfaces showed a strong positive correlation for MSE to total esterase activity in Figure 2.

An entirely different pattern was observed for the immunoblots analyzed with the anti-CE antibody (Figure 3B). When the monocytes were differentiated on PS, there was less CE detected when MDM were re-seeded on ^{14}C -HDI321B than either of the other two reseeding surfaces. This correlated well with the findings in Figure 1, which showed that when PS was the differentiating surface, HDI321 showed the lowest CPM values of the three polymers. When the differentiation surface was HDI431, only HDI321 as the reseeding surface showed greater CE than MDI321. The amount of CE observed when MDM were differentiated on MDI321 and reseeded onto HDI431 was greater than either of the other two reseeding surfaces.

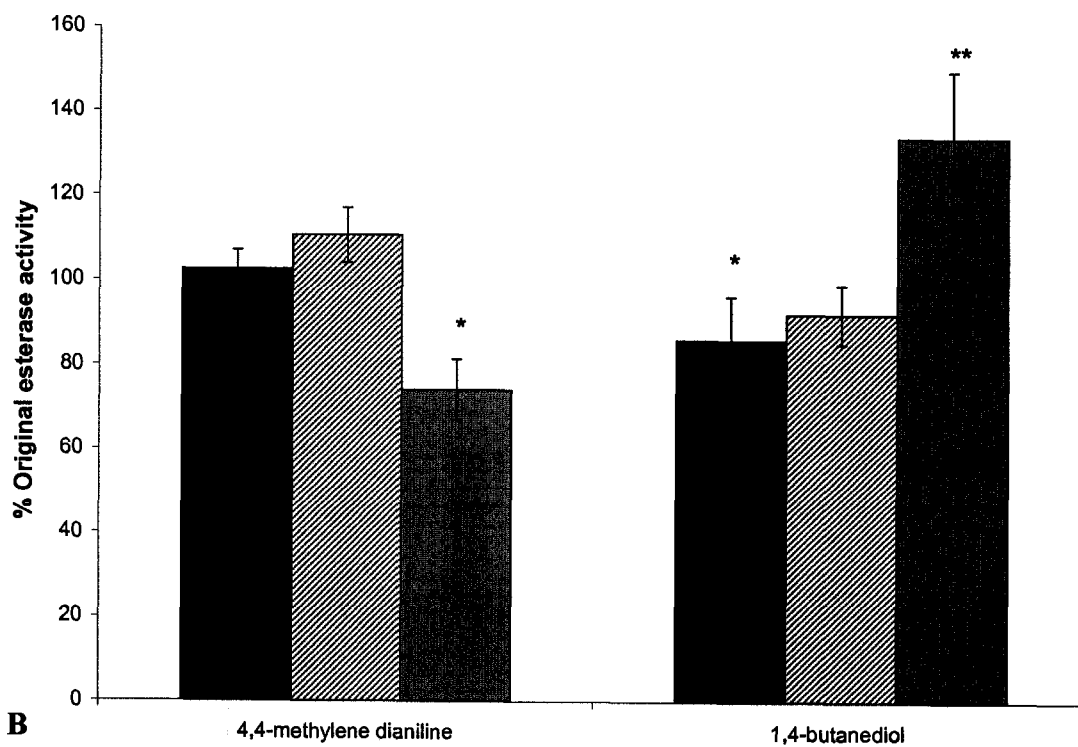
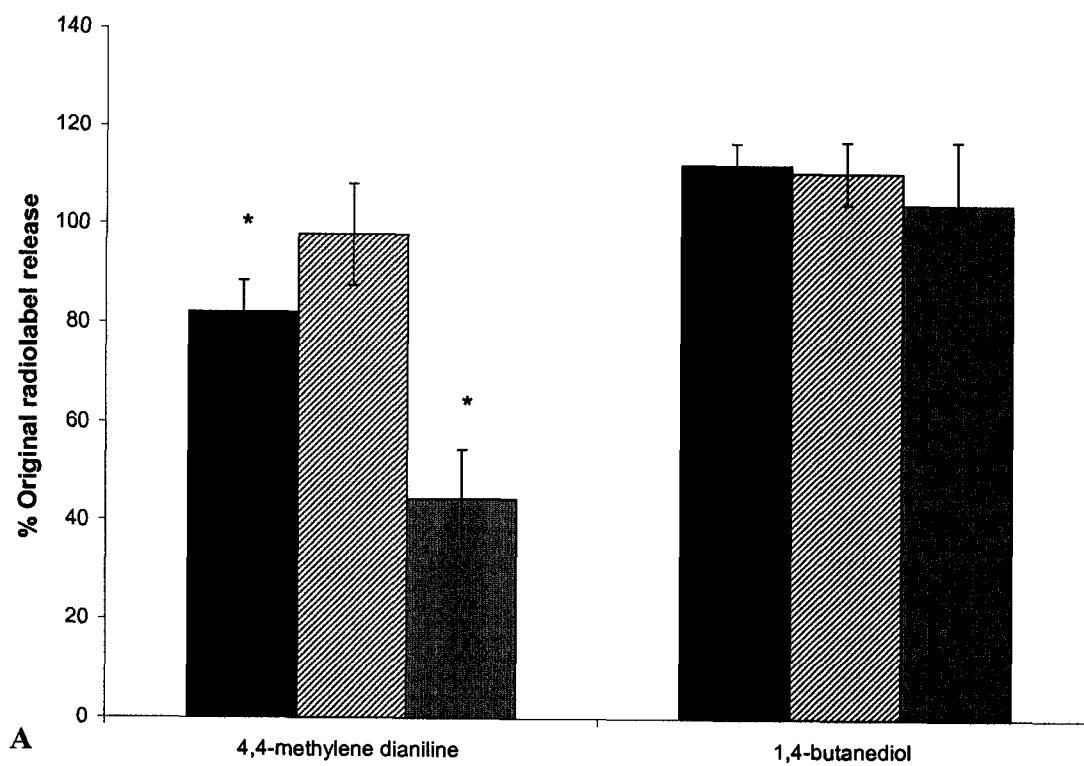
Effect of degradation products

When assessing differentiation surface effects, it must be considered that PCNU surfaces are degradable by MDM and PS is not. Given the particular sensitivity of MDI321

towards the promotion of hydrolysis on the other two surfaces it was of interest to study degradation products associated with this polymer. Although studies investigating products resulting from the incubation of cells with the polyurethanes from cells have not yet been carried out, there were many degradation products identified from the digestion of ^{14}C -MDI321B by pure CE, ranging in MW from 90 (BD) to >800 kDa and including MDA and BD [18]. Although neither HDA nor BD was detected from the CE hydrolysis of ^{14}C -HDI321B, oligomer fragments containing both of these moieties have been identified [18]. Therefore, the simplest and most readily available products (HDA, BD and MDA) were assessed for their effect on esterase and radiolabel release (Figure 4).

When cells were differentiated on PS and then reseeded onto the three different PCNUs, there was inhibition by MDA on both radiolabel release (60%) (Figure 4A) and esterase activity (30%) (Figure 4B) from MDM on MDI321. Although MDA could not be a product of the degradation from the HDI-PCNUs, it could be generated in the differentiating phase with MDI321 prior to re-seeding on HDI-PCNUs. Hence, the influence of MDA on HDI-PCNUs was also evaluated, in order to determine if MDA was having a cytotoxic effect or an effect on the pathway involved in esterase synthesis and secretion, rather than by product inhibition via a feedback loop. A small but significant inhibition of radiolabel release by MDA was observed from MDM on HDI431 (20%) (Figure 4A) with BD (25 $\mu\text{g}/\text{mL}$) having no significant effect on CPM values (Figure 4A). However, MDA had no effect on esterase activity for HDI431 but BD did show a small and significant inhibition (10%) for this activity (Figure 4B).

Figure 4 - Effect of degradation products on radiolabel release and esterase activity from PCNUs. Butanediol (BD) or 4,4'-methylene dianiline (MDA) was added to MDM re-seeded on ¹⁴C-HDI431 (black), ¹⁴C-HDI321B (diagonal), and ¹⁴C-MDI321B (gray). Radiolabel release (4A) and esterase activity (assayed with p-nitrophenylbutyrate)(4B) was measured after 48 hours of incubation as described in detail in **Materials and Methods** and the values expressed as a percent of values obtained from MDM incubated in media only (* indicates value of lower statistical significance; ** indicates value of higher statistical significance).



The concentration of MDA, which gave the maximum inhibitory effect on radiolabel release was 250 $\mu\text{g/mL}$ ($p < 0.0001$). Increasing the concentration to 500 $\mu\text{g/mL}$ did not increase the effect further and a significant decrease in cell viability occurred which was not evident at the lower concentrations (data not shown). This was true of the value for BD as well. Increasing the concentration beyond 25 $\mu\text{g/mL}$ had no further effect. The vehicle, ethanol, used for dissolving MDA and BD had no effect on radiolabel release or esterase activity when compared to MDM in media only.

DISCUSSION

Effect of the differentiating surfaces on biodegradation:

It is well established that different PUs (PESUs, PEUUs and PCNUs) [5, 8, 21] are degraded to various extents by the esterase activity found to be associated with MDM. Also, the effect of the material surface on the synthesis and secretion of potential degradative activities during the activation process of MDM has been shown [14]. However, until this study, the effect of the material surface on the monocyte differentiation process had not been investigated or appreciated by any group studying biodegradable polyurethanes. Therefore, in this study, the experimental set up was designed to assess the effect that differences in the material chemistry have on the monocyte differentiation process to mature MDM. The effects that the surface had on monocyte differentiation were assessed by assaying the MDM-mediated degradation following re-seeding and measuring the amount of esterase protein and activity generated. Although this model system was not an exact replica of the *in vivo* situation,

the findings using this approach are important and will affect the design of many future experiments.

The surface that the monocytes were differentiated on significantly affected the subsequent radiolabel release that was measured from the surface onto which the fully differentiated MDM were re-seeded. In other studies, ^{14}C -MDI321B was shown to be the least degradable by either MDM [14] or CE [11]. The MDM on MDI321 were more spread out and had fewer nuclei per cell than on HDI431 [14]. In addition, MDM secreted and synthesized more MSE when re-seeded onto HDI431 than either PS or MDI321 [14]. Based on these observations, MDI321 had appeared to be the more biocompatible surface. However, in the current study, when monocytes were differentiated on MDI321 (Figure 1), both ^{14}C -HDI431 and ^{14}C -HDI321B were degraded significantly more than when monocytes were differentiated on any of the other 3 surfaces. This implies that MDI321 has an activation effect on MDM, which promotes these cells to degrade vulnerable substrates. This may not only include biomaterials as shown in this study but also matrix tissues. There was no significant difference between any of the four differentiating surfaces when MDI321 itself was the surface reseeded with MDM (Figure 1), reflecting this latter material's relatively good biostability that has previously been reported on [11]. However, it should be noted that MDI321 is suspected of being susceptible to the activation of the respiratory burst by protein kinase C. Following the release of reactive oxygen species, MDI321 was readily degraded [22]. MDI's susceptibility to hydrolysis by esterase post-exposure to HOCl and H_2O_2 was further shown in previous work [15].

Although HDI321 was readily degraded by CE in previous work [10], it appeared that HDI321 as the reseeding surface in this study was relatively more stable to MDM-mediated degradation in comparison to HDI431 and in some cases MDI321 (Figure 1). In another study, MSE (known to be inhibited by NaF [19]) was the esterase activity in MDM, which appeared to be contributing more to HDI321 degradation as NaF (1.0 mM) inhibited the degradation observed by more than 50% ($p=0.005$). Given the known differences in the relative potential biodegradative activity between esterases [5], the differences in enzyme sensitivity may partially explain the differences between the CE study [10] versus the MDM data in the current study.

Previously, the stability of MDI321 relative to HDI431 was related to the extensive hydrogen-bonding of the urethane groups [9]. The physical structure of the MDI321 surface when compared to the HDI PCNUs showed remarkable differences by AFM when compared to the HDI PCNUs [21] and FTIR/ATR, suggesting the presence of more urethane (i.e. polar) moieties at the surface of MDI321 and increased phase mixing when compared to the other two HDI materials. In the current study it is hypothesized that these polar segments possibly contributed to the activation of the monocytes as they attached to the surface and differentiated to MDM as was described in a previous study [23]. Certainly in addition to the effects described above, the chemical nature of the material (i.e. aromatic versus linear aliphatic) and its susceptibility to hydrolysis must also be considered [9].

Effect of the differentiating surfaces on esterases

Assaying esterase activity with PNB only partially explains radiolabel release. There is no positive correlation between CPM measured and the PNB activity. Other studies suggest that in addition to PNB activation, alternative pathways contribute to biodegradation. Previously, phenylmethylsulfonyl fluoride, an esterase inhibitor, only inhibited radiolabel release by 50% [4, 24]. Moreover, in a recent study, it was found that MDI321 activated MDM to release reactive oxygen species. These agents also permitted radiolabel release to proceed by subsequent enzymatic hydrolysis [22].

Previous studies have suggested that esterase activity, in addition to being the most degradative to PUs [6], may indicate a foreign body reaction [19]. In general, the above information is an important factor to consider in culturing cells of many types, since it is often assumed that PS is biocompatible and therefore is presented as the control surface. Clearly, PS as a differentiating surface has an activating effect on cells, which should not be ignored.

As a result of these findings, it appears that on the differentiating material surfaces, which are highly activating (PS and MDI321) (Figures 1 and 2), it is CE protein that can be related to radiolabel release (CPM versus CE protein had correlation coefficients of 1.000, $p < 0.0001$ (i.e. compare trend for white bars in Figure 1 to white bars in Figure 3B for PS; and compare trend for gray bars in Figure 1 to gray bars in Figure 3B for MDI321). When trying to relate degradation (radiolabel release) to total esterase activity and the amount of each esterase, it is now apparent that material factors are involved

which influence cell function. It is difficult to determine which esterase activity is being assayed when PNB is being used since the substrate is non-specific and measures the activity of MSE and CE as well as proteins with esterolytic activity.

It has been found previously that both esterases were candidates for ^{14}C -HDI431 degradation based on the effects of specific reagents known to inhibit (sodium fluoride [19]) or stimulate (sodium taurocholate [25]) MSE and CE respectively [24]. In the current study it was observed that the MSE and CE activity resulting from cell differentiation on the two HDI surfaces are positively correlated to each other (correlation coefficient = 1.000; $p < 0.0001$), suggesting that their potential availability for promoting degradation is similar. When comparing MDI321 to HDI321, entirely different esterase specificities were obtained with regard to the effects of these reagents. Degradation of ^{14}C -MDI321B by MDM was stimulated 1.5 fold by sodium taurocholate (1.0 mM) supporting the data in this study that MDI321 activates CE thus promoting degradation, whereas ^{14}C -HDI321B degradation was inhibited by this agent ~ 30% (Data not shown). This suggests that the surfaces on which the MDM were re-seeded affected the specificity of the esterase, which elicited the degradation. The subtle changes in chemical and perhaps physical morphological characteristics resulting from the chemical differences for a specific PCNU is now believed to influence the synthesis and secretion of enzymatic activities which impact on its stability i.e. biocompatibility affects biostability [14].

In summary, degradation, esterase activity and the amount of each esterase exhibited by the MDM after reseeded were influenced by the surfaces on which the monocytes were differentiated. The PNB activity correlates with MSE protein on all PCNU differentiating surfaces. CPM release correlates to CE protein on the highly activating surfaces (PS and MDI321) whereas CPM release is not proportional to MSE protein. MSE versus CE proteins are positively correlated on the low activating surfaces, the HDI PCNUs. These data further support that there are multiple pathways responsible for the observed radiolabel release as described above [5, 22].

Effect of degradation products

When assessing the effects of MDA on MDI321, significant inhibition on both radiolabel release and esterase activity was found (Figure 4). However, MDA had no effect on esterase activity for HDI431 but BD did show a small and significant inhibition (10%) for this activity (Figure 4B). This contrasting response shows again that total esterase activity is not a good measure of MDM related biodegradation potential even though it is known to be involved in the process. In contrast to HDI431, there was a small but significant (~30%) increase in esterase activity from MDM lysates that were on MDI321 when BD was added to the re-seeded culture medium. The difference between the inhibitory effect for BD with HDI431 and the stimulatory effect on MDI321 could possibly be explained by the different effects of sodium taurocholate and sodium fluoride described above, where each showed different controlling effects on the esterase activity for MDM depending on whether the substrate was HDI321 or MDI321. Again, this

clearly indicated the activating role of material in the cell's ability to release biodegradative activities.

The above findings provide some new considerations in regards to the relative stability of MDI321. MDA itself, may be influencing biostability by having an effect via more than one mechanism, either by a product inhibition feedback loop on the activities capable of inducing the degradation and/or on the synthesis and secretion of the activities themselves. It was interesting to note that HDA which was not detected in the earlier study where CE was used to degrade the HDI-PCNUs [18], showed no effect on either radiolabel release or esterase activity in the MDM cell system on any of the PCNUs (data not shown). This would suggest that this breakdown product is relatively benign to these cells in comparison to MDA which has been extensively reported on as being a potential carcinogen [26].

CONCLUSIONS

The above findings add another dimension to our understanding of biostability as well as biocompatibility. The PCNU, which was the least degradable (^{14}C -MDI321B), once even partially degraded, may have inhibited its own degradation, perhaps by inhibiting the specific esterase activity that causes its degradation (Figure 4B). In addition, Feaster et al. [27] showed that aryl carbamates may inhibit enzyme activity by occupying binding sites of the enzyme. Given the findings in Figures 1-4, it may be possible that the esterase activity that adsorbed to the ^{14}C -MDI321B surface was partially inhibited by the hard

segment components at the polymer surface. These two hard component related events may be in part why ¹⁴C-MDI321B was degraded the least of the PCNUs.

Another important outcome of the “degradation product” study is that the activating effect of MDI321 observed in Figures 1 and 2 must be primarily surface induced rather than modulated by degradation products since the latter inhibits or shows minimal effects on the biodegradation of the HDI polymers (Figure 4A), when compared with the surface’s promotion of degradative activity (Figures 1 and 2).

In summary, using a variety of methods it has been possible to provide more evidence for the influence of material surfaces with regard to their biodegradation by MDM. This is an important element of “environmental biodegradation” that has received relatively little attention up to this point. It is clear that the degradation of a specific material is a multi-factorial process, dictated by the material’s susceptibility to hydrolysis, the effect of specific products generated during this course of action, as well as the material’s inherent ability to regulate enzyme synthesis and release. In addition to the varying effects on the degradation of a material, this study highlights to anyone doing *in vitro* cell culture work that cells are highly sensitive to the surface onto which they are seeded and that this must always be kept in mind when comparing *in vitro* data sets.

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5.0 MANUSCRIPT #4

The influence of biodegradable and non-biodegradable material surfaces on the differentiation of human monocyte-derived macrophages

STATEMENT OF AUTHOR CONTRIBUTIONS

Donna Lee M. Dinnes was the primary author and sole contributor to the experimental data for this manuscript. **Dr. J. Paul Santerre** is a co-investigator of Dr. Rosalind S. Labow, and principal investigator on the CIHR operating grant that funded this research. In addition, he has contributed to the editing and suggestions in the preparation of this manuscript. Dr. Santerre's Polymer Chemistry laboratory at University of Toronto synthesized the model PCNU materials used for the following studies. **Dr. Rosalind S. Labow** is the supervisor of Donna Lee M. Dinnes and contributed to experimental design and editing / suggestions for this manuscript.

SUMMARY

The previous study outlined in Manuscript #3, "The human macrophage response during differentiation and biodegradation on polycarbonate-based polyurethanes: Dependence on hard segment chemistry", demonstrated that once MDM were differentiated on various PCNU or TCPS surfaces, they possessed differences in degradative potential and protein expression when trypsinized and reseeded again to different material surfaces. Although this study had suggested an effect of material surface on the differentiation of MDM, it did not assess any parameters of the MDM cell function or morphology *during* this differentiation period. The following manuscript outlines the first study of MDM during their differentiation time course on PCNU materials in comparison to TCPS as a

control. The results of this study demonstrated that the material surface upon which MDM are exposed to when maturing has significant effects on cell morphology and function, most strikingly in the changes in expression of the macrophage marker CD68.

The influence of biodegradable and non-biodegradable material surfaces on the differentiation of human monocyte-derived macrophages

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Keywords: monocyte, macrophage, differentiation, biomaterial, polyurethane, cytoskeleton, biodegradation, foreign body response

ABSTRACT

Monocyte-derived macrophages (MDM) and multinucleated foreign body giant cells (FBGC) are the primary cell types that remain at the cell-material interface of polyurethane- (PU) based medical devices as a result of chronic inflammatory responses. *In vitro* studies have demonstrated that MDM possess degradative potential toward PU which can result in device failure. Since most studies have followed the degradation potential, morphology and function of these cells only once fully differentiated, the current study investigated the influence of a non-degradable control tissue culture grade polystyrene (TCPS) surface relative to two degradable model polycarbonate-urethanes (PCNU), of slightly different chemistry, on various parameters of MDM morphology and function during a 14 day differentiation time course. Differentiation of human monocytes isolated from whole blood on PCNU materials resulted in increased cell attachment, decreased multinucleation, and significant decreases in cell spreading when compared to cells differentiated on TCPS. Actin-stained podosome-like cell adhesion structures were increased in PCNU adherent cells, accompanied by alteration in β -actin and vinculin protein expression. Both model PCNU materials inhibited the full expression of the macrophage differentiation marker CD68 compared to TCPS which may indicate that PCNU materials suppress full maturation of MDM. The degradative potential of these cells was altered by the material surface they were exposed to as measured by esterase activity and protein expression of monocyte-specific esterase. This was also supported by physical material degradation evident in scanning electron microscopy images that illustrated holes in the PCNU films generated by the presence of differentiating MDM. It was concluded from these studies that PCNU materials

significantly alter the function and morphology of differentiating MDM. This must be taken into consideration when studying cell-material interactions since these cells will receive cues from their immediate environment (including the biomaterial) upon differentiation thereby affecting their resulting phenotype.

INTRODUCTION

Monocytes are phagocytic cells with the capacity to function in highly diverse environments, depending on the cues these cells receive. Upon the initiation of a foreign body response, chemokines, often secreted by neutrophils at the site of inflammation, help to initiate the recruitment of monocytes that will differentiate into macrophages. A foreign body response occurs upon blood contact with implanted biomaterials, used in the manufacture of medical devices (Williams, 1976). One of the most common classes of biomaterials used in medical applications is polyurethanes (PU). These segmented, flexible, thermoplastic polymers have been in frequent use as a result of their desirable physical and chemical properties combined with their moderate biocompatibility. A more recent generation of PU, polycarbonate-based polyurethanes (PCNU) (Stokes et al., 1995), although less susceptible to oxidation and hydrolysis than the polyether- and polyester-based PU, still show the ability to be degraded in both *in vivo* (Bucky et al., 1994; McCarthy et al., 1997; Christenson et al., 2004) and *in vitro* (Labow et al., 2001a; Matheson et al., 2002) studies [reviewed in (Santerre et al., 2005)], in addition to initiating inflammatory responses (Ma et al., 2002; Dinnes et al., 2005).

Macrophages possess the ability to fuse and form multinucleated foreign body giant cells (FBGC) (Anderson, 2000), and have been shown to be the primary cell type present at the site of long-term PU implant materials (Anderson, 1993), the hallmark of the chronic inflammatory response to implanted materials. Several studies have assessed the macrophage response to and effect on model PCNU materials (Dadsetan et al., 2004; Matheson et al., 2004; Christenson et al., 2005; McNally and Anderson, 2005; Dinnes et

al., 2006). However, very few studies have investigated the influence of PU materials on the initiation of the differentiation process that directs a monocyte along the pathway to the phenotype of a mature monocyte-derived macrophage (MDM).

In vitro models used to study the function of mature MDM in contact with materials have used IL-4 and IL-13 along with GM-CSF (granulocyte/macrophage-colony stimulating factor) to accelerate the differentiation of monocytes to MDM and also induce FBGC formation (DeFife et al., 1997; Jenney et al., 1998). In other work, an *in vitro* model of mature MDM and FBGC, similar in phenotype to the above cytokine activated cells, was developed through adherence-induced activation via tissue culture grade polystyrene (TCPS) and serum-supplemented medium, without the addition of further cytokines (Labow et al., 1998). MDM differentiating over a 14 day period in this model induced a population that was positive for the macrophage marker CD68 in 95% of the cells, and contained less than 5% of the cells positive for the lymphocyte marker CD3 (Boynton et al., 2000). This demonstrated that monocytes were differentiated to a relatively pure macrophage population with any residual lymphocytes (that do not adhere to TCPS) removed after only 3 days of culture. The 14 day maturation period has been previously established in this model with MDM exhibiting the well established characteristics of increased cytoplasmic area (Papadimitriou and Ashman, 1989), protein synthesis (Nakagawara et al., 1981) and multinucleation (Anderson, 2000) typical of terminally differentiated MDM. In addition, MDM after 14 days of maturation elicited maximal degradative capacity toward PU materials after gentle trypsinization and reseeded to model PU materials (Labow et al., 2001a; b; Matheson et al., 2002; Matheson et al.,

2004). Others have also shown that monocyte to macrophage differentiation without cytokine supplementation was possible and were able to demonstrate the expression of macrophage and not monocyte nor dendritic cell CD markers at day 14 (Ammon et al., 2000).

Although a monocyte will begin to differentiate upon contact with and respond to these foreign material surfaces, it is believed that they will not recognize the surface chemistry of the biomaterial itself but the multitude of proteins that have first adsorbed onto this surface. Proteins adsorb within seconds to minutes (Eskin et al., 2004), and the proteins that adsorb along with their conformation once adherent, will be dependent on the material chemistry and topography differences and therefore translate the biomaterial surface properties to the cells (Massa et al., 2005).

The PCNU materials used in the current study differ only slightly in chemistry; however they show differences in surface topography (Tang et al., 2002), degradability by esterase enzymes (Labow et al., 2001a; Labow et al., 2002) and MDM (Matheson et al., 2002; Labow et al., 2005) as well as differences in the effect on MDM cell morphology (Matheson et al., 2004; Dinnes et al., 2006) particularly in actin-based cytoskeleton structures.

The current study has investigated the effect of two different model degradable PCNU materials and compared them to a control non-degradable TCPS surface during the 14 day period of monocyte to MDM differentiation. The effects of these different surfaces

on MDM morphology, cell attachment, cell spreading and multinucleation were evaluated. Changes in esterase activity as well as protein expression of monocyte-specific esterase (MSE) and cytoskeletal proteins, and most important changes in the expression of the macrophage marker CD68 were investigated for characterization of the effects that PCNU materials elicit during MDM maturation, and that will subsequently affect their foreign body response. Despite these studies, no assessment has been made of phenotype, function or morphology of MDM *during* their course of differentiation on PCNU.

MATERIALS AND METHODS

Unless otherwise specified, all reagents were purchased from Sigma-Aldrich Canada Ltd (Oakville, ON, CAN).

Preparation of material surfaces

For this study, two model PCNUs were synthesized using a poly(1,6 hexyl carbonate)diol (PCN) soft segment and a 1,4-butanediol (BD) chain extender in combination with either 1,6-hexane diisocyanate (HDI) or 4-4'-methylene bis-phenyl diisocyanate (MDI) as the hard segment, as described elsewhere (Tang et al., 2002). The HDI-containing PCNU had a stoichiometric ratio 4:3:1 (HDI:PCN:BD – referred to as HDI) whereas the MDI-containing PCNU had a stoichiometric ratio of 3:2:1 (MDI:PCN:BD – referred to as MDI) as described in detail previously (Tang et al., 2002). TCPS was used as a relative control surface since this is the most commonly used material surface in cell culture.

The synthesized PCNUs were dissolved overnight in a 10% dimethylacetamide solution (w/v), centrifuged and filtered (0.45 μm Teflon filter; Chromatographic Specialties, Toronto, ON, CAN), then coated as a thin layer (100 μL) onto 15 mm diameter glass cover slips (Fisher Scientific, Ottawa, ON, CAN) under sterile conditions in a laminar flow hood, as previously described (Labow et al., 2001b). The slips were dried overnight at 50°C, purged for 24 hours and then dried under vacuum for 72 hours. PCNU coated glass slips were placed into 24-well TCPS tissue culture plates. Either TCPS cover slips (for microscopy samples) or the surface of the multiwell plate was used as the control surface for all experiments. Prior to using each surface for an experiment, the wells were filled with 1 mL of phosphate buffered saline (PBS) (0.00147 M KH_2PO_4 , 0.137 M NaCl, 0.00806 M Na_2HPO_4 , 0.00268 M KCl) at 37°C and 5% CO_2 with 100% humidity overnight. This process equilibrates the polymer slips with an aqueous environment to remove trace solvent that may possibly interfere with the experiment and condition the surface. The TCPS surfaces were treated in the same manner as model PCNU surfaces. The PBS was completely removed from each well prior to the addition of cell suspensions.

Human monocyte cell culture

Whole blood (120 mL per isolation) was collected from healthy human volunteers (with approval from the Research Ethics Committee at the University of Ottawa Heart Institute, Ottawa, ON, CAN) into EDTA Vacuette[®] tubes (Fisher Scientific). Monocytes were isolated by layering whole blood onto Histopaque[®]-1077, followed by collection of the mononuclear layer (~20% monocytes) as described in detail previously (Labow et al.,

1998). The cells were counted and analyzed for cell viability by Trypan Blue exclusion using a Vi-Cell™ Cell Viability Analyzer (Beckman Coulter Inc., Fullerton, CA, USA) and seeded (1 mL of a $6-8 \times 10^6$ cells/mL suspension) into TCPS 24-well plates (VWR, Toronto, ON, CAN) with or without PCNU-coated or TCPS coverslips. Culture medium was replaced at 24 hours, 48 hours and then every other day until day 14 with 1 mL of medium containing RPMI-1640 supplemented with 10% fetal bovine serum (FBS), L-glutamine (0.69 mM; Invitrogen, Toronto, ON, CAN), and penicillin/streptomycin (100 U/mL / 100 µg/mL; Invitrogen).

Microscopy for macrophage cell morphology

Laser scanning confocal microscopy (LSCM)

In order to assess MDM cell spreading and multinucleation after 14 days of monocyte-to-macrophage differentiation, the MDM samples were prepared for LSCM by washing each well, containing the respective HDI- or MDI-coated glass slips or TCPS control cover slips, with warmed PBS three times. The cells were then fixed with 4% paraformaldehyde (Fisher Scientific) in PBS for 10 minutes and washed with PBS prior to cell staining. Each coverslip was then incubated with rhodamine phalloidin (Invitrogen) for 30 minutes to stain for filamentous actin (F-actin), washed thoroughly with PBS and then counterstained for 5 minutes with DRAQ5™ (Cedarlane Laboratories, Hornby, ON CAN) to stain DNA. The slips were then washed briefly with PBS prior to mounting onto glass microscope slides with 90% glycerol. The cells were visualized with an Olympus 100X oil immersion objective equipped with a numerical aperture of 1.4, on an Olympus IX80 laser scanning confocal microscope operated by FV1000

software (version 1.4a). The rhodamine phalloidin and DRAQ5™ fluorochromes were excited with the 543 nm line of the green He-Ne laser and the 633 nm line of the red He-Ne laser respectively. The FV1000 software was used to measure the area (μm^2) of each cell spread onto each material surface, as determined by the outline of the cell when stained for F-actin. In addition, multinucleation or FBGC formation was determined by counting the number of nuclei stained by DRAQ5™ present in each MDM counted.

Scanning Electron Microscopy (SEM)

MDM cell spreading and morphology was assessed after 14 days of differentiation, in MDM samples that were prepared for SEM analysis. Samples were prepared by washing each well with warmed PBS three times. The cells were then fixed in 3% glutaraldehyde in PBS overnight at room temperature followed by further washing three times with PBS. The samples were then maintained at 4°C until sample analysis. The samples were post-fixed in a 1% osmium tetroxide/PBS solution followed by a graduated series of ethanol solutions as described previously (Labow et al., 1998). The samples then were placed into a Polaron® CPD7501 Critical Point Drier and then mounted on aluminum stubs and coated with 3 nanometers of platinum in a Polaron® SC515 SEM coating unit. Finally, the samples were viewed and photographed at 1000 times magnification using a working voltage of 10 kV with a Hitachi 2500 Scanning Electron Microscope (Faculty of Dentistry, University of Toronto).

Total protein and DNA determination

Whole cell lysates were prepared and analyzed for DNA as described in detail previously (Labow et al., 2001b; Matheson et al., 2004). Cold Triton-X 100 buffer (240 μ L) (0.05% Triton-X 100, 10 mM EDTA in PBS) was added to each well and the cells were lysed for 1 hour on ice. Hoechst dye (number 33258; Amersham Biosciences., Baie d'Urfe, QC, CAN) was diluted with TRIS buffer (0.01 M Tris, 0.001M EDTA, 0.2 M NaCl, pH 7.4) immediately prior to analysis. Cell lysates collected for DNA (10 μ L) were added to the dye (100 μ L) in 96 well microtiter plates (Microfluor 2 Black; VWR, Mississauga, ON, CAN). The lysates were compared to a DNA standard (Calf Thymus DNA) and each standard well to be analyzed also contained the same amount of 0.05% Triton-X 100 buffer (10 μ L) as in the lysate samples. The amount of DNA was quantified using a fluorescence microplate reader (POLARstar Galaxy, BMG Labtechnologies, Durham, NC, USA) with an excitation and emission wavelength of 360 and 460 nm respectively. The protein content of each cell lysate sample (25 μ L) was assayed using a protein dye (Bio-Rad Laboratories Ltd., Mississauga, ON, CAN) based on the Bradford method (Bradford, 1976). The protein in each sample was compared to a bovine serum albumin standard and related to the DNA content in each sample.

Immunoblotting

Proteins from whole cell lysate samples were separated by SDS-PAGE using a Protean II Cell System (Bio-Rad) with 5% stacking and 10% (for CD68 and vinculin) or 12% (for β -actin, CD14, esterase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)) separating gel. Monocytes isolated from whole blood are non-dividing cells, therefore

samples for SDS-PAGE were loaded into each well according to the DNA content in each sample. Cell lysate samples equivalent to 250 ng of DNA were loaded into each well to ensure equal loading. GAPDH was used as an additional control to ensure equal loading of protein in each well. Proteins from the resulting gels were transferred to nitrocellulose membranes (0.45 μ m, Bio-Rad) for 75 minutes at 130 V. The membranes were then blocked for 1 hour in Tris-buffered saline (TBST; 10 mM Tris, 0.1 M NaCl, 0.1% Tween-20, pH 7.5) containing either 5% non-fat dry skim milk or 5% bovine serum albumin. The membranes were then probed for 1 hour with rabbit anti-porcine esterase (Rockland Immunochemicals Inc., Gilbertville, PA, USA – reacts with MSE), mouse anti-human β -actin, mouse anti-human CD14 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), mouse anti-human CD68 (Santa Cruz), mouse anti-vinculin or mouse anti-GAPDH (Chemicon International Inc., Temecula, CA, USA) and subsequently washed 8 times with TBST. The membranes were then incubated with either anti-rabbit IgG (Rockland) or anti-mouse IgG (Pierce Biotechnology Inc., Rockford, IL, USA) conjugated to horseradish peroxidase for 1 hour followed by 8 washes with TBST. Protein bands were visualized using a chemiluminescence detection kit (Pierce). The intensity of the protein bands were quantified using Quantity One[®] software (Bio-Rad).

Esterase activity

Cell lysate samples (25 μ L) of adherent human monocytes undergoing differentiation to macrophages at 24, 48 and 72 hours, as well as 7, 10 and 14 days were assayed for esterase activity using the substrate *p*-nitrophenylbutyrate (PNB). One unit of activity was defined as the release of 1 nmol of *p*-nitrophenol ($\epsilon = 16,300 \text{ cm}^{-1}\text{M}^{-1}$) per minute at

37°C at pH 7.0 and 400.6 nm, as previously described (Labow et al., 2001b). The total intracellular esterase activity was normalized to 10 µg DNA in each sample as described above.

Statistics

Statistical analyses were performed using a one-way ANOVA and Student-Newman-Keuls test for all pairwise comparisons. The MedCalc® statistical program (version 7.2.1.0 for Windows) was used with a significance value of $p < 0.05$ for pairwise comparisons.

RESULTS

Monocyte-derived macrophage morphology

The current study investigated MDM morphology and function *during* the 14 day differentiation period applied to the macrophage model system described by Labow *et al* (Labow et al., 2001a). Figure 1 (top row) demonstrates the differences seen in F-actin-stained cytoskeletal structures in monocytes differentiated to MDM when exposed to different materials (TCPS, HDI or MDI). MDM differentiated on TCPS appeared larger and more spread out than MDM on either HDI or MDI. In addition, cells differentiated on HDI or MDI demonstrated more punctate actin structures which also appeared more aggregated on each PCNU in comparison to TCPS. The external morphology of these MDM was demonstrated more clearly seen in the SEM images (Figure 1 – bottom row) of cells differentiated on each material surface. Supporting the actin/DNA images, the MDM on TCPS also demonstrated greater cell spreading than cells on HDI or MDI.

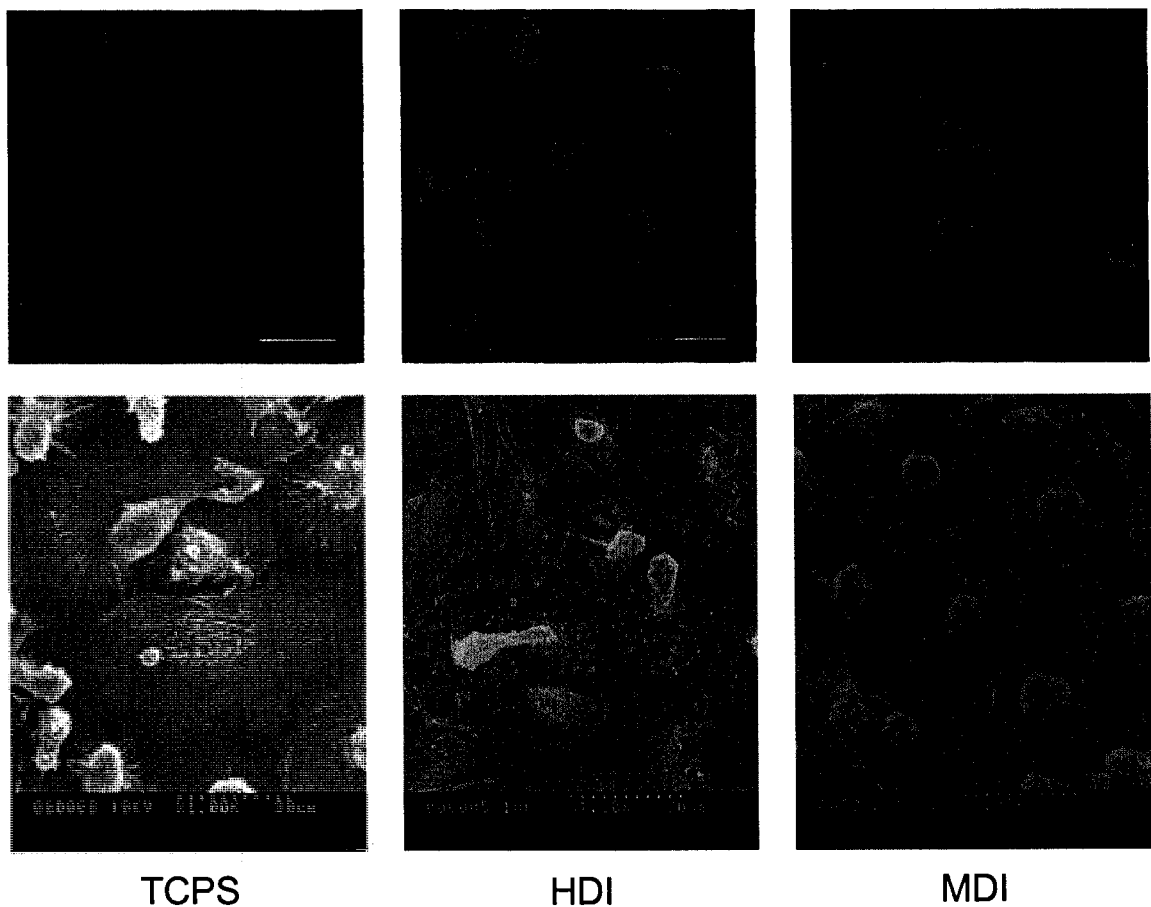


Figure 1 – Human monocyte-derived macrophage morphology is affected by differentiation material surface. Human monocytes were differentiated as described in the **Materials and Methods** on TCPS, HDI or MDI for 14 days. Cells were fixed and either stained for F-actin (red) and DNA (blue) and visualized by LSCM (top row)(scale bar = 20 μ m) or fixed and imaged by SEM (bottom row).

The surface of the MDM appeared smoother when differentiated on TCPS as compared to HDI or MDI. MDM appear more “rounded-up” on the PCNU materials, which was more obvious for MDM on MDI (right lower panel). Cells differentiated on MDI exhibited a higher degree of ruffling than either HDI or TCPS, whereas cells on HDI demonstrated features associated with membrane blebbing.

Cell spreading and multinucleation

Since the material surface on which MDM were differentiated influenced the morphology and spreading of these cells (Figure 1), the area of MDM on each surface was quantified. The outline of each MDM (350 cells in total from four independent monocyte isolations from four different donors) as indicated by staining of F-actin, was utilized for cell area (μm^2) measurements (using the FV1000 software) and confirmed differences in cell spreading on the different surfaces (Table 1). The average cell area was significantly decreased on HDI and MDI relative to cells differentiated on TCPS. In addition to cell spreading, significant differences in the number of nuclei per cell in MDM on the different surfaces were determined with values for cells on each surface statistically different from each other (Table 1).

Cell attachment (DNA) and intracellular protein

Figure 2A illustrates the DNA content, expressed as μg DNA per well, from MDM lysates taken over the 14 day time course. Monocytes and macrophages are non-proliferating cells and therefore the amount of DNA in a well, which correlated to the number of viable cells as determined by Trypan Blue exclusion (Vi-Cell™)(data not

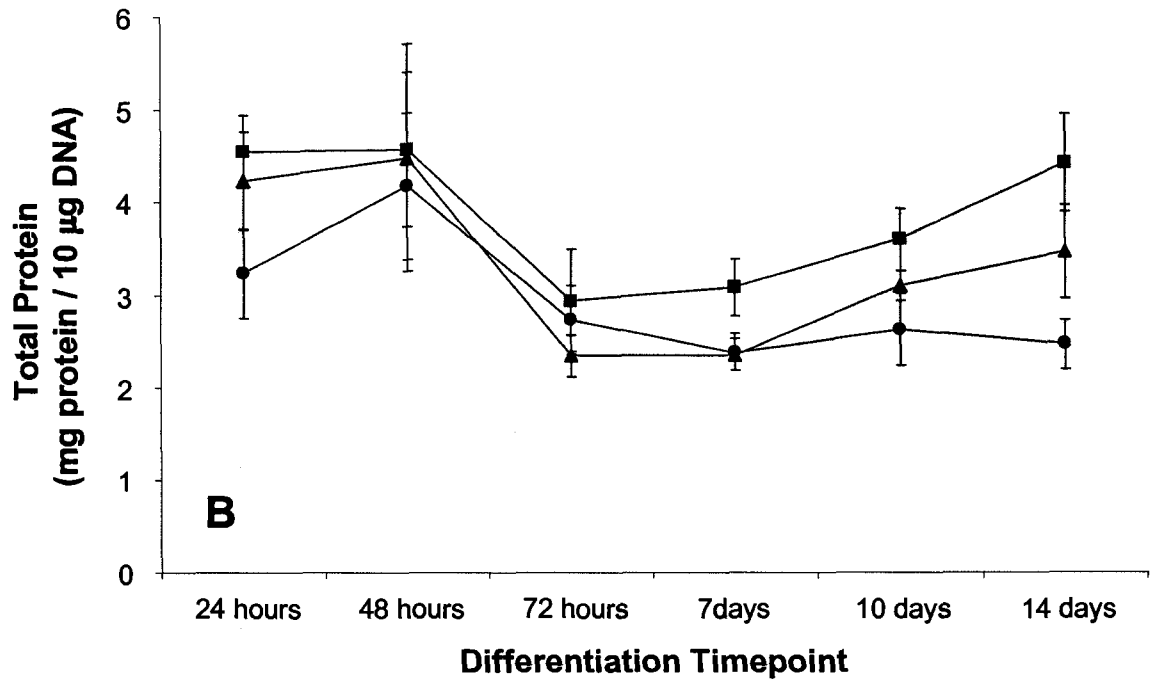
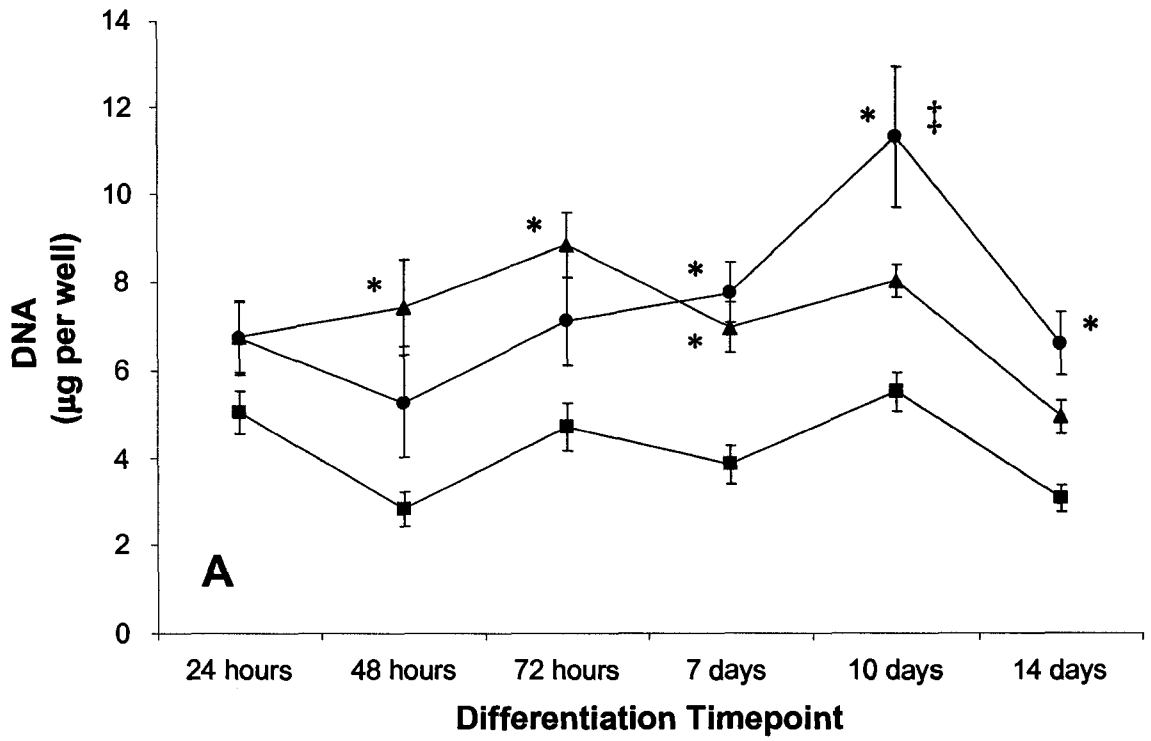
Table 1 – Effect of differentiation surface on human macrophage cell spreading and multinucleation

Material Surface	Cell Area (μm^2)	# nuclei per cell
TCPS	1459.43 \pm 36.72	1.31 \pm 0.04 **
HDI	1015.74 \pm 31.02 *	1.02 \pm 0.01 **
MDI	1077.53 \pm 26.17 *	1.16 \pm 0.02 **

* - indicates a significant decrease relative to TCPS

** - indicates a significant difference between each of the surfaces

Figure 2 – The effect of material surface on cell attachment (DNA) and intracellular protein in differentiating monocyte-derived macrophages. Freshly isolated human monocytes induced to differentiate towards macrophages over a 14 day period on TCPS (■), HDI (▲) or MDI (●) were analyzed for DNA content (A) or intracellular protein (B) per well. * significant increase relative to cells on TCPS ($p < 0.05$), ‡ significant difference between MDI and HDI ($p < 0.05$).



shown), was used to determine cell attachment. Of particular interest is that the DNA content was always the lowest in cell lysates from MDM differentiating upon TCPS. Following initial attachment of the cells (24 hours) there were no significant differences between the DNA content in cell lysate samples from each material surface. However, by 48 hours and until 7 days of differentiation, HDI containing wells demonstrated greater cell numbers (DNA content) relative to TCPS wells. The MDI-based PCNU also exhibited increased cell number relative to TCPS but only by day 7 of differentiation. Nonetheless, this value was maintained until the end of the 14 day time period. Furthermore, MDI also elicited greater cell numbers relative to HDI at 10 days of MDM differentiation. Upon analysis of total protein content in MDM during differentiation on TCPS, HDI or MDI (Figure 2B) there were no material differences throughout the entire differentiation time course. The protein level in cell lysates from each material surface followed the same pattern throughout each time point of differentiation, demonstrating a small decrease in protein along 24 to 72 hours post monocyte attachment which then returned to the baseline (24 hour) protein amounts by the end of the differentiation period.

Structural protein expression

Since significant differences in cell spreading were demonstrated in MDM differentiated on PCNU materials in comparison to TCPS (Table 1), and differences in the presentation of actin-based structures at the site of macrophage attachment to material surfaces were evident in differentiating MDM on material surfaces composed of different material chemistry (Figure 1), the expression of the structural proteins, β -actin and vinculin, were

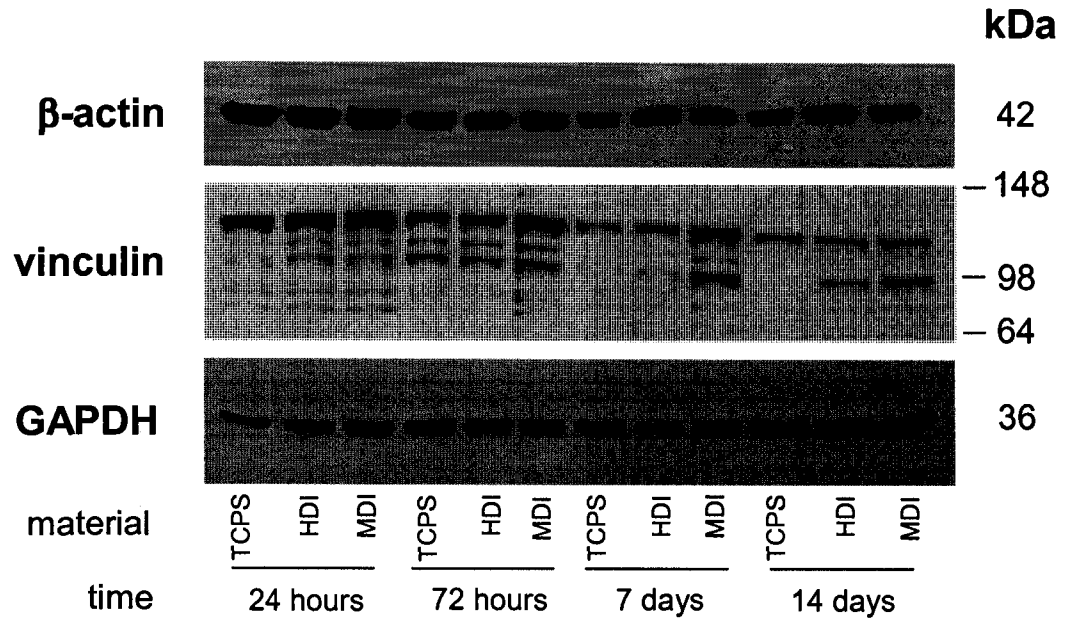
quantified during the 14 day time course of MDM differentiation on the different materials. Figure 3A shows representative immunoblots illustrating changes in β -actin (top) and vinculin (middle) protein expression over the 14 day differentiation time course on TCPS, HDI or MDI. GAPDH protein (bottom) was used as an additional loading control. During the 14 day time course, β -actin protein expression significantly decreased until day 14 of differentiation (Figure 3B). However, no differences were seen between material surfaces. Vinculin protein expression had decreased on each material surface by 7 and 14 days of differentiation relative to vinculin protein expression on TCPS at 24 hours (Figure 3C). Although, differences in vinculin expression seemed apparent between each material surface at both 7 days and 14 days of differentiation, there was only a significantly higher protein expression in MDI-adherent MDM relative to TCPS-adherent MDM at 7 days of differentiation.

Expression of monocyte/macrophage markers CD14 and CD68

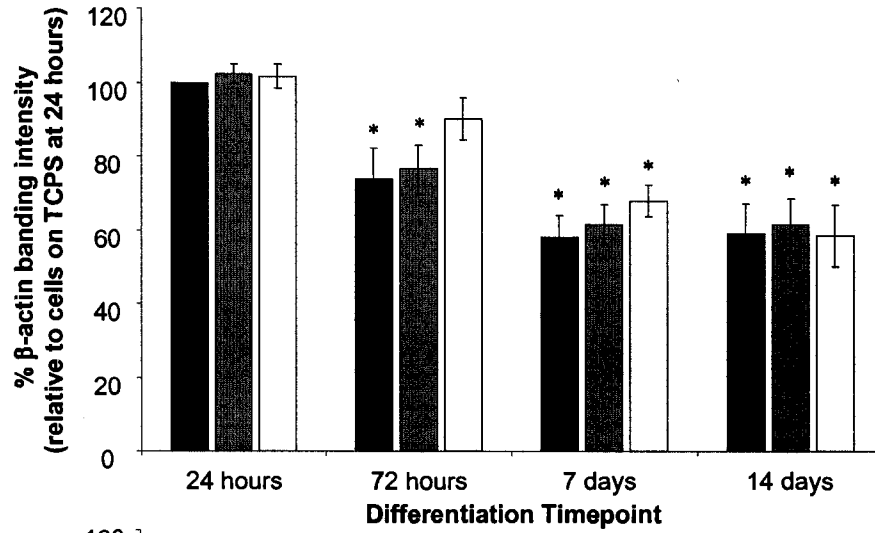
To determine if PCNU chemistry altered the MDM phenotype or maturity, protein expression of the macrophage marker CD68 was analyzed during the 14 day time course (Figure 4A – top blot). It is clearly illustrated that CD68 increased dramatically and significantly on TCPS from 24 hours to 14 days (Figure 4B). However, this increased expression of CD68 was dramatically reduced for cells that were differentiated on either HDI or MDI PCNUs (Figure 4B). In addition, immunoblot analysis for the expression of the monocyte marker CD14 was performed along the 14 day differentiation time course (Figure 4A – middle blot). Freshly isolated monocytes (24 hours) displayed the CD14 marker without significant differences between material surfaces (Figure 4C). Rapid

Figure 3 – Changes in expression of β -actin and vinculin protein in differentiating monocyte-derived macrophages in response to material surface differences. Cell lysates from human MDM differentiating on TCPS (black), HDI (grey) or MDI (white) over a 14 day time course were subjected to SDS-PAGE and immunoblotting for either β -actin, vinculin or GAPDH (loading control) (A) and quantified for changes in β -actin protein expression (B) or vinculin protein expression (C) relative to differentiating monocytes cultured for a 24 hour time period on TCPS as a reference point.

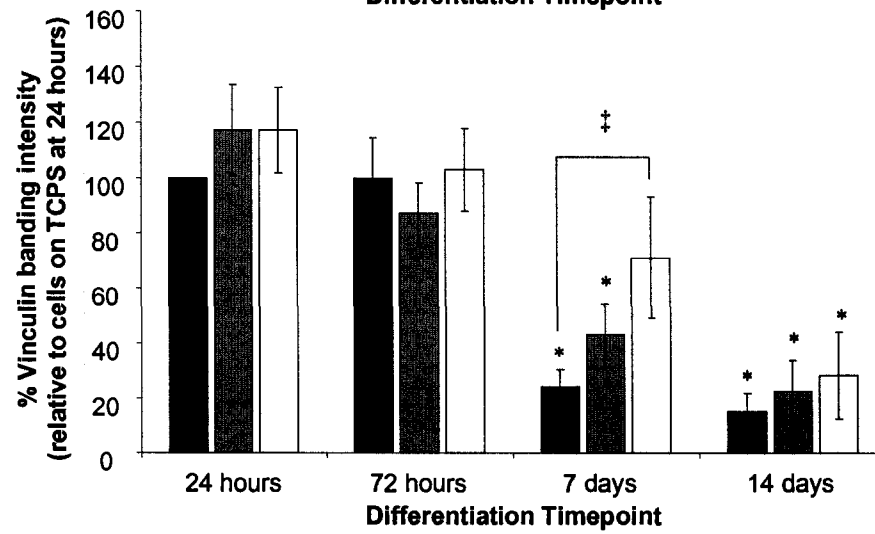
* significant decrease in protein expression relative to cells on TCPS at 24 hours ($p < 0.05$), † significant difference in vinculin protein expression between MDM differentiating on TCPS and MDI at a 7 day time point ($p < 0.05$).



A

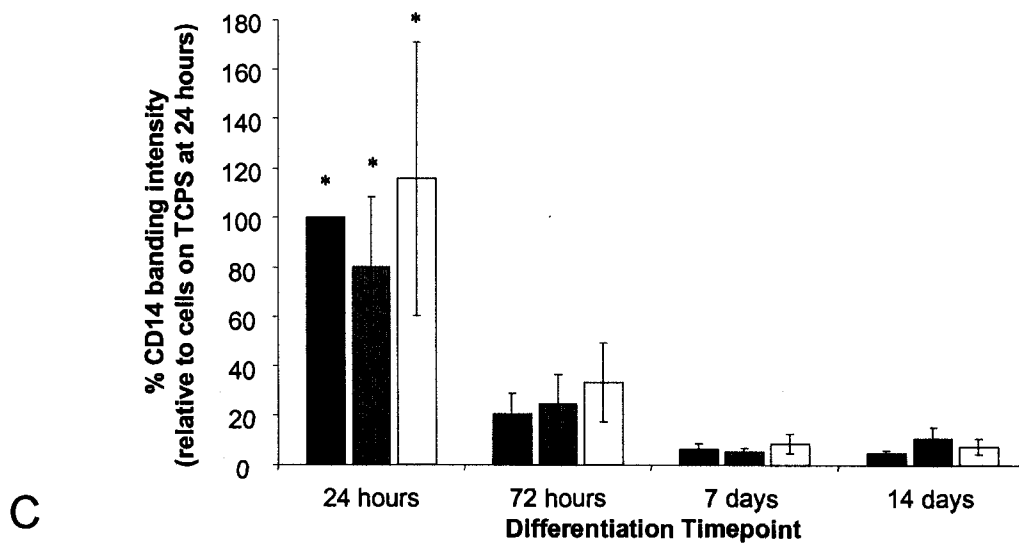
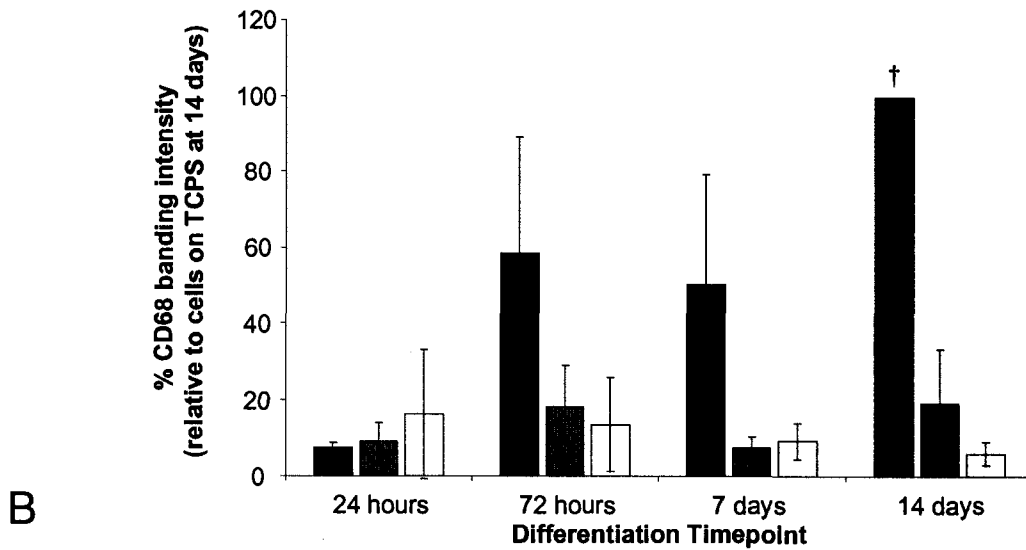
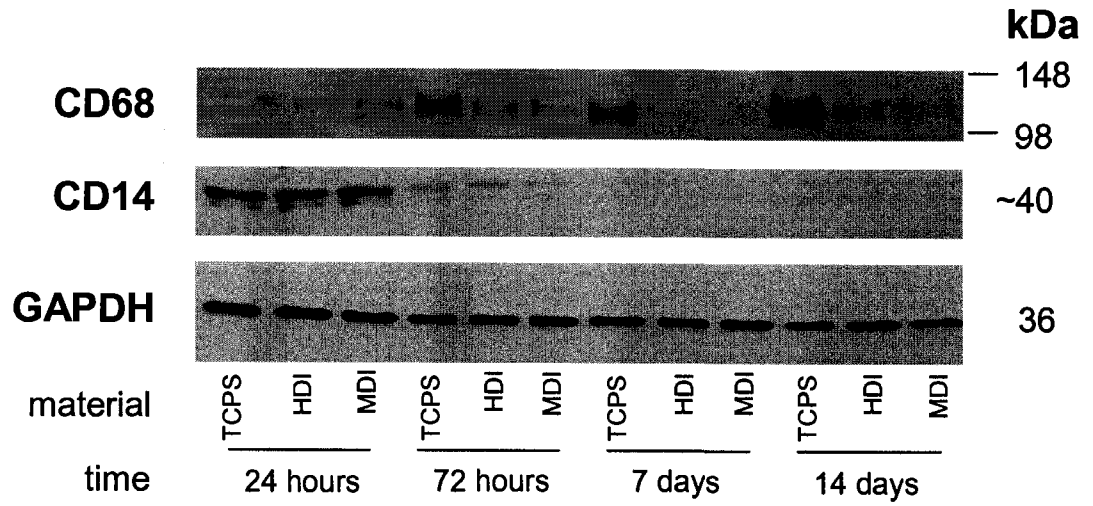


B



C

Figure 4 – Material surface chemistry influences the expression of the macrophage differentiation marker CD68. (A) Cell lysates of freshly isolated human monocytes differentiating towards MDM were analyzed by immunoblot analysis (as described in **Materials and Methods**) over a 14 day time course on TCPS (black), HDI (grey) or MDI (white) for changes in CD68 expression as a marker of monocyte to macrophage differentiation and the disappearance of the monocyte marker CD14. (B) The influence of material surface chemistry on CD68 expression in differentiating MDM and (C) the disappearance of the monocyte marker CD14 during MDM differentiation were quantified. † significantly higher CD68 banding intensity relative to cell lysates from all material surfaces at all other time points ($p < 0.05$), * significantly greater CD14 banding intensity relative to all other time points on the respective material surface.

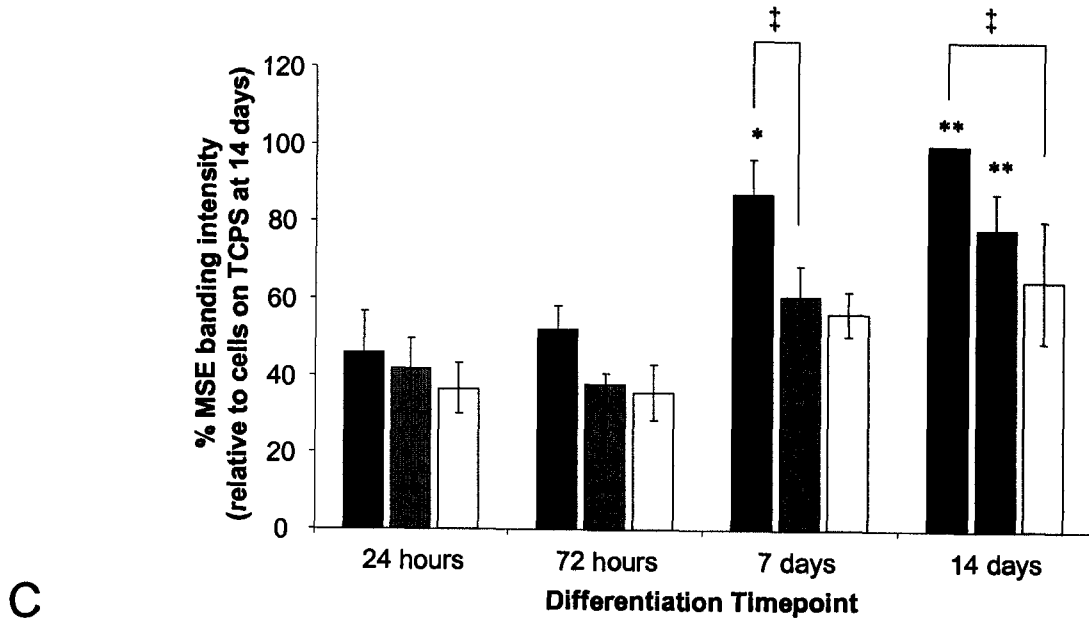
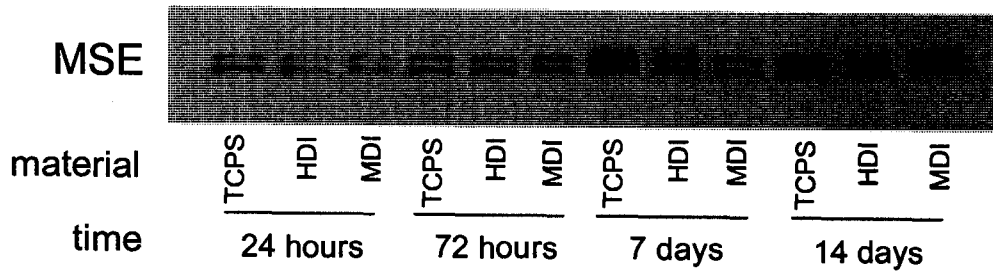
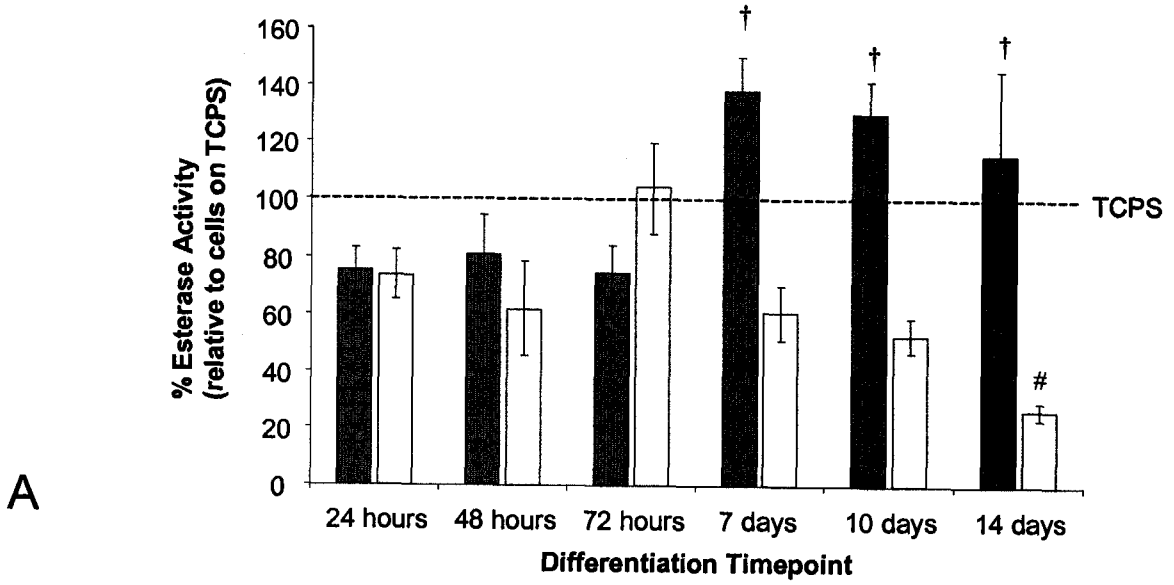


downregulation of this monocyte marker was demonstrated by 72 hours and this marker was barely detectable by 14 days of differentiation in the cells on all surfaces (Figure 4C).

Esterase activity and protein expression

Figure 5A illustrates the changes in esterase activity in cells differentiated on either HDI or MDI, relative to cells differentiated on TCPS, at each time point along the differentiation time course. For the first 72 hours of differentiation, differences were observed in esterase activity but only became significant for the different material surfaces at 7 days of differentiation. At 7, 10 and 14 days of differentiation, the esterase activity was significantly higher in MDM differentiated on HDI relative to MDI. Moreover, at 14 days of differentiation, the esterase activity in MDI-differentiated cells was significantly lower than TCPS, which did not occur at any other time point. In order to determine if the influence of differentiation surface on MDM was in esterase protein expression as well as activity, changes in MSE protein expression were evaluated in these cell lysates by immunoblot analysis with an antibody that recognizes the 40 kDa doublet of MSE protein (Figure 5B). There were no detectable differences in MSE protein expression at the early stages of differentiation (Figure 5C). However, at 7 and 14 days of differentiation, MSE protein expression was increased relative to earlier time points, significantly by 14 days on each material surface relative to cells on TCPS at 24 hours. Moreover, material differences were seen in intracellular MSE protein expression with expression significantly lower on HDI relative to TCPS at 7 days and lower on MDI relative to TCPS by 14 days of MDM differentiation.

Figure 5 – The effect of differentiation time point and material surface on esterase protein expression and activity in differentiating monocyte-derived macrophages. (A) Esterase activity was measured in cell lysates of monocytes differentiating to MDM on TCPS (dotted line), HDI (grey bars) or MDI (white bars) over a 14 day time course as described in **Materials and Methods**. (B) Cell lysates from differentiating MDM during the 14 day time course were immunoblotted for MSE protein and (C) quantified for changes in expression between MDM differentiating on TCPS (black), HDI (grey) or MDI (white) as described in **Materials and Methods**. † significant difference in esterase activity between HDI and MDI, # significant difference in esterase activity between MDI and TCPS, * significant increase in MSE protein expression relative to cells on TCPS at 24 hours, ** significant increase in MSE protein expression relative to cells on the same material surface at both 24 hours and 72 hours, ‡ significant differences in MSE protein expression between material surfaces ($p < 0.05$).



DISCUSSION

The current study investigated the changes in characteristics of primary human monocytes in contact with PCNU biomaterials differentiating along the macrophage lineage. Although the maturation of human monocytes to MDM has been well characterized particularly in terms of expression of CD markers (Ammon et al., 2000), and studies have shown changes in the degradative potential towards PCNU materials in MDM following differentiation on PCNU materials (Labow et al., 2005), no study has been conducted examining the influence of degradable PU materials (specifically PCNU) on differentiating MDM with regard to morphology, cellular attachment, cytoskeleton changes, degradative potential and expression of monocyte and macrophage markers *during* the maturation process. Changes in these parameters would ultimately result in a modulation of the MDM foreign body response to these implant materials.

In terms of morphology, the arrangement of the F-actin cytoskeleton of MDM has been shown to be sensitive to the material surface to which they are adherent. MDM differentiated for 14 days on TCPS, then trypsinized and reseeded to TCPS or PCNU materials demonstrated changes in cell spreading, as visualized by F-actin staining, in addition to alterations in the appearance of F-actin-based structures (Matheson et al., 2004). Furthermore, using proteomic techniques, β -actin was identified in MDM differentiated on HDI and demonstrated altered protein expression in comparison to cells differentiated on TCPS (Dinnes et al., 2006). Since PCNUs induced changes in the cytoskeleton and morphology of MDM, post transformation to macrophages, the effect of the material surface in expression of cytoskeletal proteins was investigated in this study

in MDM differentiating on PCNU materials. Although *striking* differences in actin-based structures were not evident in MDM differentiated on TCPS versus HDI or MDI, it was apparent that MDM were more spread out over a control TCPS surface following maturation (Figure 1), which is a typical characteristic of mature macrophages in tissue culture (Papadimitriou and Ashman, 1989). When compared to cells on TCPS, MDM differentiated on either HDI or MDI appeared more “rounded up” with increased blebbing especially prominent in SEM images of cells on HDI (Figure 1). Although membrane blebbing is quite often associated with cell death, particularly by apoptosis (Fadeel, 2004; Zimmermann and Green, 2001), several studies have shown that membrane blebbing is a feature of secretion by ectocytosis (Stein and Luzio, 1991; Lee et al., 1993; Mehul and Hughes, 1997), which may relate to the fact that HDI is the model PCNU degraded most by MDM and candidate esterases (Labow et al., 2002; Matheson et al., 2002; Matheson et al., 2004; Labow et al., 2005). The MDM may attempt to envelope the TCPS surface since it is non-degradable, while the MDM goes into a different functional state on a material that it can degrade.

Cell spreading was quantified (Table 1) and the differences demonstrated by cell area measurements supported the cell spreading changes illustrated in Figure 1. MDM fuse to form FBGC as a result of chronic inflammation (Anderson, 2000). This mechanism would require close proximity of adjacent cells in order to facilitate the fusion of membranes between two cells. It is possible that the rounding up feature produced on either HDI or MDI may reduce the degree of FBGC formation, due to the increased distance between cells. Multinucleation, as measured by the number of nuclei per cell,

was assessed to determine the propensity of cells to fuse and form FBGC. In support of the theory that MDM differentiation on PCNU may reduce cell fusion as a result of rounding up, cells on HDI or MDI had significantly less nuclei per cell than MDM differentiated on TCPS (Table 1). Upon examination of actin and DNA stained images, MDM differentiated on HDI rarely contained more than 1 nucleus, cells on MDI with 2 nuclei were encountered but rarely greater, and TCPS cells often contained 2 nuclei with the occasional cell containing up to 8 nuclei (data not shown).

In addition to cell spreading, cell attachment (as measured by DNA content per well) was quantified to determine if the differentiation surface affected the ability of cells to initially attach or remain attached to each material surface. Although some cells fuse and multiple nuclei may be contained in one cell, the DNA content per well would give an indication of the number of cells that did attach or have remained attached to each surface. Over the 14 day differentiation time course, the lowest DNA values were encountered on TCPS (Figure 2A). Higher DNA values, indicating increased cell attachment, on the PCNU surfaces relative to TCPS may have been due to the differences in surface chemistry and polarity of these materials. As mentioned earlier, the differences in material chemistry and surface features influence the serum proteins that adsorb to each surface, subsequently affecting cell attachment (Rudee and Price, 1985; Massa et al., 2005). It is also possible that since MDM on TCPS spread their cytoplasm to such a great degree, some may have fused to form FBGC as a result of the proximity of adjacent cells, but some cells may have detached from the surface because of the lack of area on the tissue culture surface for cell attachment. Although MDM differentiated

on HDI were the smallest as indicated by cell area (Table 1), this was only the area of the base of the MDM spread directly over the material surface. SEM images (Figure 1) demonstrated that although TCPS cells were more spread along the culture surface, the rounded up cells on HDI and MDI had a more “piled up” cell body, indicating that the cells were not necessarily smaller in total cell volume, but were less spread over the material surface. No significant differences were detected in MDM protein between cells differentiated on each surface (Figure 2B). Although previous proteomics studies have indicated that the protein profiles in differentiating MDM were altered by differentiation surface (Dinnes et al., 2006), this study demonstrated that overall protein amount did not significantly change.

In order to quantify the differences that were seen in the cytoskeleton of differentiating MDM, immunoblot analysis of MDM cell lysates for β -actin was performed during the time course of their 14 day differentiation. Actin and actin-binding proteins are essential in such functions as cell motility, cell shape, phagocytosis as well as cell adhesion [reviewed in (Revenu et al., 2004)]. Podosomes and other focal adhesion complexes require supporting proteins such as vinculin which participates in the attachment of actin-based filaments to the cell membrane (Goldmann and Ingber, 2002; Bailly, 2003). Vinculin also facilitates the connection of integrin receptors, suggested to facilitate cell attachment to biomaterial surfaces (McNally and Anderson, 2002), to the actin cytoskeleton (Geiger et al., 2001). Podosomes are dot-like adhesion structures that contain a central core of actin surrounded by proteins such as vinculin (Linder and Aepfelbacher, 2003). Podosome-like structures were evident especially in PCNU

differentiated MDM (Figure 1), therefore the changes in expression of β -actin and vinculin were quantified. Although immunoblots demonstrated that both β -actin and vinculin protein expression decreased in MDM over the differentiation time course (Figure 3A), significant differences between cells on TCPS versus PCNU were only evident in vinculin protein expression (Figure 3B). This could be explained by the fact that MDM may be highly motile both in initiating cell attachments and spreading over material surfaces, which requires an overabundance of actin monomers to facilitate constant actin polymerization (Welch et al., 1997). However, firm cell adhesion may occur at later time points which would require vinculin for formation of podosomes. Differences in podosome-like structures were seen between TCPS-adherent and both HDI- and MDI-adherent MDM with a greater number and more even distribution of dot-like F-actin structures that are representative of podosomes. This could explain the changes in vinculin protein expression between material surfaces at later time points (Figure 3B). Although vinculin expression decreased on all surfaces during the 14 day time course, at day 7 of differentiation vinculin expression was significantly greater in MDM lysates from MDI-adherent cells relative to TCPS-adherent cells (Figure 3B). Although this significant difference in vinculin protein expression between TCPS and MDI is not clearly reflected in the number of podosome-like structures, studies have also shown that vinculin is required for the control of cell shape and spreading (Goldmann and Ingber, 2002). Differences in MDM morphology were indicated in Figure 1, and it is a strong possibility that the differences in vinculin protein expression were linked in part to the altered shape and cytoplasmic rearrangement of the cells in response to surface chemistry differences.

As a result of the obvious differences seen in morphology and cell attachment, the capability of cells to mature and express the macrophage marker CD68 was investigated in MDM on each of the surfaces. Previous studies establishing this MDM cell model demonstrated that 95% of mononuclear cells isolated from whole blood by 72 hours on TCPS expressed the CD68 macrophage marker and were negative for the lymphocyte marker CD3 (Boynton et al., 2000). These findings demonstrated that with this cell model, non-adherent lymphocytes were removed with repeated media changes by 72 hours and that a mature macrophage population (CD68⁺) was present by 72 hours. Consistent with the previous study, MDM in the current study differentiated on TCPS highly expressed CD68 by 72 hours (Figure 4A). However, monocytes that were differentiated on HDI or MDI had a significantly lower expression of CD68 and did not increase during differentiation (Figure 4B). Monocytes typically display the differentiation marker CD14 and as these cells mature into macrophages, the expression of CD14 has been shown to decrease (Ziegler-Heitbrock and Ulevitch, 1993; Kruger et al., 1996). To determine if cells on HDI and MDI surfaces were suspended in a monocyte phenotype, cell lysates were probed with CD14 antibodies. The CD14 marker significantly decreased by 72 hours in monocytes differentiating on each material surface indicating that the cells were no longer immature monocytes (Figure 4C). It is unlikely that the PCNU surfaces induced differentiation of monocytes to a different cell type, but rather prevented the expression of CD68. Monocyte-derived dendritic cells typically display cytoplasmic veils protruding from the cell body (Jeras et al., 2005), and osteoclasts are typically highly multinucleated cells with a ruffled border that increases the cell area for bone resorption (Boyle et al., 2003). The morphology of the cells

presented here did not exhibit morphological features of either dendritic cells or osteoclasts and it is therefore likely that the PCNU materials did not induce maturation to a separate cell lineage, but altered the expression of the particular marker CD68.

Previous studies with mature (14 day differentiated) MDM differentiated on TCPS showed significant differences when reseeded on HDI and MDI with respect to esterase activity, synthesis and release as well as material degradative potential (Labow et al., 2002; Matheson et al., 2002; Matheson et al., 2004; Labow et al., 2005; McBane et al., 2005). As previously demonstrated, both esterase activity and protein expression did increase during differentiation with the different material surfaces causing significant alterations in the monocyte response with progression to mature MDM (Figure 5). Although esterase activity did not correlate with MSE protein expression, the assay used to measure activity is not specific and could be measuring other esterases (e.g. cholesterol esterase) known to be synthesized and released from mature MDM as previously shown (Matheson et al., 2002; Matheson et al., 2004).

Previous studies have provided evidence that esterases secreted from MDM are responsible for the *in vitro* biodegradation of PU materials (Labow et al., 1998; 2001a; b; Labow et al., 2002). However, it is likely that there are multiple proteins and chemicals that contribute to this response. Ectocytosis, illustrated by blebbing of MDM on HDI (Figure 1) could be a potential method of release of degradative and/or signaling proteins into the extracellular space as a part of this foreign body response, particularly cytoplasmic proteins that lack a signal sequence (Lee et al., 1993). Biodegradation, as

indicated by holes generated in the material surface, was previously illustrated when mature MDM were trypsinized and reseeded onto HDI (Labow et al., 2001a). Despite these findings, this is the first study to demonstrate by SEM analysis that cells differentiated on a PCNU material without activation by trypsinization could generate “pitting” with distinct holes in a model PCNU material (Figure 6).

CONCLUSIONS

When a biomaterial is implanted into the body, the foreign body reaction elicits the recruitment of neutrophils and monocytes. This study describes a model relevant to the *in vivo* situation where monocytes attach to the material surface following their recruitment and are significantly altered by the surface chemistry and topography to which they are exposed. Understanding the characteristics of the MDM phenotype generated during differentiation is necessary in order to direct the subsequent course of the tissue-material interaction for the successful outcome of the desired function of the medical device.

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Figure 6 – Human MDM differentiated on HDI demonstrate biomaterial degradation. Scanning electron microscopy image of human MDM differentiated on HDI for 14 days demonstrating pitting/holes (as indicated by arrows) in the material surface.

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6.0 MANUSCRIPT #5

Material surfaces affect the protein expression patterns of human macrophages: A proteomics approach

STATEMENT OF AUTHOR CONTRIBUTIONS

Donna Lee M. Dinnes is the primary author and main contributor to the experimental data in this manuscript. All experiments were performed by Donna Lee M. Dinnes with assistance and training from University of New South Wales PhD student **Helder Marçal**. Helder Marçal contributed to the experimental design of the proteomics experiments in this manuscript. Donna Lee M. Dinnes carried out cell culture for these experiments at the University of Ottawa Heart Institute. All preparation of samples and proteomics techniques were performed in the research lab of **Dr. Stephen M. Mahler**, also the supervisor of Helder Marçal, in conjunction with the Bioanalytical Mass Spectrometry Facility. **Dr. J. Paul Santerre** is a co-investigator of Dr. Rosalind S. Labow, and principal investigator on the CIHR operating grant that funded this research. In addition, he has contributed to the editing and suggestions in the preparation of this manuscript. Dr. Santerre's Polymer Chemistry laboratory at University of Toronto synthesized the model PCNU materials used for the following studies. **Dr. Rosalind S. Labow** is the supervisor of Donna Lee M. Dinnes and contributed to the experimental design of MDM cell experiments performed.

SUMMARY

The following manuscript entitled "Material surfaces affect the protein expression patterns of human macrophages: A proteomics approach" is in press with the *Journal of*

Biomedical Materials Research: Part A. The study was a direct result of a Short-Term Research Visit awarded to Donna Lee M. Dinnes from the CIHR Institute of Musculoskeletal Health and Arthritis. Experiments contained within this manuscript were conducted at the University of New South Wales in the research lab of Dr. Stephen Mahler, in conjunction with the Bioanalytical Mass Spectrometry Facility of the University of New South Wales. The aim of this study was to take a proteomics approach to assess the intracellular proteins that were involved in the MDM response to PCNU surfaces. These methods allow an explorative approach to determine multiple proteins that may be modulated in an MDM in response to adherence to PCNU surfaces. These studies have brought to our attention pathways and proteins that have thus far not been considered to contribute to the MDM foreign body response. As outlined in the following introduction, as far as the authors are aware, this is the first study to use proteomic methods to assess human macrophages at the cell-biomaterial interface. Preliminary results, yet unpublished, from similar studies assessing the profiles of secreted proteins are detailed in Appendix VI.

Material surfaces affect the protein expression patterns of human macrophages: A proteomics approach

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Keywords: monocyte-derived macrophage, polyurethane, inflammation, proteomics, 2-dimensional electrophoresis, MALDI-ToF

ABSTRACT

Monocyte-derived macrophages (MDM) are key inflammatory cells and are central to the foreign body response to implant materials. MDM have been shown to exhibit changes in actin cytoskeleton, multinucleation, cell size and function in response to small alterations in polycarbonate-urethane (PCNU) surface chemistry. Although PCNU chemistry has an influence on *de novo* protein synthesis, no assessments of the protein expression profiles of MDM have yet been reported. The rapid emerging field of expression proteomics facilitates the study of changes in cellular protein profiles in response to their microenvironment. The current study applied proteomic techniques, 2-dimensional electrophoresis (2-DE) combined with MALDI-ToF (matrix assisted laser desorption ionisation – time of flight) mass spectrometry, to determine differences in MDM protein expression influenced by PCNU. Results indicated that MDM responded to material chemistry by modulation of structural proteins (i.e. actin, vimentin and tubulin). Additionally, intracellular protein modulation which requires proteins responsible for trafficking (i.e. chaperone proteins) and protein structure modification (i.e. bond rearrangement and protein folding) were also altered. This study demonstrated for the first time that a proteomics approach was able to detect protein expression profile changes in MDM cultured on different material surfaces, forming the basis for utilizing further quantitative proteomics techniques that could assist in elucidation of the mechanisms involved in MDM-material interaction.

INTRODUCTION

Recent studies have focused on identifying the cellular mechanisms responsible for regulating the release of biological constituents known to degrade polyurethane (PU) surfaces¹. Monocyte-derived macrophages (MDM) are likely to be the major contributor to degradation of PU materials since they remain at the cell-material interface for the lifetime of implanted devices², fusing to form foreign body giant cells, which is a hallmark of chronic inflammation³. Several studies have demonstrated the destructive capacity of inflammatory MDM toward model PUs⁴⁻⁶ where biodegradation was assessed visually, using scanning electron microscopy and quantitatively, measuring the products liberated from radiolabelled PUs. In order to piece together the pathways that are involved in the biodegradation process, numerous signaling and enzymatic pathways have been assessed for their involvement⁷. Furthermore, studies have demonstrated a role for serine proteases/esterases⁸, phospholipase A₂ (PLA₂)⁹ and protein kinase C¹⁰ in biodegradative processes of inflammatory cells. Although these pathways each contribute to the process of PU biodegradation, a global approach to assess the effect of material chemistry on the protein profile of these inflammatory cells has not yet been examined. Hence, a proteomics approach which allows for the identification of additional proteins and, subsequently, inflammatory and degradative pathways that have perhaps not yet been considered in the MDM responses to PU surfaces is warranted.

In order to achieve an assessment of the MDM response to PU with respect to changes in protein expression and proteome profiles, the current study thus employed the tools of proteomics. Proteomics involves the study of all proteins expressed by cells¹¹ with the

human MDM proteome being defined as all *proteins* expressed by the MDM *genome*¹². The first detailed 2-dimensional electrophoresis (2-DE) map of primary human MDM was published recently in 2004¹³. This study identified proteins isolated from the MDM proteome and secretome (the profile of proteins secreted by MDM into the surrounding culture medium) of primary human MDM cultured on a tissue culture grade polystyrene (PS) substrate. For separation of proteins within a complex mixture, the techniques of 2-DE combined with matrix assisted laser desorption ionisation - time of flight mass spectrometry (MALDI-ToF MS) was used for protein identification in order to generate proteome and secretome expression profile maps of MDM¹³. 2-DE has long been accepted as a popular method of protein separation¹⁴. The combination of these proteomics techniques (2-DE and MALDI-ToF MS) constitutes the most common proteomics method for identifying multiple proteins within a cell type and has continually increased in reliability and reproducibility with advances in methods and automation^{11,14,15}.

Prior to the above mentioned study¹³, a reference map of the human MDM proteome had not been generated. These reference MDM proteome maps generated have created a basis from which investigations into macrophage responses can be explored. Other studies have applied proteomics approaches to determine the effect of various stimuli on human monocytes^{16,17} as well as assessing putative monocyte differentiation pathways. U937 cells, a promonocytic cell line that can be differentiated to a macrophage-like cell¹⁸, have been used as a model for macrophage studies⁶. In studying monocyte differentiation, proteomic analyses of U937 cells have been generated assessing the

protein profiles of undifferentiated cells in comparison to PMA (phorbol 12-myristate 13-acetate) differentiated U937 macrophage-like cells¹⁹ as well as U937 cells differentiated to a macrophage-derived foam cell phenotype²⁰. Additionally, primary human monocytes have been utilized in proteomics studies to assess differences between the undifferentiated state and that of monocyte-derived dendritic cells^{21,22}. To date however, studies have not been conducted to investigate the influence of material surfaces on the proteome of human monocytes differentiating along the macrophage lineage. Moreover, very few studies have applied proteomics techniques to investigate the degradation of biomaterials (such as PU) and these studies have mainly been limited to characterizing the profile of proteins that adsorb onto various biomaterial surfaces using MALDI-ToF MS alone^{23,24}.

Using the proteomics approach of combining 2-DE and MALDI-ToF MS, an initial investigation into the differences in protein expression profiles were compared between MDM exposed to PS as the control non-degradable surface and a model polycarbonate-urethane (PCNU) which was synthesized using 1,6-hexane diisocyanate (HDI), a polycarbonate (PCN) polydiol and butanediol (BD) chain extender in a stoichiometric ratio of 4:3:1 respectively (HDI431). This model PCNU demonstrated high degradability by candidate esterases^{8,25} and MDM⁵. In addition to its degradability, HDI431 also demonstrated a significant effect on MDM morphology and function²⁶. HDI431 (relative to PS) induced a significant decrease in cell area, increase in MDM multinucleation and foreign body giant cell formation consistent with inflammation and a foreign body response^{2,3}. In addition, punctate filamentous actin (F-actin) rich structures were evident

throughout the entire cell-material interface of HDI431-adherent cells that were sparse only at the edges of PS-adherent cells²⁶. Moreover, differentiation of monocytes on HDI431 yielded MDM that exhibited significantly less degradative potential when trypsinized and re-seeded on different PCNUs²⁷. These studies have demonstrated that material surface chemistry can significantly alter the morphology and function of MDMs, particularly with respect to degradative capacity. The proteomics approach (2-DE combined with MALDI-ToF MS) in the current study, allowed for the broad characterization and comparison of protein profiles associated with human MDM cultured on PS or PCNU surfaces during a differentiation or biodegradation time point. Although key degradative enzymes were not identified, this study strengthens and contributes to the understanding that MDM are principally influenced in morphology, cell structure and function by material surface chemistry.

MATERIALS AND METHODS

All materials were purchased from Sigma-Aldrich Ltd. (Oakville, ON, CAN or Sydney, NSW, AUS) unless otherwise specified.

Preparation of material surfaces

The model PCNU used in this study was synthesized with a PCN soft segment, BD chain extender and HDI in a 4:3:1 ratio (HDI:PCN:BD)(referred to from here as HDI) as described in detail previously²⁵. A 10% PCNU/dimethylacetamide solution (w/v) was used to coat custom made 15 mm diameter round glass cover slips (Fisher Scientific, Ottawa, ON, CAN), that fit precisely in a 24-well plate as previously described⁵. The PS

surface of the 24-well tissue culture plate was used as a control non-degradable surface. Prior to use, surfaces were incubated with phosphate buffered saline (PBS) at 37°C and 5% CO₂ with 100% humidity overnight to remove any traces of solvent and equilibrate the PCNU slips to an aqueous environment.

Monocyte-derived macrophage cell culture

Whole blood (120 mL per isolation) was collected from healthy human volunteers (with approval from the Research Ethics Committee at the University of Ottawa Heart Institute, Ottawa, ON, CAN) into EDTA Vacutainers[®] (Becton Dickinson, Toronto, ON, CAN). Monocytes were isolated by layering whole blood onto Histopaque[™] 1077 as described in detail previously⁴. The mononuclear layer (~20% monocytes) was collected and the cell suspension ($6-8 \times 10^6$ cells/mL) seeded into PS 12-well plates (VWR, Toronto, ON, CAN). Cells were fed every other day with 2 mL of media containing RPMI-1640 supplemented with 10% fetal bovine serum (FBS), L-glutamine (0.69mM), penicillin/streptomycin (100 U/mL / 100 µg/mL; Invitrogen, Toronto, ON, CAN) until day 14 of differentiation. Differentiated mature MDM were then lifted by gentle trypsinization as previously described⁵. This method of gentle trypsinization has proven to be a mode of “activation” in this model cell system since PU degradation is seen only by mature MDM cells post-trypsinization and not from differentiating MDM alone²⁸. The adherent differentiated MDM were treated briefly with 0.25% Trypsin/EDTA, gently resuspended with 5% FBS in Hanks balanced salt solution, centrifuged at 450 x g and resuspended in media with 10% FBS as a 1×10^6 cells/mL suspension. The MDM cell suspension (1mL) was seeded into PS 24-well plates alone and into wells containing HDI

coated slips and allowed to attach for 2 hours. For culture of MDM to be used for microscopy, cells were seeded into wells containing 13 mm diameter round PS cover slips (Sarstedt Inc, Montreal, ON, CAN). Subsequent to attachment (2 hours), the medium was aspirated, and the wells were washed twice with serum-free medium (the same medium used above without the addition of FBS) and replaced with 1 mL of fresh serum-free medium, in order to reduce FBS interference during proteomic analysis, and further incubated for 24 hours. No difference in MDM cell viability or morphology was observed after incubation with serum-free medium (data not shown).

Alternatively, in order to assess changes in monocyte protein profiles during the progression of differentiation to MDM on PS versus HDI, cells were also cultured on each surface following monocyte isolation and cell lysates were collected at day 7 of the differentiation period. For this procedure, following monocyte isolation, 1 mL of the cell suspension from the mononuclear fraction ($\sim 6-8 \times 10^6$ cells/mL) was seeded into PS 24-well plates alone or in wells with HDI coated slips and the culture medium (containing 10% FBS) was changed every other day. Only $\sim 20\%$ of this mononuclear fraction is typically monocytes that will subsequently adhere to the material surface. For culture of MDM to be used for microscopy, cells were seeded into wells containing 13 mm diameter round PS cover slips, rather than the surface of the PS 24-well plate. On day 6, the medium was aspirated, and wells were washed twice with serum-free medium and further incubated for 24 hours.

Fluorescence microscopy for cellular morphology

MDM that were cultured on PS or HDI slips as stated above were prepared for fluorescence confocal laser scanning microscopy to examine differences in cellular morphology. MDM either differentiated for 7 days on PS coverslips or HDI-coated glass slips or mature MDM trypsinized and reseeded for 24 hours on PS or HDI were washed with warmed PBS and permeabilized with 0.5% Triton-X 100/EDTA for 5 minutes. Each slip was then washed with PBS and fixed with 4% paraformaldehyde for 10 minutes. The slips were washed again with PBS before incubating slips with rhodamine phalloidin (Molecular Probes, Burlington, ON, CA), which stains F-actin, for 30 minutes in a humid chamber. Slips were then washed in PBS before and after incubating slips with DRAQ5™ (Axxora, San Diego, CA, USA) as a counterstain for DNA. Slips were then mounted onto microscope slides (Fisher Scientific) with 90% glycerol and covered with 22 mm diameter round glass cover slips. Cells were visualized with an Olympus 100X oil immersion objective with a numerical aperture of 1.4, on an Olympus IX80 laser scanning confocal microscope operated by FV1000 software (version 1.4a). The rhodamine phalloidin and DRAQ5™ fluorochromes were excited with the 543 nm line of the He/Ne green laser and the 663 nm line of the He/Ne red laser respectively.

Sample preparation for proteomic analysis

All sample preparation was conducted under laminar flow to minimize keratin contamination. Differentiating MDM cultured for 7 days, or trypsinized (14 day differentiation) MDM reseeded for 24 hours were lysed as previously described²⁶. As a slight modification, 1mM PMSF (phenyl methyl sulfonyl fluoride) was added to the 0.5%

Triton-X 100/EDTA lysis buffer to prevent protein degradation. For each experiment, depending on the mononuclear cell count of each donor at the time of isolation, ~20 wells of differentiating monocytes or ~10 wells of trypsinized and reseeded MDM were pooled together from each surface for protein precipitation in order to analyze protein expression from the same number of cells. Cell lysates were collected into polypropylene centrifuge tubes (Sorvall, Newtown, CT, USA) and 90% (w/v) trichloroacetic acid (TCA) added in a 1:10 ratio (v/v) and incubated on ice for 3 hours. Precipitated samples were centrifuged for 15 min at 30,000 x g, the supernatants were discarded and cell pellets resuspended in 40 mM acetic acid/ethanol (1 mL) and transferred to microcentrifuge tubes, then centrifuged at 18,000 x g for 15 min. The pellets were resuspended in 1 mL of ice cold 100% acetone and sonicated for 1 min and finally centrifuged at 18,000 x g for 15 min. The supernatants were discarded and the final pellets air dried for 1 hour to ensure all traces of acetone were removed and samples were stored at -80°C. Precipitated protein pellets were resolubilized in buffer containing 8 M urea, 100 mM DTT (dithiothreitol), 4% (w/v) CHAPS (3-[(3-Cholamidopropyl)-Dimethylammonio]-1-Propane Sulfonate), 0.2% (v/v) carrier ampholytes (pH 3-10; Bio-Rad, Hercules, CA, USA), 40 mM Tris Base (pH 7), 0.02% (w/v) bromophenol blue. Samples were then vortexed and sonicated for 30 sec before beginning 2-DE.

Two dimensional electrophoresis

Isoelectric focusing was carried out by passive rehydrating of linear gradient Ready Strip™ IPG strips (18 cm, pH 3-10; Bio-Rad) with 350 µL of resolubilized protein samples for 8 hours. Rehydrated strips were loaded onto a horizontal Multiphor II

electrophoresis unit (Amersham Biosciences, Castle Hill, NSW, AUS) and focused for 100 kVh (kilovolt-hours) with cooling at 15°C. Focused IPG strips were then equilibrated for 30 minutes with slow shaking in equilibration solution containing 6 M urea, 2% (w/v) SDS (sodium dodecyl sulfate), 0.375 M Tris-HCl (pH 8.8), 20% (v/v) glycerol, 100 mM DTT and 2.5% (w/v) acrylamide. Equilibrated IPG strips were laid onto the second dimension (8-18% polyacrylamide gradient gels - 20 cm x 20 cm x 2 mm) using an Ettan™ DALTsix electrophoresis unit (Amersham Biosciences). Proteins were separated in the second dimension by applying a current, 40 mA/gel for two hours followed by 50 mA/gel for a further 10 hours, at 15°C. Each gel was then fixed in a solution of 10% (v/v) methanol and 7% (v/v) acetic acid for a minimum of 2 hours and then stained overnight with SYPRO Ruby® (Bio-Rad) fluorescent protein stain and subsequently destained in the above fixing solution for 3 hours^{29,30}. Protein detection was achieved with a Molecular Imager FX™ System using Quantity One® (Bio-Rad) software at a pixel resolution of 200 µm and 618 nm wavelength. For protein visualization, gels were stained with Coomassie G-250 overnight and destained in 1% acetic acid before manual excision of visible protein spots. Gel pieces were transferred into 1.5 mL microcentrifuge tubes and stored at 4°C until use.

Protein identification by MALDI-ToF MS

Excised gel pieces were first destained by washing twice in 120 µL 25 mM NH₄HCO₃ containing 50% (v/v) acetonitrile for 30 min followed by drying in a Speedvac® (Thermo Electron, Milford, MA, USA). In-gel digestion of proteins was achieved by reswelling gel pieces with 15 µL of 20 ng/µL sequencing grade trypsin (Promega, Annandale, NSW,

AUS) in 25 mM NH_4HCO_3 (pH 7.8) for 1 hour at 37°C followed by the addition of 20 μL of 10 mM NH_4HCO_3 and further incubation for 18 hours at 37°C. Gel pieces were then sonicated for 20 min to liberate peptide fragments from the gel and briefly centrifuged at 15,000 x g for 30 sec. The trypsin extracts were concentrated and desalted using C_{18} ZipTips[®] (Millipore, North Ryde, NSW, AUS) and eluted with 2 μL of matrix (8 mg/ml α -cyano-4-hydroxy cinnamic acid in 80% acetonitrile (v/v), 0.1% (v/v) trifluoroacetic acid) onto the MALDI target plate and dried at room temperature. Peptide mass fingerprinting (PMF) was performed using a Voyager-DE[™] STR mass spectrometer (AME Bioscience, Torøed, Norway) with delayed extraction in reflectron mode with an accelerating voltage of 20,000V. Spectra were obtained over the m/z range of 800-3500 Da and internal calibration was conducted using trypsin auto digestion peaks at 842.51 Da and 2211.11 Da on all spectra. The peptide mass spectra obtained were used to generate peptide mass lists to submit queries to the Swiss-Prot protein knowledgebase (restricted to mammalian proteins) using the Mascot search engine (www.matrixscience.com). Search parameters were set to allow a tolerance of one missed trypsin cleavage, mass tolerance of ± 100 ppm, and to allow for oxidation to methionine residues. Identified proteins were accepted when query matches contained four or more peptides and those peptide masses matched with statistically significant Mowse (molecular weight search) scores as generated by the Mascot search engine.

RESULTS AND DISCUSSION

The current study is the first to integrate proteomic techniques with the study of cell-material interactions considering the effect of material chemistry on monocyte

differentiation to mature MDM, as well as mature trypsinized MDM that exhibit degradation of PCNUs. The rationale for these time point result from previous studies by Labow et al^{4-6,8,27,28}. The *in vitro* model of degradation established involves monocyte to macrophage differentiation solely by adherence to PS for 14 days in typical serum supplemented culture medium. Alternative *in vitro* models have applied combinations of cytokines such as IL-4 and IL-13 and GM-CSF in order to accelerate monocyte to macrophage differentiation and foreign body giant cell formation^{31,32}. In a previous study, primary human monocytes were found to display the macrophage marker CD68 in 95% of the population by 72 hours after adherence to PS without additional cytokine supplementation in the culture medium³³. Several resulting studies have demonstrated 14 days of monocyte to macrophage differentiation to be optimal for subsequent PU degradation assays^{5,28}. Moreover, an extensive study of monocyte to macrophage differentiation without cytokine supplementation, characterized the expression of several monocyte and macrophage markers through 14 days of differentiation, demonstrating the expression of macrophage and not monocyte nor dendritic cell CD markers at day 14³⁴. Additional characteristics of differentiated MDM include increased cytoplasmic area³⁵, protein synthesis³⁶ and multinucleation³ which were all evident in the current cell system. Essentially, this 14 day MDM model system was selected based on the combination of two factors: the number of MDM obtained following trypsinization and the amount of esterase activity (a measure of biodegradative capacity for PCNU) in each MDM described in detail previously²⁸.

The current study evaluated protein profiles of monocytes undergoing differentiation towards the macrophage lineage (7 day differentiation) at a time point when macrophage markers are displayed (> 3 days)³³. The 7 day time point was selected on the basis that it is halfway along the optimal 14 day time point of differentiation in the model system currently used to study biodegradation, and thereby assessing the MDM undergoing differentiation as it relates to the model system previously described²⁸. *In vivo*, monocytes are recruited to the site of the biomaterial and would then differentiate upon this surface. It is evident that this material would thus influence the characteristics/phenotype of the mature MDM. As outlined in a recent review of PU degradation, the combination of the differentiation state of a monocyte along with the chemistry of the material surface upon which it is adherent influences the numerous cellular mechanisms resulting in the release of an unknown biological element(s) that play a role in PU degradation¹. As a model of *in vitro* macrophage-mediated biodegradation, mature MDM (as described above) were trypsinized and re-seeded onto ¹⁴C-radiolabelled PCNU surfaces in order to measure degradation⁵. Material degradation was measured after 48 hours post-trypsinization and reseeded to the PCNU surface^{5,6,8}. Therefore, in addition to investigating the proteome profiles of differentiating MDM, the current study assessed the protein profile changes in MDM between PS and HDI during this period of material degradation (at 24 hours).

Previously, it was demonstrated that the material surface upon which these MDM were differentiated had an influence on the biodegradative capacity of these cells when reseeded onto radiolabelled model PCNU and analyzed for candidate enzyme protein and

activity in addition to release of radioactivity from ^{14}C -PCNU²⁷. Therefore, there must be significant intracellular differences in the protein expression of the MDM that are influenced by PCNU during monocyte to macrophage differentiation. Several previous studies have indicated a role for esterase enzymes in the degradation of PU materials^{4,5,8,25,26,28}. These enzymes in addition to PLA₂, another enzyme under investigation for an association with macrophage mediated PCNU biodegradation⁹, were not identified in this study. Both of these proteins are likely low abundance proteins and were therefore not detected by MALDI-ToF MS. Sensitivity remains one of the drawbacks of this approach. In general, only protein spots that are stained darker by Coomassie staining will likely be detected by MALDI-ToF MS. Therefore, many less intense proteins spots visualized by a much more sensitive SYPRO Ruby[®] or silver stain will not be identified by this method. An additional drawback of the 2DE approach is incomplete separation of proteins. Since the mixture of proteins in a complex sample, such as whole cell lysate, contains such a great number of proteins, there will often be more than one protein in a spot that appears to the naked eye as one spot. If one protein in this spot is a high abundance protein, it may mask any other lower abundance proteins in the same protein spot or protein spots of close proximity. This would be the case with actin, which possesses very similar MW (~50-60 kDa) and pI (~5-6) as monocyte specific esterase, therefore leading to the possibility that candidate esterases were undetectable because of the great abundance of actin expressed in these samples. Regardless of the fact that degradative enzymes were not identified in this study, the results below indicate significant cell morphology, structure and function adaptations in response to material differences. These may not reflect direct changes in the degradative

capacity of MDM in response to PCNU, however, indicate functional changes that may facilitate the movement and release of key enzymes from cytoplasmic or vesicular compartments to the extracellular space to gain access to the foreign body.

MDM proteome profile changes in response to PCNU during differentiation

Using the technique of 2-DE provided visualization of the MDM proteome map which displayed differences in protein profiles obtained when monocytes were differentiated on either PS or HDI. Figure 1A and 1B represent images of SYPRO Ruby[®] stained 2-DE gels generated from MDM differentiated on PS or HDI respectively for 7 days. Equivalent amounts of MDM protein (900 µg) from the same donor were run on each gel. A visual inspection of the protein spots revealed observable differences in the MDM proteome maps between PS- and HDI-adherent cells. The numbering of the spots in Figures 1 and 3 was initiated near the top left corner of each gel and continued in approximately a clockwise manner. Upon inspection of the protein spots expressed in MDM between PS and HDI during differentiation, it is evident that the majority of proteins are increased in expression in cells adherent to HDI (Figure 1B) compared to the protein profile on PS (Figure 1A). In order to further characterize the proteome profiles generated, protein spots of interest were excised from the gels and identified by PMF (MALDI-ToF MS). Proteins from each gel that were successfully identified are listed for MDM differentiating on PS (Table 1) and HDI (Table 2). The results in Tables 1 and 2 list the spot number for each protein spot indicated on the 2-DE gel image, accession number from Swiss-Prot (<http://www.expasy.org/sprot/>) for each protein identified,

Figure 1 – 2-D electrophoresis gel images of cell lysates from primary human monocytes differentiating to macrophages on either A) PS or B) HDI for 7 days. Equal amounts of protein (900 µg) were subjected to isoelectric focusing on linear 18 cm pH 3-10 IPG strips, followed by 8% - 18% gradient SDS-PAGE. Gels were stained with SYPRO Ruby[®] to obtain images and then subsequently stained with Coomassie G-250 in order to excise spots for peptide mass fingerprinting by MALDI-ToF mass spectrometry. Spots that were positively identified (Tables 1 & 2) by peptide mass fingerprinting are indicated by arrows.

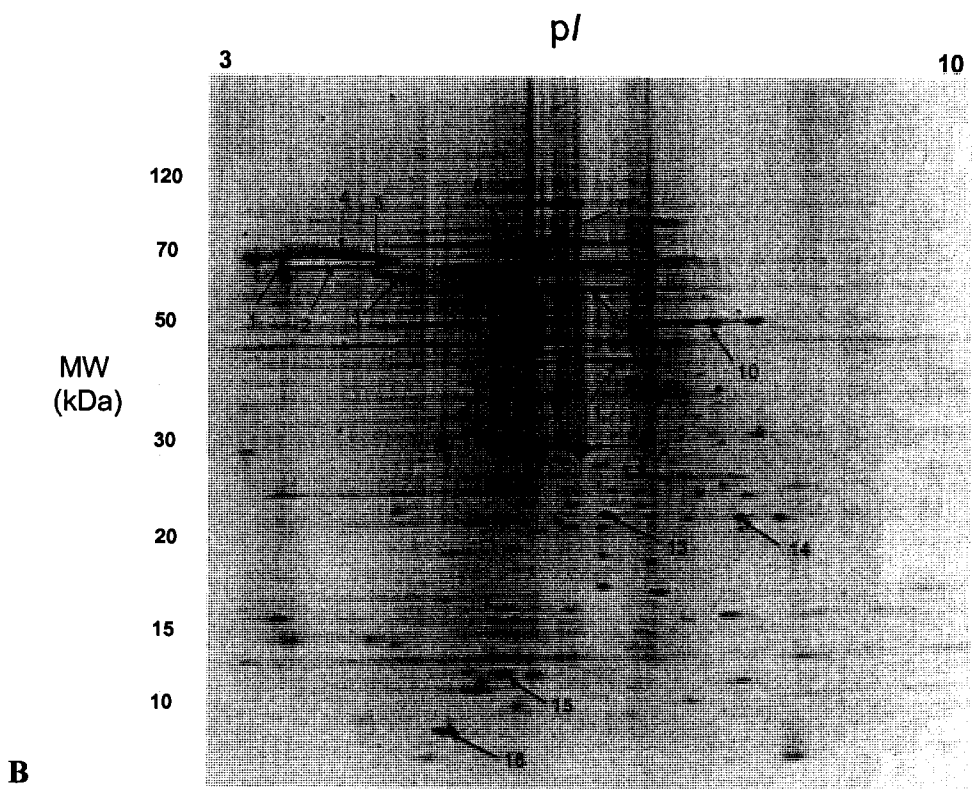
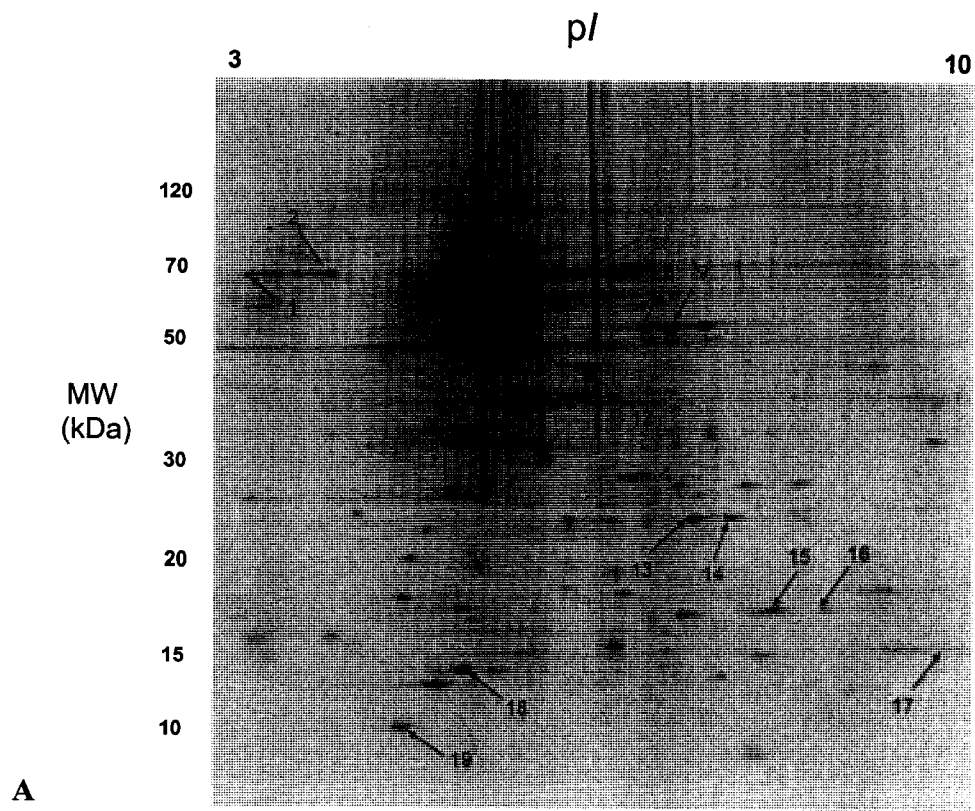


Table 1 – Identified proteins from cell lysate protein spots excised from a 2-D electrophoresis gel (Figure 1A) of human monocytes differentiating on PS to macrophages (7 days)

Spot Number	Accession Number (SwissProt)	Protein Name	MW (kDa)	pI	Function
1	P27797	Calreticulin precursor	48.1	4.3	chaperone
2	P27797	Calreticulin precursor	48.1	4.3	chaperone
3	P07437	Tubulin β -1 chain	49.6	4.8	microtubules
4	P49065	Serum Albumin precursor	68.9	5.8	FBS contamination
5	P08238	Heat shock protein HSP-90 β	83.1	5.0	chaperone
6	P63017	Heat shock cognate 71kDa protein	70.9	5.4	chaperone
7	P08670	Vimentin	53.5	5.1	cytoskeleton
8	P06733	Alpha enolase	47.2	7.0	multifunctional
9	P06733	Alpha enolase	47.2	7.0	multifunctional
10	P60709	Actin, cytoplasmic 1 (β -Actin)	41.7	5.3	cell motility
11	P60709	Actin, cytoplasmic 1 (β -Actin)	41.7	5.3	cell motility
12	P40121	Macrophage capping protein	38.5	5.9	actin remodelling
13	Q06830	Peroxiredoxin 1	22.1	8.3	redox regulation
14	Q8HXP7	Superoxide dismutase	22.2	6.9	destruction of toxic radicals
15	P62937	Peptidyl-prolyl cis-trans isomerase A	17.9	7.8	protein folding
16	P62937	Peptidyl-prolyl cis-trans isomerase A	17.9	7.8	protein folding
17	P07737	Profilin-1	14.9	8.5	actin polymerization
18	P09382	Galectin-1	14.6	5.3	regulation of differentiation
19	Q9H299	SH3 domain-binding glutamic acid-rich-like protein 3	10.4	4.8	modulate glutaredoxin activity

Table 2 – Identified proteins from cell lysate protein spots excised from a 2-D electrophoresis gel (Figure 1B) of human monocytes differentiating on HDI to macrophages (7 days)

Spot Number	Accession Number (SwissProt)	Protein Name	MW (kDa)	pI	Function
1	Q62725	Nuclear transcription factor Y subunit γ	37.2	4.9	gene transcription (β -actin)
2	P27797	Calreticulin precursor	48.1	4.3	chaperone
3	P02545	Lamin A/C	74.1	6.3	nuclear lamina component
4	Q9NSV4	Diaphanous protein homolog 3	98.6	5.8	recruits profilin to membrane
5	Q9NSV4	Diaphanous protein homolog 3	98.6	5.8	recruits profilin to membrane
6	P02769	Serum Albumin precursor	68.9	5.8	FBS contamination
7	P63017	Heat shock cognate 71 kDa protein	70.9	5.4	chaperone
8	P08670	Vimentin	53.5	5.1	cytoskeleton
9	P30101	Protein disulphide isomerase	56.8	6.0	disulphide bond rearrangement
10	P06733	Alpha enolase	47.2	7.0	multifunctional
11	P40121	Macrophage capping protein	38.5	5.9	actin remodelling
12	P07339	Cathepsin D precursor	44.6	6.1	intracellular protein breakdown
13	O35165	27kDa golgi SNARE protein	24.7	6.8	protein transport
14	Q06830	Peroxiredoxin 1	22.1	8.3	redox regulation
15	P09382	Galectin-1	14.6	5.3	regulation of differentiation
16	Q9H299	SH3 domain-binding glutamic acid-rich-like protein 3	10.4	4.8	modulate glutaredoxin activity

protein name, along with the theoretical MW (kDa) and pI and proposed function (as outlined by Swiss-Prot).

The identified proteins that demonstrated changes between surfaces fall into 2 general categories: proteins involved in structural organization of the MDM and those involved in protein restructuring, folding and trafficking. A comparison of these proteins between each surface was made in Figure 2 demonstrating the spot intensity differences between PS and HDI. Although it was not possible to report significant changes in amount of specific proteins, this method of visually comparing obvious protein profile differences is widely accepted in the proteomics field^{13,19,21}. Although most of the proteins identified increased in expression on HDI versus PS during differentiation, proteins responsible for the direct structural integrity of the cell, β -actin (Figure 2A – both spots identified as β -actin) and tubulin (microtubules) (Figure 2B) were shown to decrease on HDI. Vimentin, a component of the cytoskeleton, was also identified on PS, however, appears to be unchanged between the material surfaces (Figure 1A – spot 7, Figure 1B – spot 8). Proteins that are involved in the structure of the cell by modifying actin, macrophage capping protein (Figure 2C) (reversibly blocks the barbed ends of actin filaments), or regulating β -actin gene expression, nuclear transcription factor Y (a – Figure 2D), were also identified with an increase evident on HDI. Also involved in the structural integrity of the cell, lamin A/C (b – Figure 2D), which showed increased expression on HDI, is a nuclear lamin component responsible for the framework of the nuclear envelope. This protein may play an important role in macrophages, since they fuse to form multinucleated cells³. Profilin-1, a regulator of actin polymerization, was also identified

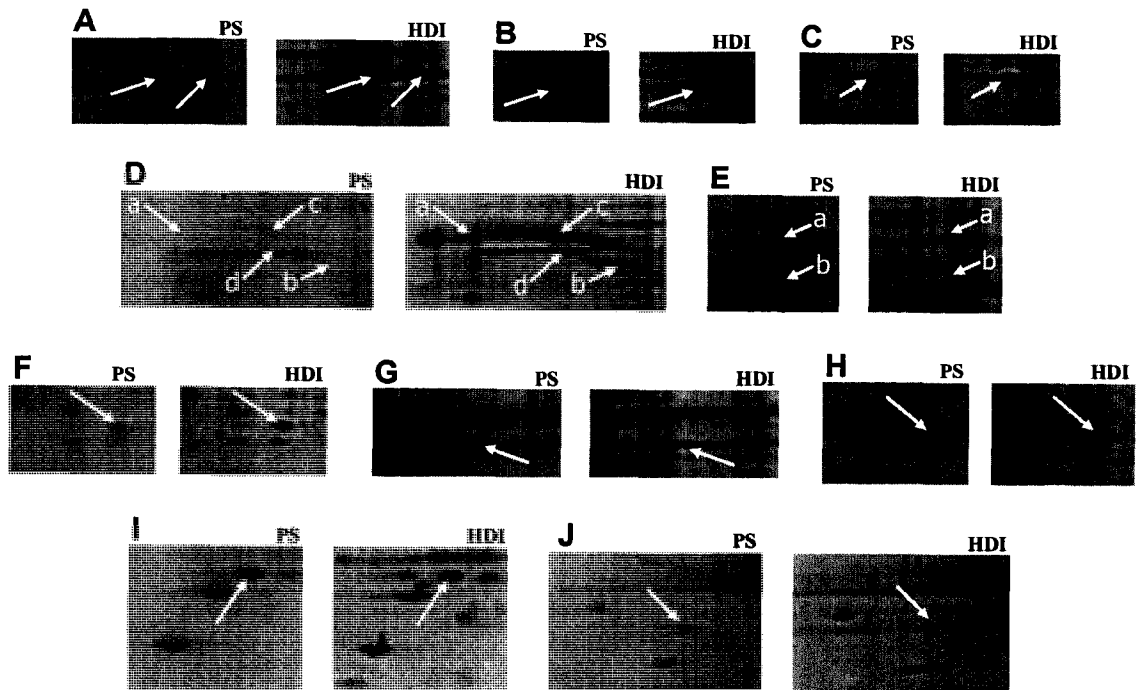


Figure 2 – Comparison of spot intensity between proteins identified by MALDI-ToF mass spectrometry in protein spots excised from 2-DE gels of cell lysates from monocytes differentiated to MDM on PS or HDI for 7 days (Figure 1A and 1B). The intensity of specific spots identified were compared for relative intensity between the spots located on the PS 2-DE gel (Figure 1A) versus spots located on the HDI 2-DE gel (Figure 1B). A – β -actin, B – tubulin β -1 chain, C – macrophage capping protein, D – (a) nuclear transcription factor Y, (b) lamin A/C, (c) diaphanous protein homolog 3 and (d) calreticulin, E – (a) HSP-90 β and (b) heat shock cognate 71kDa protein, F - 27kDa golgi SNARE protein, G – protein disulphide isomerase, H – cathepsin D precursor, I – galectin-1, J – protein spot with decreased intensity on HDI relative to PS.

in MDM on PS (Figure 1A – spot 17), however, appeared unchanged between surfaces. Despite this, a protein responsible for recruiting profilin-1 to the cell membrane to promote actin polymerization, diaphanous protein homolog 3 (c – Figure 2D), is barely detectable on PS though largely expressed on HDI. Changes in MDM protein profiles demonstrate that there are significant changes in the cytoskeletal machinery responsible for cell structure between MDM differentiated on PS versus HDI. These structural differences in MDM exposed to different material surfaces supported previous findings in which F-actin was fluorescently labeled and quantified to measure MDM cell spreading²⁶ between PS and HDI surfaces or relative intensity of F-actin between various PU material surfaces³⁷.

In addition to identifying several proteins associated with cell structure, proteins involved in protein modulation/restructuring and trafficking were identified and differences in protein expression were demonstrated. Several chaperone and transport proteins including calreticulin (b – Figure 2D), heat shock protein-90 β (a – Figure 2E), heat shock cognate 71kDa protein (b – Figure 2E) and 27kDa golgi SNARE protein (Figure 2F) were shown to be differentially expressed between surfaces. Proteins such as protein disulphide isomerase (Figure 2G) and cathepsin D precursor (Figure 2H) that have functions of disulphide bond rearrangement and lysosomal protein breakdown respectively were both increased in expression on HDI relative to PS. Furthermore, proteins involved in protein folding, peptidyl-prolyl cis-trans isomerase A (Figure 1A - spot 15 and 16), was identified on PS, and although this protein appears as though it may decrease in expression on HDI, a conclusion cannot be made without further sensitive

quantification techniques. Another protein of interest was galectin-1 (Figure 2I), which is involved in regulating cellular differentiation, and demonstrated increased expression on HDI. This suggests that the differentiation state or phenotype of MDM on HDI is altered relative to PS. Although the majority of proteins in these MDM profiles increased in expression on HDI, spots such as that indicated in Figure 2J demonstrate that proteins also decreased in expression between the surfaces, confirming that differences in protein spot intensity were not due to protein samples being loaded unequally.

MDM proteome profile changes in cells undergoing material degradation

In addition to the differences exerted on MDM between surfaces during differentiation, changes in the MDM proteome profile were also evident at 24 hours post-reseeding. At this time point, MDM have been shown to be exerting their biodegradative potential towards PCNU^{5,6}, with maximal degradation demonstrated at 48 hours. Figure 3A and Figure 3B represent SYPRO Ruby[®] stained 2-DE gels of human MDM from the same donor and protein amount (200 µg) from trypsinized MDM reseeded onto PS and HDI respectively. Noticeable differences are seen in the proteome maps of these cells, however, the general trend of protein expression is the reverse of differentiating MDM in that the majority of proteins decreased in expression on HDI (Figure 3B) relative to cells reseeded onto PS (Figure 3A). The proteins identified by PMF are indicated in Table 3 (PS) and Table 4 (HDI). Similar to the proteins identified during MDM differentiation, the list of proteins generated are predominantly responsible for actin/cytoskeletal structure and protein remodeling and trafficking. In this case, however, the influence that HDI had on the majority of proteins was in decreasing the expression of these proteins

Figure 3 – 2-D electrophoresis gel images of cell lysates from differentiated trypsinized human monocyte-derived macrophages that have been reseeded onto either A) PS or B) HDI for 24 hours. Equal amounts of protein (200 µg) were subjected to isoelectric focusing on linear 18 cm pH 3-10 IPG strips, followed by 8% - 18% gradient SDS-PAGE. Gels were stained with SYPRO Ruby[®] to obtain images and then subsequently stained with Coomassie G-250 in order to excise spots for peptide mass fingerprinting by MALDI-ToF mass spectrometry. Spots that were positively identified (Tables 3 & 4) by peptide mass fingerprinting are indicated by arrows.

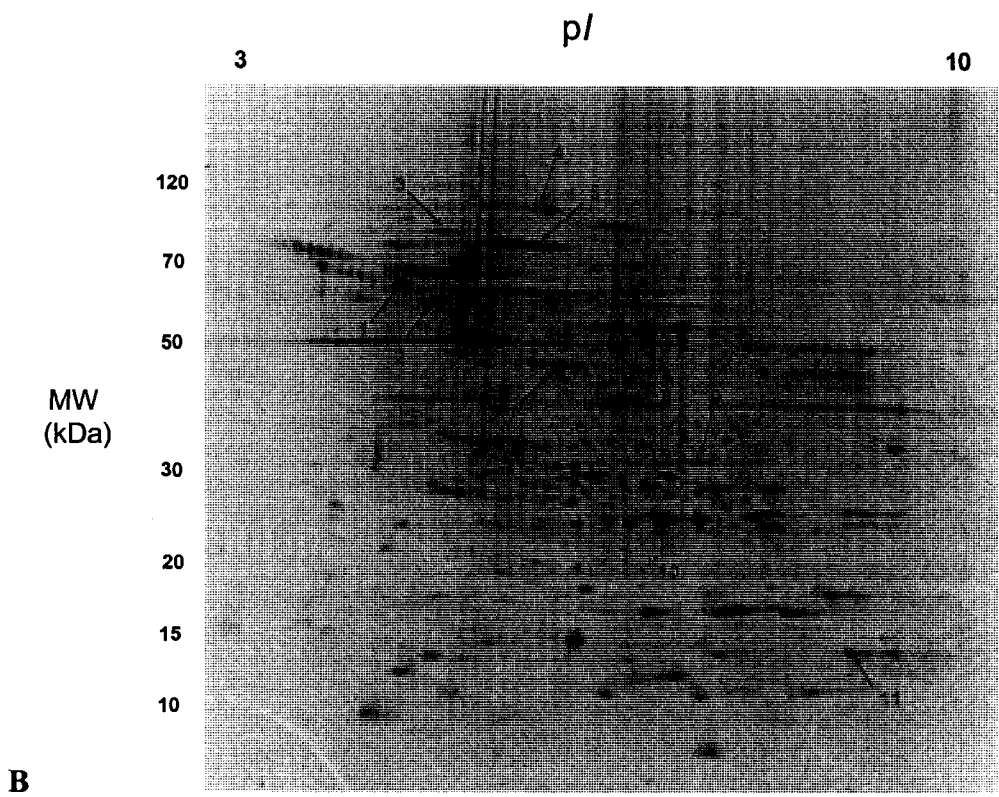
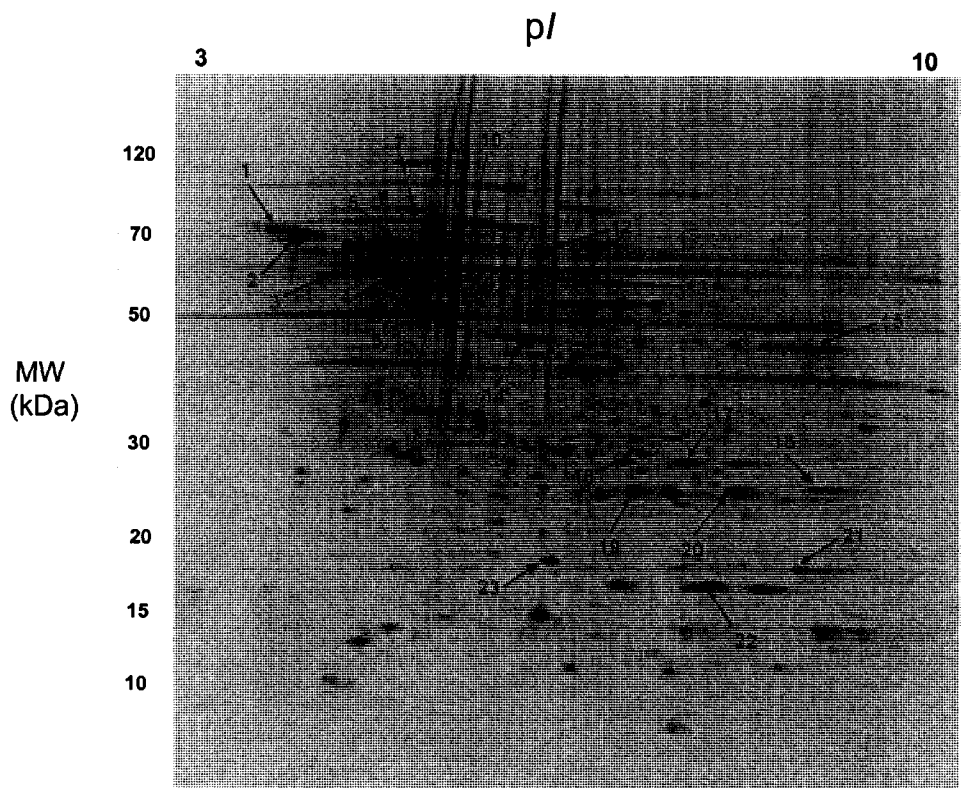


Table 3 – Identified proteins from cell lysate protein spots excised from a 2-D electrophoresis gel (Figure 3A) of trypsinized MDM reseeded on PS for 24 hours

Spot Number	Accession Number (SwissProt)	Protein Name	MW (kDa)	pI	Function
1	Q9Y6U3	Adseverin	80.4	5.5	actin remodelling
2	P27797	Calreticulin precursor	48.1	4.3	chaperone
3	Q6P602	Tubulin	49.7	4.8	microtubules
4	P07437	Tubulin β -2 chain	49.6	4.8	microtubules
5	P06576	ATP synthase β chain	56.5	5.3	ATP synthesis
6	P08670	Vimentin	53.7	5.1	cytoskeleton
7	P11021	78kDa glucose regulated protein precursor	72.3	5.1	protein complex assembly
8	P60709	Actin, cytoplasmic 1 (β -Actin)	41.7	5.3	cell motility
9	P08670	Vimentin	53.7	5.1	cytoskeleton
10	P38646	Stress-70 protein	73.7	5.87	cell proliferation / chaperone
11	P30101	Protein disulfide isomerase	56.7	6.0	disulphide bond rearrangement
12	P08670	Vimentin	53.7	5.1	cytoskeleton
13	P28838	Cytosol aminopeptidase	52.6	6.3	intracellular protein turnover
14	P40121	Macrophage capping protein	38.5	5.88	actin remodelling
15	P04075	Fructose bisphosphate aldolase A	39.3	8.39	glycolysis
16	P60175	Triosephosphate isomerase	26.7	6.4	glycolysis
17	P25774	Cathepsin S precursor	37.5	8.6	lysosomal thiol protease
18	Q06830	Peroxiredoxin 1	22.1	8.3	redox regulation
19	Q06830	Peroxiredoxin 1	22.1	8.3	redox regulation
20	Q06830	Peroxiredoxin 1	22.1	8.3	redox regulation
21	P23528	Cofilin-1	18.5	8.2	actin remodelling
22	P62937	Peptidyl-prolyl cis-trans isomerase A	17.9	7.8	protein folding
23	P23528	Cofilin-1	18.5	8.2	actin remodelling

Table 4 – Identified proteins from cell lysate protein spots excised from a 2-D electrophoresis gel (Figure 3B) of trypsinized MDM reseeded on HDI for 24 hours

Spot Number	Accession Number (SwissProt)	Protein Name	MW (kDa)	pI	Function
1	P07237	Protein disulphide isomerase precursor	57.1	4.8	disulphide bond rearrangement
2	P07437	Tubulin β -2 chain	49.7	4.8	microtubule
3	P11021	78kDa glucose-related protein precursor	72.3	5.1	protein complex assembly
4	P06396	Gelsolin precursor	85.7	5.9	actin remodelling
5	Q8VEB1	G protein-coupled receptor kinase 5	69.2	8.5	GPCR phosphorylation
6	P28838	Cytosol aminopeptidase	52.8	6.3	intracellular protein turnover
7	P40121	Macrophage capping protein	38.5	5.9	actin remodeling
8	P00918	Carbonic anhydrase II	29.1	6.8	reversible hydration of CO ₂
9	O95755	Ras-related protein Rab-36	36.3	8.1	protein transport
10	Q06830	Peroxiredoxin 1	22.1	8.3	redox regulation
11	P07737	Profilin-1	15.1	8.4	actin remodelling

relative to cells adherent to PS post-trypsinization, a process shown to initiate biodegradation following reseeding of these inflammatory cells on PCNU surfaces⁵.

As seen during MDM differentiation, the structural components vimentin (a – Figure 4A and a – Figure 4B), tubulin (b – Figure 4A) and β -actin (Figure 4C) decreased in expression on HDI relative to PS. At this time point, the proteins responsible for remodeling actin decreased in expression, in contrast to the increased expression demonstrated during MDM differentiation. The multifunctional protein gelsolin is involved in both capping the barbed ends of actin as well as severing preformed actin filaments. Decreased expression of gelsolin was shown on HDI relative to PS (a – Figure 4D). Macrophage capping protein (Figure 4E), previously described above, and cofilin-1 (Figure 4F – both spots identified as cofilin-1) which directly binds to actin to polymerize and depolymerize actin in a pH sensitive manner, both decreased in expression on HDI relative to PS. The proteins calreticulin (Figure 4G) and protein disulphide isomerase (c – Figure 4A and c – Figure 4B) mentioned above and cytosol aminopeptidase (b – Figure 4B), responsible for processing and turnover of intracellular proteins and stress-70 protein (a chaperone protein) (b - Figure 4D) all decrease in expression on HDI. The proteins peptidyl-prolyl cis-trans isomerase and protein disulphide isomerase mentioned above were too similar in protein expression between PS (Figure 3A, Table 3) and HDI (Figure 3B, Table 4) to properly quantify without sensitive quantification software. Proteins that were not identified in protein samples during differentiation such as adseverin (an actin filament severing protein), 78kDa glucose regulated protein precursor (protein complex assembly in endoplasmic reticulum) and Ras-related protein Rab-36

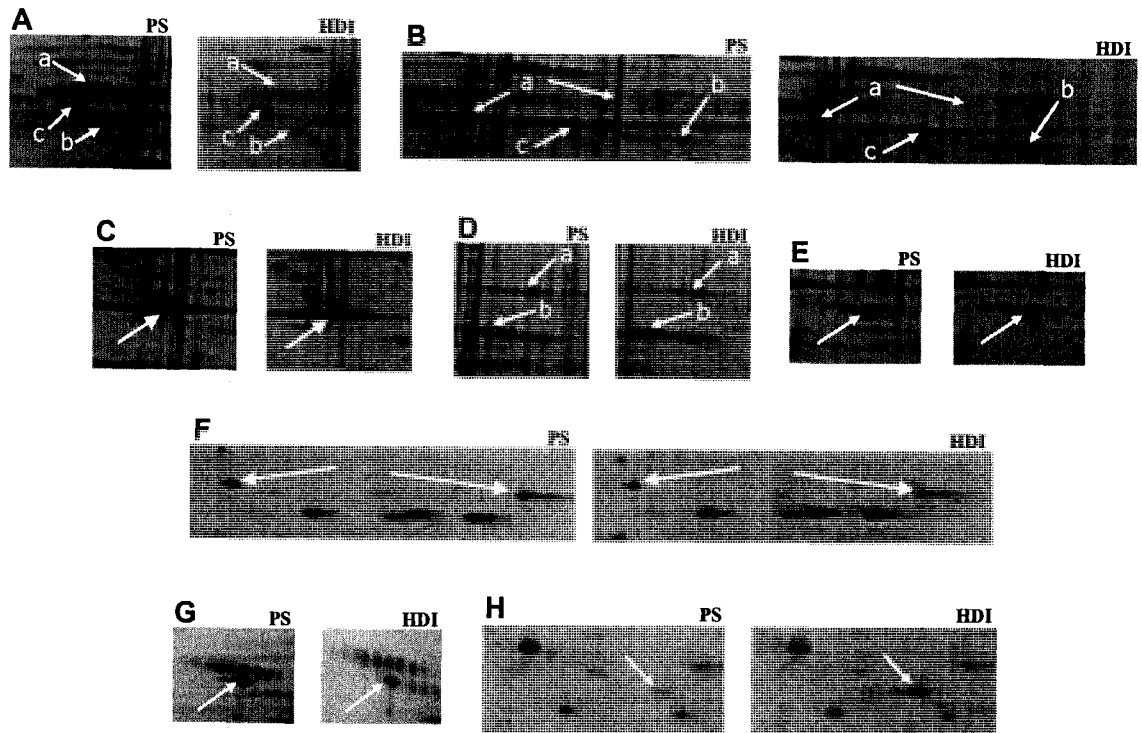


Figure 4 – Comparison of spot intensity between proteins identified by MALDI-ToF mass spectrometry in spots excised from 2-DE gels of cell lysates from trypsinized MDM reseeded on PS or HDI for 24 hours (Figure 3A and 3B). The intensity of specific spots identified were compared for relative intensity between the spots located on the PS 2-DE gel (Figure 2A) versus spots located on the HDI 2-DE gel (Figure 2B). A – (a) vimentin, (b) tubulin β -2 chain and (c) protein disulphide isomerase precursor, B – (a) vimentin, (b) cytosol aminopeptidase and (c) protein disulphide isomerase, C – β -actin, D – (a) gelsolin precursor and (b) stress-70 protein, E – macrophage capping protein, F – cofilin, G – calreticulin, H – protein spot with increased intensity on HDI431 relative to PS.

(involved in protein transport via vesicular trafficking) fall into the categories of actin remodeling and protein restructuring and trafficking. However, differences in these protein expression profiles between surfaces were also difficult to accurately detect. Since the majority of proteins expressed in the mature MDM proteome decrease on HDI relative to PS following reseeding, it is important to point out this was not universally true. Specific proteins demonstrated increased expression on HDI (Figure 4H) and ensured that the general decrease in protein expression of isolated spots was not due to unequal protein loading.

Material effects on macrophage morphology

Although it is difficult to assign an explanation for the differences observed in the cytoskeletal and protein remodeling and trafficking proteins, the striking morphological changes of MDM adherent to these two surfaces can be clearly observed in the fluorescence microscopy images of MDM differentiating on PS (Figure 5A) and HDI (Figure 5B), as well as in trypsinized MDM reseeded to PS (Figure 5C) and HDI (Figure 5D). In comparing MDM morphology during differentiation (Figures 5A, 5B) both surfaces present MDM with punctate actin structures. However these are more evenly dispersed throughout the cell-material interface of HDI-adherent cells. Punctate actin structures tend to be more grouped and less evenly dispersed on PS. MDM on PS are larger and more spread than HDI-adherent cells that are smaller and rounded up. The cells on PS are quite close to each other and even overlapping, perhaps indicating a greater potential to begin to fuse and form foreign body giant cells, a typical inflammatory response that has been suggested to be a self-protective mechanism for

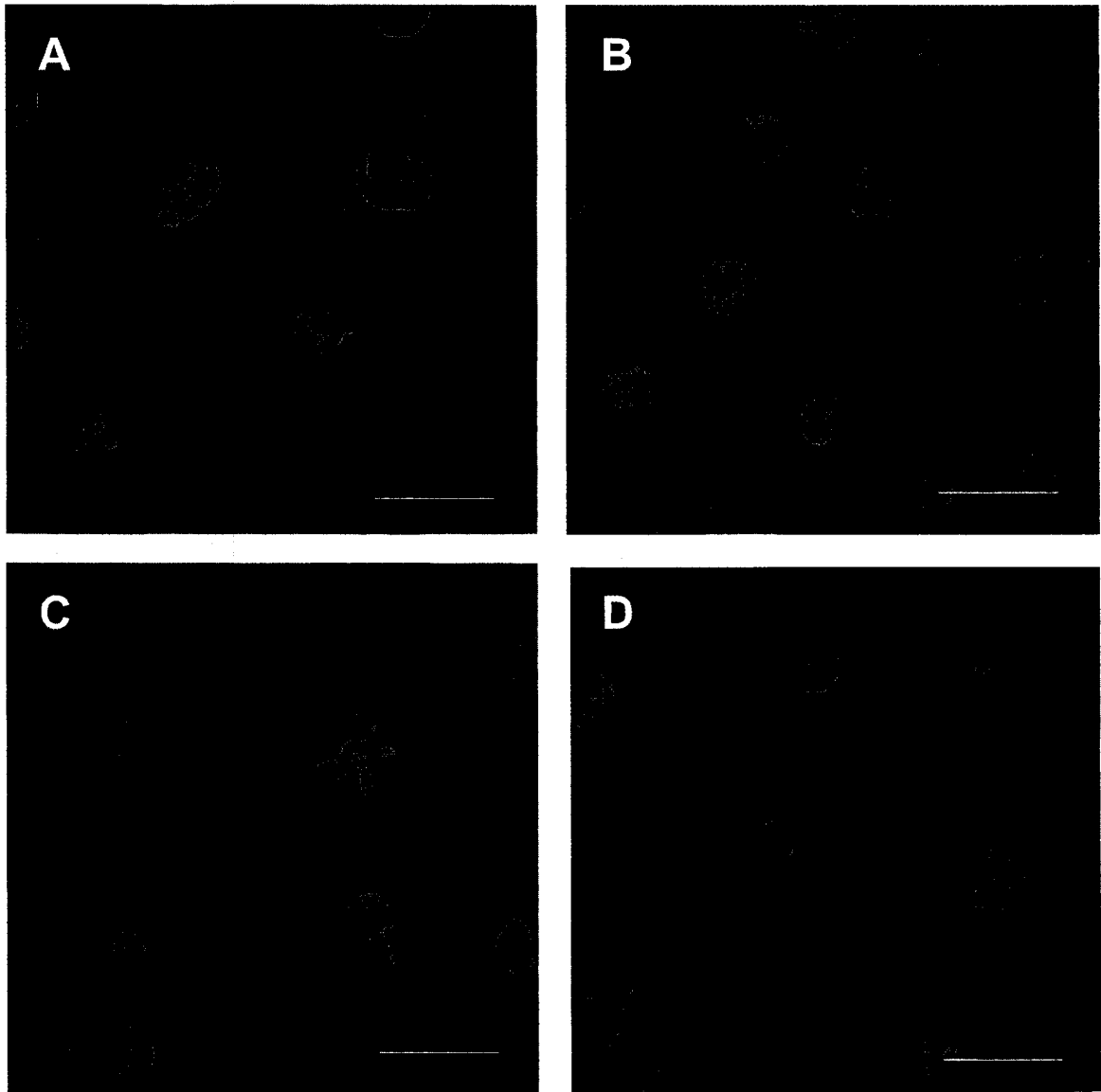


Figure 5 – MDM cytoskeleton organization is dependent on material surface. Representative fluorescence microscopy images of MDM stained with rhodamine phalloidin for F-actin (red) and DRAQ5™ for DNA (blue). Presented are representative fluorescence images of MDM differentiated for 7 days on either (A) PS or (B) HDI or trypsinized MDM reseeded onto either (C) PS or (D) HDI for 24 hours. Scale bar = 20 μm.

MDM³⁸. The morphological differences are not as evident in trypsinized reseeded MDM (Figures 5C, 5D). The punctate actin structures are no longer as evenly dispersed as was evident in differentiating MDM on HDI. In addition, the cells adherent to HDI are smaller than those adherent to PS. These morphological differences confirmed results seen in a previous study where phase contrast, scanning electron microscopy and fluorescent microscopy assessed the morphological differences in MDM adherent to PS and HDI 48 hours after trypsinization and re-seeding and confirmed that MDM were significantly larger on PS than HDI²⁶. In addition, other studies have demonstrated significant F-actin reorganization during monocyte-to-macrophage progression³¹ and in response to material differences³⁷ in cytokine induced MDM.

Associations between protein expression and material biodegradation

Another possible explanation for two proteins identified on HDI lies in a possible association between material surface activation of PLA₂ and the cytoskeleton. Gelsolin (Figure 3B – spot 4) and G protein-coupled receptor kinase 5 (Figure 3B – spot 5) are of interest and relevance to a previous study⁹, which investigated the role of PLA₂ association with biodegradation of PCNUs. The activation of PLA₂ in macrophages on HDI was significantly greater than macrophages on PS, with inhibitors of PLA₂ decreasing both the activation of this pathway (as assessed by arachidonic acid release) as well as subsequent material degradation by macrophages⁹. Gelsolin has been demonstrated to be regulated by lysophosphatidic acid³⁹, a downstream product of PLA₂ mediated membrane phospholipid hydrolysis⁴⁰. This could potentially provide a link between PLA₂ activation seen by PCNU in this previous study and cytoskeleton

remodeling evident by changes seen in the current and other studies^{26,37}. The protein G protein-coupled receptor kinase 5 is responsible for phosphorylating activated forms of G protein-coupled receptors. Although the MDM receptor(s) that are responsible for recognition of PCNU surfaces are as yet unknown, the identification of this protein may indicate that the receptor hypothesized, in the schematic diagram in Figure 6 by Dinnes et al.⁹, to be involved in surface recognition may be a G protein-coupled receptor. The current proteomics approach did not identify any potential membrane receptors responsible for material degradation. This may lie in the fact that in separating proteins by 2-DE, many of the proteins at the 'extreme' ends of MW and *pI* and also hydrophobic proteins such as membrane proteins will not be completely solubilized and therefore not successfully incorporated into the IPG strips. As a result, the 2DE + MALDI-ToF MS approach may provide an incomplete proteome. Despite these drawbacks, this method has provided support for pursuing further techniques in determining the full proteome profile of MDM at a cell-material interface.

CONCLUSIONS

The global expression of proteins by MDM in contact with different material surfaces was investigated using the proteomics techniques of 2-DE and MALDI-ToF MS. This study provides a new perspective in examining the effects of material surface chemistry on MDM structure and function. By assessing a time course of PCNU biodegradation by MDM, in comparison to MDM responding to PS (a non-degradable control surface), and the associated MDM differentiation on each surface, it has been possible to identify proteins that are involved in the cellular mechanisms that influence these cells to respond

so specifically to each surface. Collectively, the proteins identified can be categorized into two broad areas: proteins involved in structural cell remodeling (actin/cytoskeleton/microtubule formation and rearrangement) and those responsible for protein folding, trafficking (chaperone proteins) and restructuring. By the identification of proteins that are shown to have altered expression between PS and HDI, the results demonstrated that MDM undergo cellular remodeling and restructuring in order to generate an appropriate inflammatory foreign body response to the material surface to which the MDM was exposed. These changes are not only reflected in the MDM proteome, but also in the morphological changes exhibited by these cells during differentiation and biodegradation in response to material surface changes. This study demonstrated for the first time that a proteomics approach is useful in demonstrating protein expression profile changes in MDM cultured on different material surfaces. These results support further detailed proteomics techniques with the ability to quantify changes in protein expression that should assist in the elucidation of the mechanisms of the MDM response to material surfaces.

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7.0 DISCUSSION

A foreign body response (FBR), whether acute or chronic, to medically implanted PU materials is an inevitable consequence. The design of synthetic polymers is continually advancing with an aim of tailoring material surface chemistry or modifications for highly specific applications. Few biomaterials have achieved the ability to prevent attachment of inflammatory cells, such as neutrophils and macrophages, however significant progress is being made in the design of biomaterials that can prevent cell attachment or direct the infiltration and/or attachment of specific cell types [Ernsting et al., 2005; Cao et al. 2006; Croll et al., 2006; Milner et al., 2006]. Although efforts continue to modify and engineer improved PU materials, the cellular responses to and cell-mediated degradation of PU materials, particularly PCNU materials, still is not completely understood. Understanding how and why cells attach in addition to how cells respond to and degrade biomaterials is an essential base of knowledge required for the most efficient design of medical implant materials for their intended use.

The basis for this research project originates from a study conducted with human neutrophils cultured on PEUU materials [Labow et al., 2001a]. In this study, human neutrophils were cultured on ^{14}C - PEUU materials along with several chemical inhibitors of various neutrophil activation and signaling pathways including PLA₂ enzymes. As a result of this study, PLA₂, PKC and esterases were justified as potential candidates for the participation in the acute FBR (neutrophil) to PU materials. Recent studies in the Labow group have focused on examining the chronic response (macrophages) to PCNU [Labow et al., 2001b; c; 2002; 2005; Matheson et al., 2002; 2004]. The PLA₂ pathway(s) were

further validated as potential candidates in the FBR in a more recent study establishing the U937 cell line as a model for macrophage-mediated PCNU degradation [Matheson et al., 2002]. In this study, one particular inhibitor of sPLA₂ resulted in a decrease in U937 cell-mediated and human primary MDM-mediated ¹⁴C-PCNU degradation. These studies warranted further elucidation of the inflammatory response of human macrophages to PCNU materials in addition to the involvement of PLA₂ enzymes in macrophage-mediated PCNU degradation.

PLA₂ participation in PCNU degradation and the macrophage foreign body response

The first manuscript in this thesis described initial experiments carried out to further investigate the specific categories of PLA₂ enzymes (sPLA₂, cPLA₂ or iPLA₂) that may participate in the mechanisms of U937 cell-mediated PCNU degradation. Multiple chemical inhibitors were used to target each of the categories of PLA₂ enzymes. Although inhibition of each type of PLA₂ resulted in a significant reduction of U937 cell-mediated ¹⁴C-PCNU degradation, it appeared that sPLA₂ and cPLA₂ were the greater participants in this response. This was further supported by PLA₂ activation studies performed through ³H-AA release assays, demonstrating that each of the model PCNU materials used in the above studies could stimulate ³H-AA release (PLA₂ activation) from U937 cells. Furthermore, when PLA₂ inhibitors were cultured with ³H-AA labeled U937 cells, a sPLA₂ inhibitor (ARIST) elicited the most profound effect on PCNU-mediated PLA₂ activation, whereas an iPLA₂ inhibitor elicited minimal effects. This likely participation of sPLA₂ and cPLA₂ enzymes and not iPLA₂ can be explained by the fact that sPLA₂ and cPLA₂ are primarily involved in membrane hydrolysis [Balsinde et al.,

2002] with cPLA₂ possessing high specificity for AA-containing membrane glycerophospholipids [Leslie, 2004]. In addition, cPLA₂ and sPLA₂ have been demonstrated to cross-talk [Murakami et al., 2000], therefore leading to the potential in participating in other signaling pathways. Conversely, iPLA₂ typically participates in membrane remodeling, contributing much less to AA release from cell membranes [Murakami et al., 1998].

So, do PLA₂ enzymes have a direct or indirect role in material degradation? Although a PCNU chain is not a typical substrate for PLA₂ enzymes, their natural substrate is the center, *sn*-2 ester bond of membrane glycerophospholipids [Six and Dennis, 2000]. Since a PCNU chain contains a carbonate linkage, PLA₂ enzymes could theoretically act on this linkage to result in PCNU chain cleavage (see Figure 1.6 – section 1.3.3.1). The esterolytic enzyme CE was able to degrade both the carbonate and urethane linkages of PCNU chains, again not the natural substrate for this enzyme, depending on the surface chemistry and phase separation of these polymers [Tang et al., 2001a; b; 2002; 2003]. Although chemical inhibition of PLA₂ enzymes reduced PCNU degradation, commercial preparations of sPLA₂ did not lead to material degradation suggesting an indirect participation in degradative mechanisms. Considering cPLA₂ is not a secreted protein, it cannot participate in direct hydrolysis of the polymer chain.

Activation of PLA₂ enzymes, and the release of the eicosanoid AA, is the initial step in a pathway resulting in the production of highly inflammatory mediators. Further experiments described in Manuscript #2 also indicated that not only do PCNU materials

activate PLA₂, but also result in increased expression of PLA₂ protein, specifically cPLA₂ but not sPLA₂. cPLA₂, as mentioned, is known to have the highest specificity for AA-containing phospholipids [Leslie, 2004] and would be sufficient to account for the ³H-AA release observed with U937 cells when stimulated by PCNU materials. Furthermore, PCNU materials induced a change in location of U937 cell cPLA₂ (group IV) protein as indicated by immunofluorescence microscopy images. Although it is clear that there is a change in location towards the outer cytoplasm of these macrophages, further studies are necessary to determine the exact location of this mobilization of cPLA₂ upon stimulation by adherence to PCNU. The role of sPLA₂ in the FBR to PCNU most certainly cannot be ruled out. As mentioned, sPLA₂ inhibitors reduced degradation and PCNU-mediated ³H-AA release. However, despite several efforts, sPLA₂ protein could not be detected in the conditioned media of U937 cells. Intracellular sPLA₂ (group V) was detected and did not change in expression between material surfaces, but as a secreted protein it is possible that differences in sPLA₂ protein may only be evident in conditioned media. sPLA₂ once secreted could thus potentially contribute to further inflammatory and/or cell signaling pathways that may participate in the FBR via extracellular signaling, particularly through the sPLA₂ receptor [Ohara et al., 1995; Fonteh et al., 2000; Hanasaki et al., 2002].

Harnessing the proteomics toolbox to examine the foreign body response

It is clear that in a relatively unknown system, such as the cellular response to PCNU materials, to continue selecting individual macrophage proteins and pathways to investigate that might have a potential role in the FBR to PCNU, and also subsequent degradation, would be a considerable task. Although the complex mechanisms of the

macrophage FBR to PCNU is slowly being understood, the tools of proteomics could be extremely advantageous in a “shot gun” approach to identify multiple proteins and subsequently pathways that may participate.

Initial studies assessing changes in the MDM proteome (Manuscript #5) and secretome (Appendix VI) in response to differentiation upon and degradation of PCNU materials were performed with some of the standard techniques in proteomics. The initial approach typically taken when commencing proteomics approaches includes protein separation by 2-DE and identification of protein spots by MALDI-ToF MS. Results presented in Manuscript #5 detail intracellular proteins (MDM proteome) identified, which were found to change between TCPS (control surface) and HDI431 (PCNU biomaterial). The proteome was explored in MDM that were both differentiating on these surfaces or in MDM reseeded onto each surface undergoing biodegradation. Although these studies were preliminary, they were one of the few initial studies to begin exploring the proteome of mammalian cells at a cell-biomaterial interface [Derhami et al., 2001; El-Amin et al., 2003; Li et al., 2005].

Several proteins were identified and found to change in expression between HDI431 and TCPS. The majority of proteins that were identified were high abundance proteins such as cytoskeletal and microtubule proteins in addition to actin remodeling proteins. Other proteins that presented with altered expression were several chaperone proteins and proteins involved in general maintenance of intracellular proteins (involved in protein breakdown and protein folding). The key proteins, CE and MSE as well as the PLA₂s,

were not identified despite the fact that protein spots at the appropriate MW and pI were located on the 2-DE gel. The inherent draw backs of using the 2-DE + MALDI-ToF MS approach were outlined in the discussion of Manuscript #5, one of these being the lack of high sensitivity of MALDI-ToF MS. Despite this, the approach taken is a first step when launching into proteomics approaches to elucidating the mechanisms of polymer degradation and the FBR.

One of the advantages of separating proteins by 2-DE is the generation of a “picture” of the proteome. Even if proteins are not identified from this “picture” it can give a clear indication as to whether there are significant differences to pursue, and perhaps even a particular MW or pI range to focus on and fractionate for future studies. An additional issue in proteomics in general is the presence of serum in *in vitro* culture conditions. Since serum proteins adhere to biomaterial surfaces and even TCPS (see section 1.3.4), these proteins can become mixed in lysate samples when cells are lifted from the culture surface or in conditioned medium samples when medium is removed from the well. However, the presence of a much greater amount of FBS proteins in the conditioned medium 2-DE gels of samples from HDI431 relative to TCPS is a positive result (Appendix VI). This indicates much greater adsorption of serum proteins to the HDI431 surface than TCPS, that may then lift from the material surface into the conditioned medium during the 24-hour serum free period, and may also account for the greater cell attachment seen in macrophage differentiation studies illustrated in Manuscript #4. As mentioned in section 1.3.6, proteomics studies have been done to characterize the complement of proteins that adsorb onto a material surface that is reflective of the

properties of the material and may also influence the cell types that may attach and how well they may attach and respond to each surface. For example, serum albumin has been identified here in the MDM proteome samples (Manuscript #5 – Table 2, spot #6 – pg.203) and also secretome samples (Appendix VI) and shown to drastically increase in the HDI431 secretome sample compared to TCPS perhaps influencing MDM cell attachment to PCNU materials in particular.

These studies have re-established that proteomics studies are an appropriate vehicle for pursuing investigations of the cell-biomaterial interface [Gallagher et al., 2006]. Little has been done in this area and therefore is “wide open” for future discoveries. A key area lacking in the field of biomaterials includes characterization and a better understanding of receptors through which macrophages attach to and also initiate signaling pathways upon recruitment to a material surface. Proteomics techniques may also be the tools to help us unravel these mechanisms of macrophage attachment to biomaterials and initiation of the secretion of degradative enzymes.

PCNU induces morphological and functional changes in differentiating macrophages

Upon recruitment of monocytes to a medical implant device, these cells will differentiate into MDM under the influence of the surrounding microenvironment, a large component of which will include the surface of the biomaterial. Although, certain studies have addressed the concept of cellular differentiation/maturation on biomaterial surfaces [Rhodes et al., 1997; Godek et al., 2004; Babensee and Paranjpe, 2005; Soria et al., 2006; Qu et al., 2006], specifically exploring macrophage behaviour *during* cellular

differentiation is essential in interpreting the FBR to various surface and chemistry differences in PCNU materials. Closely related to the studies here were several studies by the Anderson research group that have explored cytokine-induced *in vitro* fusion of MDM and formation of FBGC on silane modified surfaces [Jenney et al, 1998] and surface-modified PU and non-modified commercial PU materials [Kao et al., 1994; Jones et al., 2004; Dadsetan et al., 2004]. These studies have characterized the influence of these surfaces on macrophage adherence, cell viability and potential for FBGC formation. However, studies have not been conducted to characterize the phenotype and degradative capacity of these cells *during* their maturation period. Moreover, the MDM/FBGC system used by the Anderson group employs cytokine induced differentiation, whereas the system used herein involves MDM differentiation solely by serum-supplementation and cell attachment to the material surface. Therefore, these studies sought to characterize monocyte to macrophage differentiation during the process of cell attachment and maturation on PCNU materials compared to typical TCPS that is used widely in cell culture.

The initial study, in Manuscript #3, to assess material surface chemistry influences on differentiating MDM primarily considered these cells functionally in terms of degradative capacity towards ¹⁴C-PCNU materials. MDM were characterized only *after* they had fully “matured” over 14 days on PCNU materials and the TCPS control surface. The MDM that were differentiated on each material surface were then subjected to typsinization in order to reseed these cells again onto each of the model material surfaces. This considerably tedious study demonstrated that the material surface upon which MDM

differentiated subsequently affected the ability to degrade materials onto which they were reseeded. Even slight differences in chemistry between the PCNU materials (i.e. HDI431 vs. HDI321) could significantly affect the degree to which this MDM could degrade the following surface it encountered. These were validated with respect to degradative capacity (esterase activity and ^{14}C -PCNU radiolabel release) as well as intracellular CE and MSE protein expression. This was a key study to justify further exploration into the effects of these material surfaces on the phenotype and function of MDM *during* their differentiation time course.

The successive study built upon this knowledge gained and assessed parameters of function and morphology of maturing MDM *during* the differentiation time course. An excellent model has been established using differentiated (on TCPS) primary human MDM cells that are then gently trypsinized (activated) and reseeded to PU surfaces for the study of MDM-mediated degradation [Labow et al., 2001c]. However, this model is clearly not ideal for examining the progressive differentiation of monocytes to macrophages. The study conducted in Manuscript #4 aimed to further characterize the differentiating macrophages that were shown in Manuscript #3 to be sensitive, in terms of function, to changes in material chemistry. Differentiating human MDM were followed *during* their differentiation time course, exposing freshly isolated monocytes to each surface, collecting cell lysates, conditioned media and fixed cells along the time course. Characterizing differentiating MDM in this way allows an assessment of phenotype and function that would be more parallel to the *in vivo* response of monocytes that would first

adhere to the surface of PCNU containing medical devices and then differentiate into mature MDM depending on the material surface they encountered.

Several differences were found between MDM differentiated on PCNU (both HDI431 and MDI321) compared to TCPS. The most notable effects were that of altered cell morphology, changes in esterase protein expression and enzyme activity as well as marked changes in macrophage marker protein expression. Morphological differences were clearly indicated in both SEM images and in MDM stained to illustrate the actin cytoskeleton. Significant reduction in MDM cell spreading on both PCNU materials relative to cells on TCPS was validated by quantitative measurements. These alterations in cell shape were accompanied by changes in the arrangement of cell adhesion structures considered to be podosomes. Podosomes are the most abundant part of the actin cytoskeleton of primary macrophages, and contain a dot-like core of actin, not seen in focal adhesions, surrounded by a ring of proteins such as vinculin or talin [Linder and Aepfelbacher, 2003]. Although the images presented were not additionally stained for vinculin, it is hypothesized that these structures are podosomes as indicated by their dot-like F-actin structure.

MDM are less spread on PCNU and their arrangements of podosomes are varied with what appeared to be increased podosome formation on PCNU. Podosomes have been illustrated to create small invaginations at the cell-substrate interface suggested to facilitate the secretion of matrix metalloproteinases [Linder and Aepfelbacher, 2003], that typically are involved in extra cellular matrix remodeling (tissue invasion) but could be

participating here in degradation of the protein layer between the cell and material. Moreover, since enzymes have been shown to be secreted via podosomes, this could provide a novel release mechanism by which esterases may gain concentrated access to the biomaterial eliciting their hydrolytic activity resulting in “pitting” seen by the actions of adherent macrophages [Labow et al., 2001b; Manuscript #4 – Figure 6]. Degradative potential (as measured by MSE protein expression and intracellular esterase activity) was hypothesized to increase over MDM differentiation time. This was indicated by decreased intracellular MSE protein in PCNU-adherent MDM compared to TCPS, and increased esterase activity suggesting activation of esterase enzymes on HDI431 and secretion of MSE into the extracellular space in response to PCNU. Supporting these findings, intracellular CE enzyme activity was demonstrated to increase over differentiation time in MDM maturing on PEUU and PESU materials (28 day vs. 8 day differentiation) during the establishment of an MDM model to further examine esterase mediated PU degradation [Labow et al., 1998]. HDI431 which demonstrated the greatest stimulation of esterase activity (Manuscript #4), also was the only surface to exhibit holes in the surface of the biomaterial by SEM analysis, which further supports SEM images from a previous study [Labow et al., 2001b].

It is possible that since these MDM are so “busy” responding to this foreign surface and attempting to degrade this material that the phenotype changes so that expression of the macrophage marker CD68 is drastically reduced. These differentiating MDM did illustrate typical down regulation of the monocyte marker CD14 upon maturation. However, they only expressed increased CD68 expression in TCPS adherent cells.

Further validation would be required to determine if these cells demonstrate a completely altered phenotype, or if these drastic changes in CD68 expression are a result of functional changes.

The FBR to PCNU materials is clearly a multi-factorial process, however a picture may be coming together that involves an interplay between PLA₂ enzymes, the actin cytoskeleton, along with its supporters and regulators, and the degradative enzymes that are released. Assessment of the MDM proteome illustrated changes in a protein, the G protein-coupled receptor kinase 5, that phosphorylates G protein coupled receptors. These receptors have been shown to activate phospholipase C that in turn initiates a pathway resulting in PKC activation, which could phosphorylate and activate cPLA₂. Furthermore, one of the products of cPLA₂-mediated membrane hydrolysis is lysophosphatidic acid (LPA). LPA has been demonstrated to downregulate gelsolin, which is an actin severing protein [Meerschaert et al., 1998]. Gelsolin was identified (Manuscript #5) and observed to decrease in PCNU adherent MDM relative to TCPS. Therefore, downregulating gelsolin and hence the actin-severing capabilities would allow actin filaments to be constructed, as would be needed for the assembly of structures such as podosomes and for continuous cell spreading and cell motility. Other supporting studies linking PLA₂ enzymes and the cytoskeleton include studies demonstrating that cPLA₂α (also known as group IV) interacts with microtubules, specifically vimentin [Nakatani et al., 2000; 2002], and also with actin facilitating translocation of cPLA₂ [Fatima et al., 2005]. Therefore, further studies should continue to examine these potential cPLA₂ interactions with cytoskeletal proteins that may facilitate the formation

of adhesion structures and novel release/secretion mechanisms (i.e. ectocytosis), contributing to the release of degradative substances which may destroy biomaterial surfaces and/or the adjacent tissues.

Conclusions

The work contained in this thesis aimed to contribute to the understanding of the macrophage FBR to PCNU materials demonstrating that:

- i) PLA₂ pathways contribute an indirect role within macrophages during the mechanisms of PCNU degradation, possibly by initiating signaling pathways triggering the release of degradative substances.
- ii) PCNU materials stimulate increased macrophage cPLA₂ protein expression and PLA₂ activation indicating the ability to induce a strong inflammatory response.
- iii) Changes in material surface chemistry during cell differentiation affects the capacity of MDM to degrade PCNU materials.
- iv) PCNU materials have a considerable influence on the morphology of differentiating MDM and function of these cells in terms of degradative capacity and ability to display markers of macrophage differentiation.
- v) Material surface differences influence changes in the expressional profile of proteins in the macrophage proteome and secretome of differentiating MDM and MDM undergoing active material degradation.

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8.0 APPENDICIES

APPENDIX I: PHOSPHOLIPASE A₂ ASSOCIATION WITH MDM-MEDIATED POLYCARBONATE-URETHANE DEGRADATION

The manuscript “Phospholipase A₂ association with macrophage-mediated polycarbonate-urethane degradation” in section 2.0 utilized the U937 cell line for studies with PLA₂ inhibitors to determine their effect on macrophage-mediated PCNU biodegradation. This cell line was used as a model for MDM for the purpose of biodegradation assays. In order to justify continuation of studies with this cell line, studies were confirmed in primary human MDM to see if similar effects could be seen. Figure AI-1 demonstrates that each of the inhibitors used in Section 2.0 (Figure 1) also resulted in significant decreases in ¹⁴C-PCNU radiolabel release (biodegradation assay) mediated by MDM. These studies were only carried out with the model PCNU which demonstrated the greatest macrophage-mediated degradation, ¹⁴C-HDI431.

In addition, ³H-AA release assays from Section 2.0 (Figure 4) were also confirmed in primary human MDM to determine if similar ³H-AA release could be stimulated in PCNU adherent MDM. Figure AI-2 confirms that ³H-AA labeled MDM were also stimulated by a PCNU surface to release ³H-AA in a significantly greater amount than PS-adherent MDM. Figure AI-2 shows a time course of ³H-AA release. Only at 10 minutes was there a greater release of ³H-AA from PCNU-adherent MDM than PS, which was also seen in U937 cells.

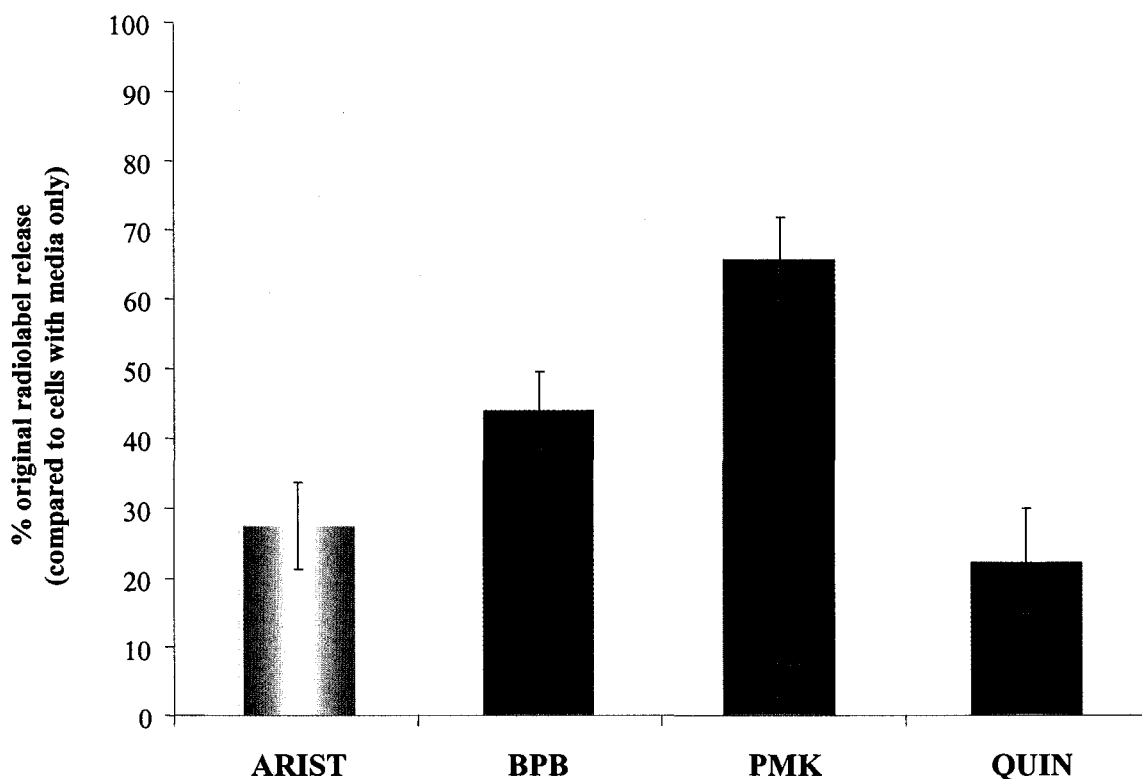


Figure AI-1 Effect of PLA₂ inhibitors on ¹⁴C-PCNU radiolabel release by MDM. Human monocytes were isolated from whole blood (see **Materials and Methods**, section 6.0) and differentiated for 14 days on PS. Cells were then trypsinized and reseeded onto ¹⁴C-HDI431 for 1 hour. Culture medium from each well was then removed and replenished with media with or without inhibitor. The cells were incubated for a further 48 hours, conditioned medium was collected for ¹⁴C scintillation counting and cells were lysed (see **Materials and Methods**, Section 2.0) to analyze for DNA to relate ¹⁴C radiolabel release to 10 µg DNA.

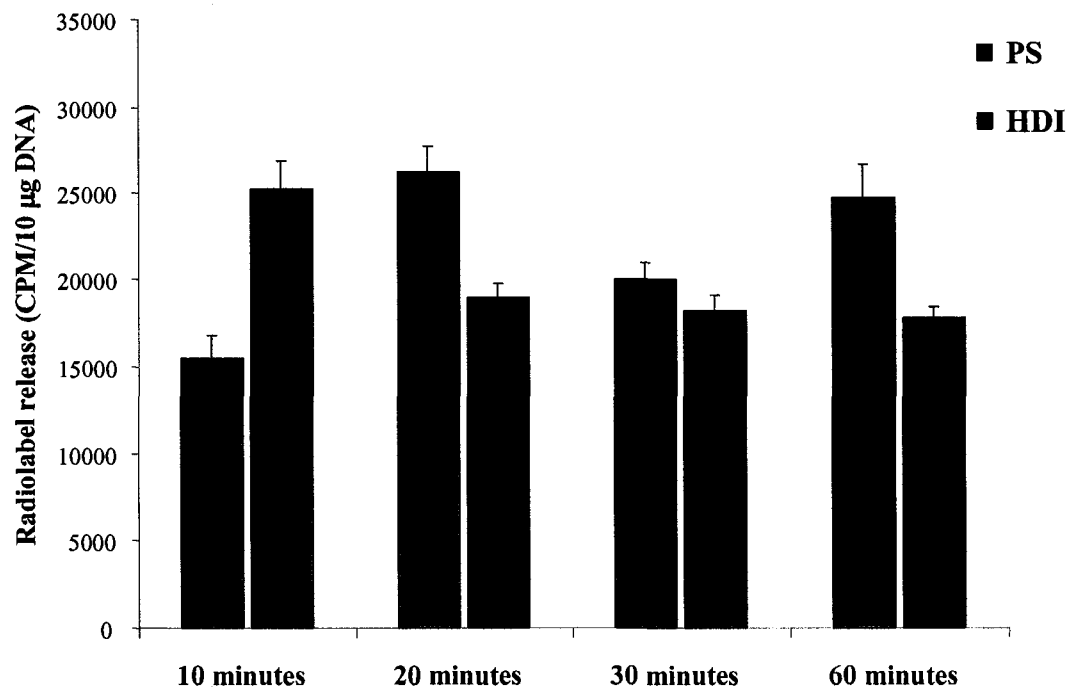


Figure AI-2 $^3\text{H-AA}$ release from PS- and HDI431-adherent MDM. Monocytes were isolated from human whole blood (see **Materials and Methods**, Section 6.0) and differentiated for 14 days on PS. $^3\text{H-AA}$ (0.1 $\mu\text{Ci/mL}$) was added to differentiating monocytes on Day 13 and allowed to incubate with cells for 24 hours. Cells were washed twice with culture media then trypsinized and reseeded (with A23187 – see **Materials and Methods**, section 2.0) onto either PS (red bars) or HDI431 (blue bars) for 10, 20, 30 or 60 minutes at which time conditioned medium was collected for ^3H scintillation counting. Cells were lysed and analyzed for DNA (see **Materials and Methods**, section 2.0) to relate $^3\text{H-AA}$ release to 10 μg DNA.

APPENDIX II – ANALYSIS OF U937 CELL ³H-AA RELEASE BY THIN LAYER CHROMATOGRAPHY (TLC)

Experiments outlined in Manuscript #1 included an assessment of the effect of material surface on PLA₂ activation measured by ³H-AA release from attached differentiated U937 cells. As outlined in the **Materials and Methods** section of the above manuscript, U937 cells were radiolabelled with ³H-AA during cell differentiation then ³H-AA release from the cells was measured after the cells were resuspended and reseeded to each material surface. It was determined that 50% of the ³H-AA added into culture was taken up by the cells (data not shown). Following this, it was also desired to determine what percentage of the ³H that was being released from the cells, as measured by scintillation counting, was due to AA. The remaining percentage of ³H released would then be downstream products of AA that would then already have been converted to prostaglandins and leukotrienes before release from the cell.

In order to determine the percentage of ³H products in the conditioned media that was AA, samples from the conditioned media of pre-labeled U937 cells were subjected to thin layer chromatography (TLC). Methods were developed to apply an appropriate solvent system that would allow the separation of the phospholipid from hydrolysis products.

METHODS

Extraction procedure

1. In a glass test tube (16x100mm) aliquot 200 μ L of conditioned media and add 800 μ L of ddH₂O.
2. Add 2.0 mL of methanol and vortex.
3. Add 1.0 mL of chloroform and 12 μ L of formic acid and vortex for 1 minute, then incubate at room temperature for 30 minutes.
4. Centrifuge samples for 20 min at 3000 rpm.
5. Remove most of the bottom (organic) layer, transfer to smaller glass test tube (12x75mm) and evaporate to dryness under nitrogen.
6. Reconstitute in 20 μ L methanol.

Thin Layer Chromatography (TLC)

7. Use 10 μ L of sample for scintillation counting to determine amount of ³H for whole sample.
8. Spot 5 μ L of sample at the origin line on the TLC plate (TLC plastic sheets Cat# 5746/7 – 20x20cm Silica gel 60 – EM Science, Gibbstown, NJ).
9. Also spot 10 μ L of an arachidonic acid standard (Cat# A3555 Sigma) to determine the distance AA migrates from the origin.
10. Place the plate with the origin at the bottom in a TLC tank with enough solvent mixture (hexane:ether:glacial acetic acid – 70:30:1) to cover the bottom of the tank and so that the bottom of the TLC plate is just immersed in solvent.
11. Leave in tank until solvent front has moved approximately $\frac{3}{4}$ up the plate.
12. Remove plate, allow to dry overnight.
13. Place dried TLC plate in TLC tank with iodine crystals to “expose” spot with AA standard.
14. Cut out AA standard spot, and the spot at same distance from origin as the standard for the conditioned media sample, also cut out the spot at the origin where conditioned media sample was spotted.
15. Place each spot that was cut out in a scintillation vial with 10 mL scintillation fluid and count the ³H radioactivity.

It was determined by scintillation counting of each sample that migrated along with the AA standard, that the amount of ^3H at that point on the TLC plate with conditioned media samples was approximately 25% of the amount of radioactivity of the entire sample that was extracted and reconstituted. Therefore 25% of the ^3H released and measured from the cells was AA and the other 75% was most likely downstream products from the conversion of AA.

APPENDIX III – sPLA₂ GROUP V ANTI-SENSE OLIGONUCLEOTIDES

It was determined in Manuscript #1, that the use of a chemical sPLA₂ inhibitor (ARIST) decreased U937 cell-mediated ¹⁴C-PCNU degradation. Since chemical inhibitors are known to be non-specific, it was then attempted to inhibit sPLA₂ through a more specific method, anti-sense oligonucleotide inhibition. Phosphorothioated oligonucleotides are very widely used in sequence-specific inhibition in anti-sense methodology [Eckstein F., *Antisense Nucleic Acid Drug Dev* (2000) 10:117-121]. Phosphorothioated oligonucleotides were designed and ordered from Sigma-Genosys. Oligonucleotides were phosphorothioated on bases 1-3 and 18-20 as indicated by lower case letters. These were designed against the mRNA sequence of human sPLA₂ group V (NCBI – NM_000929), beginning at the start codon of the mRNA sequence. Both an anti-sense and sense sequence were designed; an anti-sense nucleotide sequence to bind the mRNA and block protein transcription of this protein and a sense nucleotide sequence as a control to ensure that simply adding in an oligonucleotide into cell culture did not bind to the mRNA and/or have any detrimental or negative effects.

sPLA₂ group V anti-sense oligo

5' – tacTTTCCGGAGGAGGGtgaC – 3'

sPLA₂ group V sense oligo

5' – gtcACCCTCCTCCGGAAagtA – 3'

Efforts to “knockdown” sPLA₂ group V protein expression were attempted on several occasions using multiple concentrations, times of addition to culture and also complexing the oligonucleotide with the reagent Lipofectamine 2000™ (Invitrogen). Since each of

these attempts were relatively unsuccessful and significant decreases in ^{14}C -PCNU degradation could not be achieved with the use of these anti-sense oligos, experiments were discontinued and efforts were focused then on protein expression. It was further examined as to which PLA₂ proteins were increased in expression in response to macrophage attachment to PCNU materials (see Manuscript #2)

APPENDIX IV - cPLA₂ PROTEIN EXPRESSION IN MOUSE RAW264.7 MACROPHAGES IN RESPONSE TO PCNU

It was presented in Manuscript #2 that PCNU materials can induce significantly increased expression of cPLA₂ protein in the U937 cell line. As indicated in the Introduction and Manuscripts #1 and #2, activation of the PLA₂ pathway leads to the production of several potent inflammatory mediators. During a visiting research fellowship to the University of New South Wales (May-September 2006), work was done in collaboration with the biopharmaceutical company CBio BioPharmaceuticals Inc. (Brisbane, QLD, AUS). The model of inflammation used was PCNU-induced macrophage-mediated inflammation. The mouse RAW 264.7 macrophage cell line (ATCC[®] # TIB-71[™]) was chosen as the cell line to use for these studies in the research lab at UNSW. Therefore, increased cPLA₂ protein expression in response to cell attachment to PCNU materials was used to validate this model system of inflammation.

METHODS

The following is a brief outline of the methods employed for these experiments:

Cell Culture

- RAW 264.7 cells (an adherent cell line), were cultured in T75 flasks (Greiner Bio-One) in RPMI 1640 medium supplemented with L-glutamine and 10% heat inactivated FBS.
- At 80% confluence, RAW 264.7 cells were scraped, centrifuged and 1mL of a 8×10^6 cell suspension was reseeded into T75 flasks containing 19 mL of culture media.
- After cells were cultured for 24 hours, the cells were then scraped again, centrifuged and 1 mL of a 5.0×10^5 cells/mL suspension was reseeded into 24 well plates with or without HDI431-coated glass slips.
- Cells were then cultured for 30 minutes, 2 or 24 hours.

- At the end of the incubation period, the conditioned medium was removed and cell lysates were collected as described in the **Materials and Methods** in each of the above manuscripts.
- Cell lysates were quantified for DNA concentration using a Nanodrop[®] ND-1000 Spectrophotometer.

NuPAGE and Immunoblotting

- The volume of cell lysate equaling 250 ng of DNA was aliquoted into microcentrifuge tubes and samples were prepared to run on NuPAGE[®] Bis-Tris precasted gels by adding the appropriate amount of NuPAGE[®] LDS Sample Buffer, NuPAGE[®] Reducing Agent and ddH₂O.
- Samples were loaded into 12-well NuPAGE[®] Bis-Tris gels (4-12%, 1mm) and run at 160 V for 80 minutes in NuPAGE[®] MOPS running buffer.
- Proteins were then transferred to nitrocellulose membranes (Protran[®] - Whatman), using a semi-dry system, and NuPAGE[®] transfer buffer, with a current of 2 mA/cm² for 2.5 hours.
- Membranes were then stained with Ponceau Red to ensure efficient transfer of proteins.
- Membranes were then probed in the following manner for detection of cPLA₂ protein:
 - Wash blots in TBST
 - Block 1 hr in 5% BSA in TBST
 - Incubate with mouse anti-cPLA₂ (Chemicon) in 5% BSA for 1 hr
 - Wash 7 x 5 minutes in TBST
 - Incubate with anti-mouse IgG (Chemicon) in 5% BSA for 1 hr
 - Wash 7 x 5 minutes in TBST
 - Incubate with Immobilon Chemiluminescent substrate (Chemicon) for 5 min, remove excess substrate
 - Expose blot to film and develop

Figure AIV-1 illustrates a representative blot of mouse RAW 264.7 macrophage lysates from cells cultured on either TCPS or HDI431 (PCNU) for 30 minutes, 2 or 24 hours. As is indicated in the figure, cPLA₂ protein expression is clearly increased at each timepoint, more prominently at 24 hours. There are two points to notice that differ between this immunoblot and those illustrated in Manuscript #2. The first is that the cPLA₂ protein in this figure migrates at 85kDa as opposed to ~110 kDa. This is likely due to the differences in the gels used for electrophoresis. These samples were run on a NuPAGE Bis-Tris 4-12% gel with MOPS, whereas the samples in Manuscript #2 were run on a Tris-Glycine SDS-PAGE 10% gel. An additional difference to note was that the greatest expression of cPLA₂ protein on the PCNU in Figure AIV-1 was indicated at 24 hours, whereas in performing experiments for Manuscript #2, the greatest expression of cPLA₂ protein was at 2 hours and decreased at the 48 hour time point. This was likely due to the fact the RAW 264.7 cells used here are proliferating cells, whereas the U937 cells used in Manuscript #2 were differentiated with PMA. Therefore, proliferating and differentiated, non-dividing cells may respond differently to a stimulus such as a biomaterial.

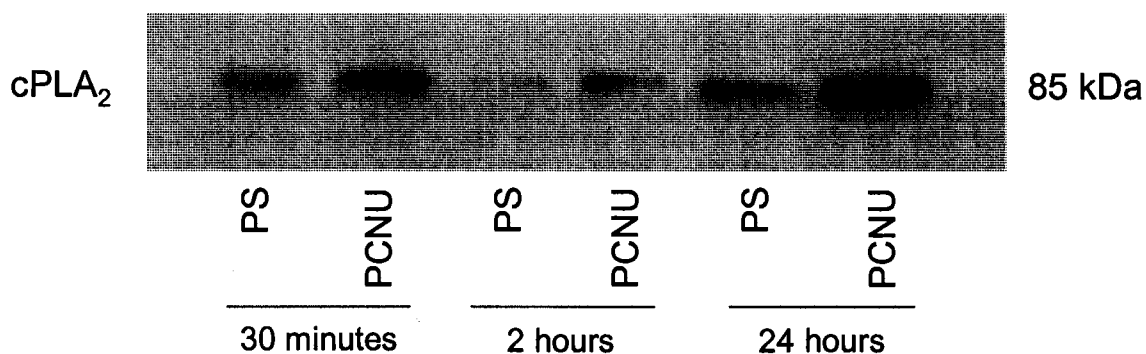


Figure AIV-1 – PCNU induces increased cPLA₂ protein expression in mouse RAW 264.7 macrophages

APPENDIX V: THE EFFECT OF CE-MEDIATED PCNU DEGRADATION PRODUCTS ON INTRACELLULAR MSE EXPRESSION IN HUMAN MDM

Note: This work was presented at the 30th Annual Meeting of the Society for Biomaterials (April 2005) Memphis, TN, USA. **Dinnes DLM**, Sa D, Santerre JP, Labow RS. Methylene dianiline (MDA) affects monocyte-derived macrophage-mediated polycarbonate-urethane degradation. Podium presentation.

In Section 4.0, the manuscript “The human macrophage response during differentiation and biodegradation on polycarbonate-based polyurethanes: Dependence on hard segment chemistry” explored the effects of PCNU degradation products 4,4'-methylene dianiline (MDA) and 1,4-butanediol (BD) on the degradative capacity of PCNU adherent MDM. As outlined in the results and discussion of the above manuscript, MDA was found to decrease MDM-mediated biodegradation (as assessed by ¹⁴C-PCNU radiolabel release assays) and esterase activity. Subsequent to these studies, however not included in this publication, MDA was then explored for effects on intracellular MSE expression. Discussed in section 1.3.3.1, MSE is a candidate enzyme in the biodegradation of PU materials.

MATERIALS AND METHODS

Refer to the **Materials and Methods** section of 4.0 for detailed protocols and methods for these experiments. Monocytes were isolated from whole blood of human volunteers and differentiated into mature MDM on TCPS. MDM were trypsinized and reseeded onto non-radiolabelled PCNU materials of composition identical to ¹⁴C-PCNU identified for this study (Table 1) and TCPS. Cells were allowed to adhere for 1 hour at which time the medium was replenished with medium alone or medium containing MDA. MDM were then incubated for 48 hours after which the medium was removed and cells were lysed and analyzed for DNA. Cell lysates were equally loaded onto SDS-PAGE gels by

DNA amount. Using methods and antibody conditions outlined in the **Materials and Methods** of Section 4.0, nitrocellulose membranes were blotted with antibodies to MSE and relative amounts quantified by Quantity One Software. Figure AV-1 shows a representative immunoblot for anti-MSE demonstrating the effect of MDA on intracellular MSE expression.

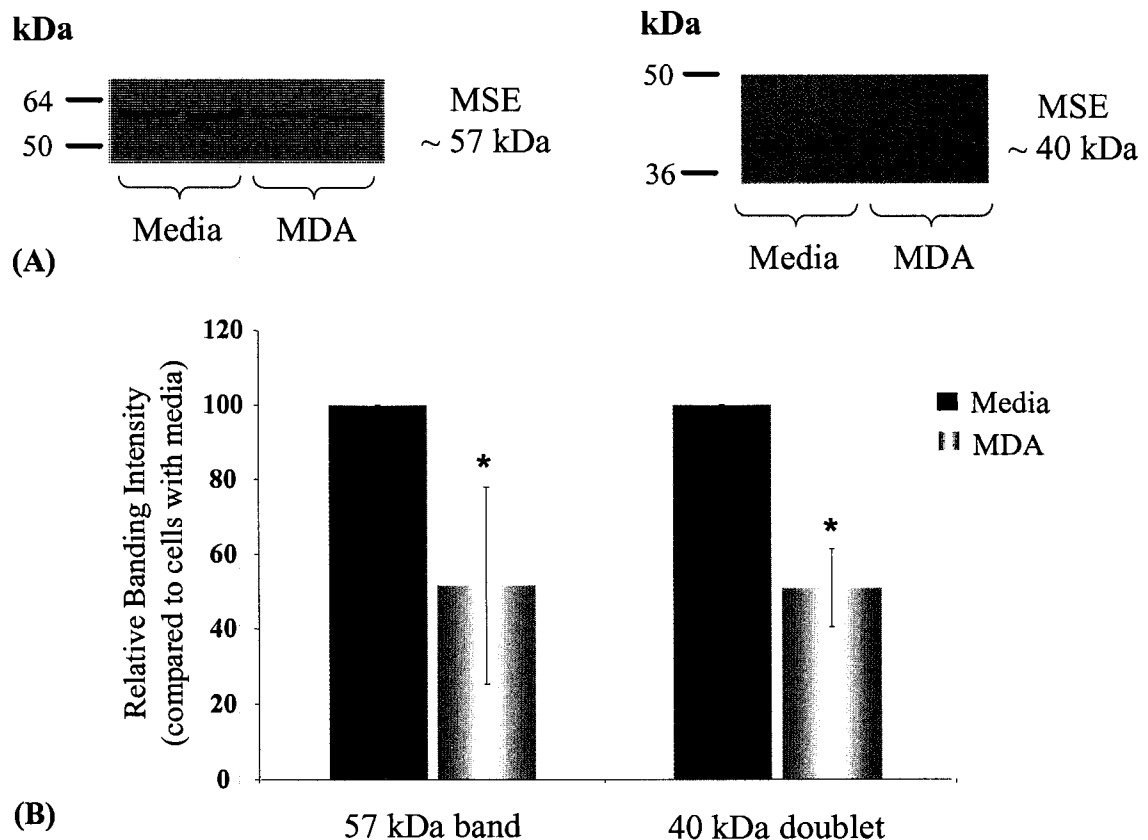


Figure AV-1 Effect of MDA on intracellular MSE expression. Differentiated human MDM were trypsinized and reseeded onto HDI431. MDM were incubated for 48 h with either culture media alone, or culture media with MDA (250 $\mu\text{g}/\text{mL}$). Cell lysates were collected and run on an SDS-PAGE gel, transferred to nitrocellulose membranes and probed with antibodies to MSE. A) Representative blots of MDM cell lysates probed with anti-MSE. Both the 57 kDa band and 40 kDa doublet demonstrated decreased expression in MDM exposed to MDA. B) Quantification of changes in MDM MSE expression in cells cultured with media alone (red bars) or media with MDA (yellow bars).

APPENDIX VI – ANALYSIS OF THE MDM SECRETOME FROM CELLS ADHERENT TO TCPS (CONTROL) AND PCNU MATERIALS

Note: These results were presented at the 25th Annual Canadian Biomaterials Society Meeting in Calgary, AB (May 2006) **Dinnes DLM**, Matheson LA, Marçal H, Mahler SM, Santerre JP, Labow RS. Use of expression proteomics to define proteins secreted from monocyte-derived macrophages on polycarbonate-urethane surfaces. Poster presentation.

Results published in the manuscript “Material surfaces affect the protein expression patterns of human macrophages: A proteomics approach” above (Manuscript #5) detailed experiments assessing the changes in intracellular proteins (or the proteome) of MDM in contact with a model PCNU (HDI431) and the control material surface TCPS. In addition to the work outlined in the above manuscript that was performed during a research visit to UNSW, experiments were also carried out to identify and detect changes in conditioned media proteins (or the secretome) of MDM under the same conditions. In order to achieve this, cells had to be cultured for a minimum of 24 hours serum-free in order to reduce the FBS protein contamination in the sample. This would also aid in the analysis of the proteome since serum proteins would inevitably adsorb to the material surface from which the cells were lysed and also to the surface of the cells.

Conditioned media samples were treated similarly to those samples outlined in Manuscript #5. Below is a brief description of sample preparation and methods used in order to produce 2-DE gels that were used to excise spots for identification. Further to identification, a software program was used to better visualize the differences in protein spots that were identified.

Preparation of conditioned media samples

1. Monocytes were isolated and cultured on either TCPS or HDI431 exactly as outlined in the **Materials and Methods** section of Manuscript #5.
2. At the end of the culture period, the conditioned medium was collected from each well and pooled
 - 1 mL of conditioned medium was collected from 10 wells either containing MDM on TCPS or HDI431
3. The conditioned medium was briefly centrifuged to pellet any non-adherent cells or cell-debris, the supernatant was collected and 1 mM phenylmethylsulfonyl fluoride was added to reduce protein degradation.
4. The conditioned media samples were then TCA precipitated and then protein pellets resolubilized in resolubilization solution (250 μ L final volume, also ensuring that there was equal amount of protein (100 μ g) in each sample for loading) as outlined in the **sample preparation for proteomic analysis** section of the **Materials and Methods** of Manuscript #5 (pg. 194).

Isoelectric Focusing (IPGphor isoelectric focusing system)

5. Resolubilized samples were loaded into IPGphor strip holders (IPGphor isoelectric focusing system – Amersham) along with 13 cm pH 3-10 Immobiline™ IPG strips (Amersham).
6. The strips were rehydrated for 12 hours (120 μ A/strip @ 20°C).
7. The strips were then focused using the following conditions:
 - 300V 1.5 hr
 - 500V 1.5 hr
 - 1000V 1hr
 - 8000V Gradient 1hr
 - 8000V 3hr
 - 50V holdtotal of 30 kVhr

IPG strip Equilibration

8. Each strip was then equilibrated in two steps; reduction and then alkylation. The equilibration solution was prepared as follows:
 - 0.5 M Tris/HCl pH 6.8 1 mL
 - Urea 3.6g
 - Glycerol (87%) 3 mL
 - SDS 0.2g
 - ddH₂O up to 10 mL

9. For the reduction step, add 0.04g DTT to 2mL of equilibration solution for each strip, reduce strip in this solution for 10 min.
10. For the alkylation step, add 0.05g iodoacetamide and 10 μ L bromophenol blue to 2mL of equilibration solution for each strip, alkylate strip in this solution for 10 min.

2nd Dimension (SDS-PAGE) and MALDI-ToF MS

11. Lay IPG strips onto 15% SDS-PAGE gels (15x15 cm), with warmed agarose solution layered on top of the gel.
12. Run gel @ 220V (limiting) for 4 hours.
13. Fix gels, stain in Sypro Ruby for imaging, destain and restain in Coomassie Blue for visualization and spot excision as outlined in detail in the section ***two dimensional electrophoresis*** of the **Materials and Methods** of Manuscript #5 (pg. 195).
14. Protein spots were excised and proteins identified by MALDI-ToF MS as outlined in detail in the section ***protein identification by MALDI-ToF MS*** in the **Materials and Methods** section of Manuscript #5 (pg. 195).

The resulting gels are illustrated in Figure AVI-1 demonstrating 2-DE gel images (Sypro Ruby stained) from MDM conditioned media of cells differentiated for 14 days on either A) TCPS or B) HDI431, or fully differentiated on TCPS then trypsinized and reseeded onto either C) TCPS or D) HDI431 for 24 hours. Protein spots that have been identified thus far are indicated by numbered arrows in Figure AVI-1. The protein ID's of these spots are listed in Table AVI-1.

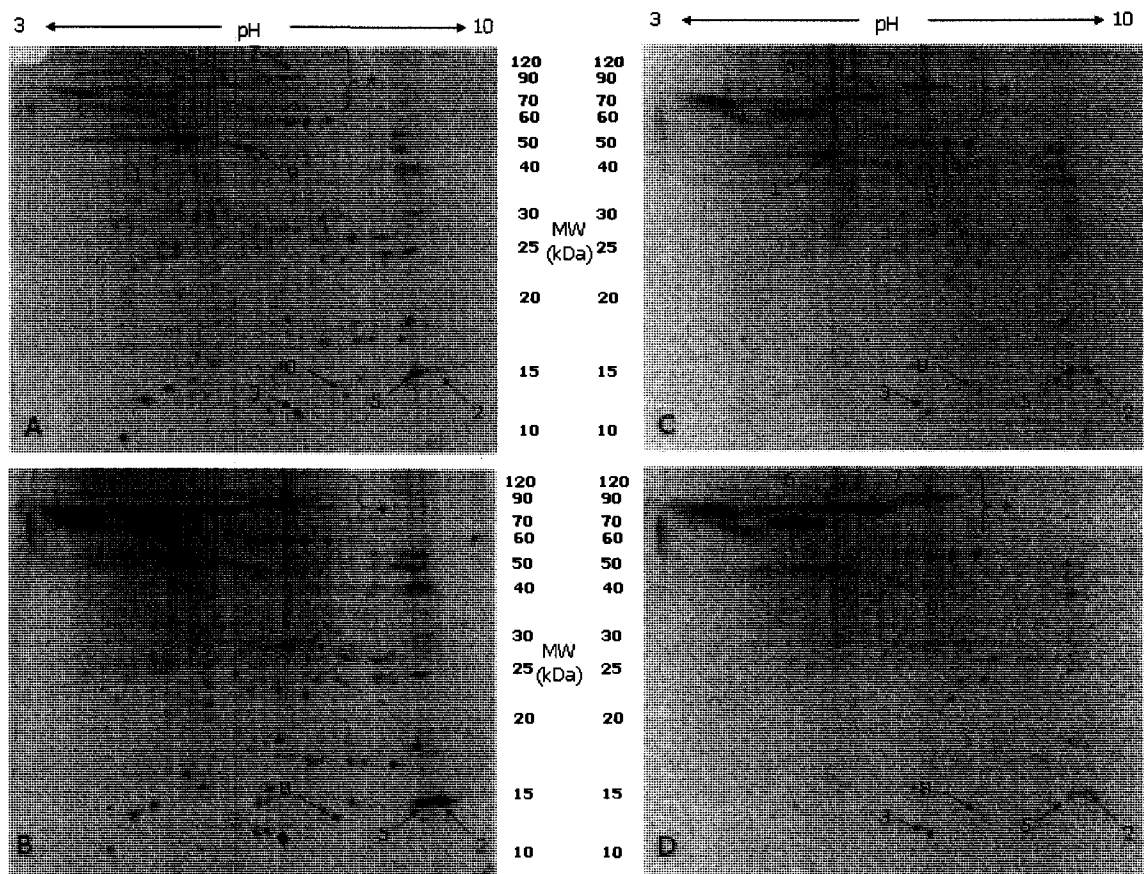


Figure AVI-1 Sypro Ruby stained 2-DE gel images of MDM conditioned media proteins (secretome). Conditioned media samples from MDM differentiated for 14 days on A) TCPS or B) HDI431 or fully differentiated on TCPS then trypsinized and reseeded onto C) TCPS or D) HDI431.

Table AVI-1 Protein identification of spots excised from 2-DE gels in Figure AVI-1.

Spot #	Accession #	Protein Name	MW	pI	Function
1	P60709	β -Actin	41.7	5.29	cytoskeletal protein
2	P61626	Lysozyme C Precursor	16.5	9.38	lysosomal protein
3	P61769	β -2-microglobulin (precursor)	137.2	6.06	β -chain - MHC Class I molecule
4	P02769	Serum albumin (bovine)	69.3	5.82	FBS protein
5	Q9H293	Interleukin-25	20.3	8.73	proinflammatory cytokine
6	P34955	α -1-antitrypsin (bovine)	46.1	6.05	FBS protein
7	Q29443	Serotransferrin (bovine)	77.7	6.75	FBS protein
8	P04080	Cystatin B	11.1	6.96	reversible cathepsin inhibitor
9	P21580	TNF- α induced protein-3	89.5	8.60	inhibits NF- κ B activation

The area indicated by the asterisk * Figure AVI-1 points out the area of the 2-DE gel that is mostly serum proteins from cell culture. These proteins very often hinder the ability to identify other proteins of similar *pI* and MW since the serum proteins are so highly abundant.

In addition to identifying proteins on these 2-DE gels, the Sypro Ruby stained gel images were analyzed using the Z3 image analysis software (Compugen). This software program allows the user to generate 3D images of the 2-DE gel which visualizes the differences in intensity of protein spots. When multiple gels are run and averaged, these differences in protein intensity can be statistically quantified.

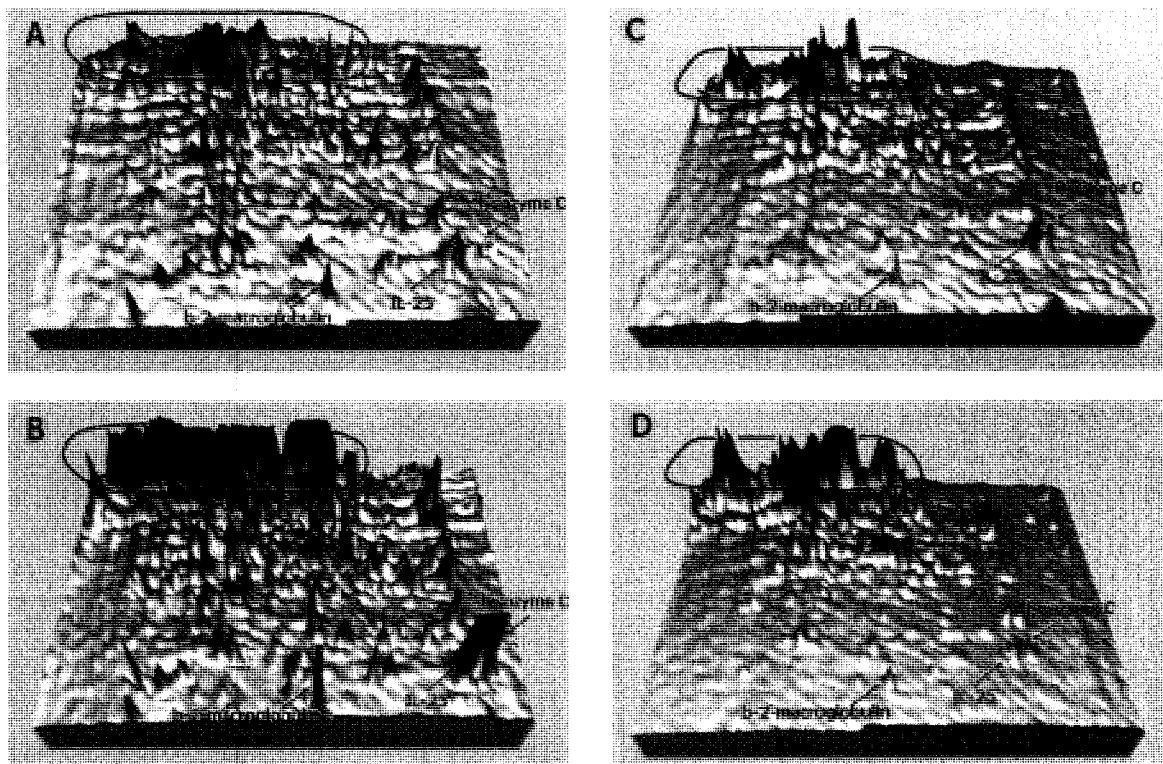
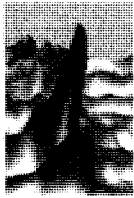


Figure AVI-2 - Image analysis of Sypro Ruby stained 2-DE gels from Figure AVI-1



- protein spots in general, excluding the serum proteins, appear to demonstrate a general increase in expression during differentiation, versus a general decrease in expression post-trypsinization and reseeded onto HDI compared to PS
- proteins of very high *pI* at each time point appear to demonstrate visible differences
- in addition, proteins of very low MW demonstrate drastic increases in MDM differentiating on HDI compared to PS, indicating the importance of small MW proteins that are likely cytokines and chemokines responsible for signaling and inflammatory responses



- proteins encompassed in the red circled area are mostly serum proteins
- this protein group, as a whole, appears to exhibit an increase in samples from HDI vs PS, both during differentiation and post-reseeding. This may be due to material differences in the initial serum protein adsorption.
- differences in these protein intensities can be seen by changes in the intensity of blue and height of the peaks



- IL-25**
- a recently identified protein of the IL-17 family of cytokines
 - induces Th2 cytokine expression (IL-4 and IL-13), eosinophilia and the chemokine eotaxin
 - demonstrates that IL-25 has an involvement in hyperreactivity that may indicate biocompatibility issues with MDM adherent to PCNU

- lysozyme**
- hydrolytic enzyme with esterase activity
 - demonstrates bacteriolytic function
 - presence in these samples indicates a potential contribution of lysosomal enzymes in the MDM responses to PCNU

- β -2-microglobulin**
- a fragment of this protein was identified at a much lower MW (processed protein?)
 - this protein comprises the β -chain of the MHC class I molecules
 - interacts with killer T lymphocytes – recognition of self versus non-self antigens

APPENDIX VII – AFFYMETRIX GENECHIP[®] ANALYSIS OF EXPRESSIONAL CHANGES IN MOUSE RAW 264.7 MACROPHAGES IN RESPONSE TO SURFACE CHEMISTRY DIFFERENCES

Some preliminary microarray experiments were performed while on the Endeavour Research Fellowship to the University of New South Wales. The purpose of this experiment was to conduct somewhat of a “fishing expedition” for genes that were up- or down-regulated in mouse RAW 264.7 macrophages in response to a change in material surface (TCPS versus PCNU) and also to determine if any of these genes would then be regulated by a therapeutic anti-inflammatory agent. In this section, results are presented of the genes that were found to be upregulated in mouse RAW 264.7 macrophages cells adherent to PCNU (HDI431) relative to the control TCPS surface.

The following is a brief overview of the methods to prepare RNA samples for microarray analysis:

Cell Culture

1. RAW 264.7 cells (cultured in the same manner as in Appendix IV)(1mL of a 5.0×10^5 cells/mL suspension) were plated into 24 well plates (Greiner) with or without HDI431 (PCNU) coated glass slips (6 wells for each condition).
2. Cells were incubated for 2 hours.
3. Culture medium was removed and cells were scraped from the material surface of each well and collected into individual microcentrifuge tubes.
4. The cells were centrifuged at 16,000 x g for 12 seconds, the supernatant removed, and the cell pellet lysed with 100 μ L of Trizol reagent.
5. The cell lysates from the same condition (TCPS x 6 wells and PCNU x 6 wells), were pooled into one microcentrifuge tube, these cell lysates were then extracted for RNA using a Trizol method.
6. The amount of RNA was quantified using the NanoDrop[®] ND1000.

7. The RNA samples were then submitted to the Ramaciotti Gene Analysis Centre (UNSW) where the integrity of the RNA samples was checked and samples were processed for Affymetrix GeneChip[®] Analysis using a Mouse Genome 430 2.0 Array.

Once the data were generated from the microarray, the results were log transformed (normalization at the probe level) and subjected to an RMA (Robust Multiple-array Analysis) data analysis. In order to determine the differences in gene expression between TCPS and PCNU, an MVA plot (difference in expression versus average intensity) (Figure AVII-1) was produced illustrating the differences in the expression of each probe set.

Figure AVII-1 illustrates the MVA plot for expressional changes between RAW 264.7 cells cultured on PCNU versus TCPS. As a general rule, differences in expression are considered significant when the change is ± 2 . In Figure AVII-1, the dots illustrated in yellow are probe sets that had >2 differences in gene expression. No genes were demonstrated to decrease by 2. The list of genes that demonstrated a difference >2 in expression on PCNU versus TCPS are listed in Table AVII-1. Although this experiment was only done once, and changes cannot be considered significant, this study demonstrated that microarray analysis may be a suitable platform to begin to assess changes in gene expression in macrophages in response to changes in material surface. These experiments may be repeated in order to allow statistical analysis of the data, or specific genes may be further investigated via other methods such as qPCR.

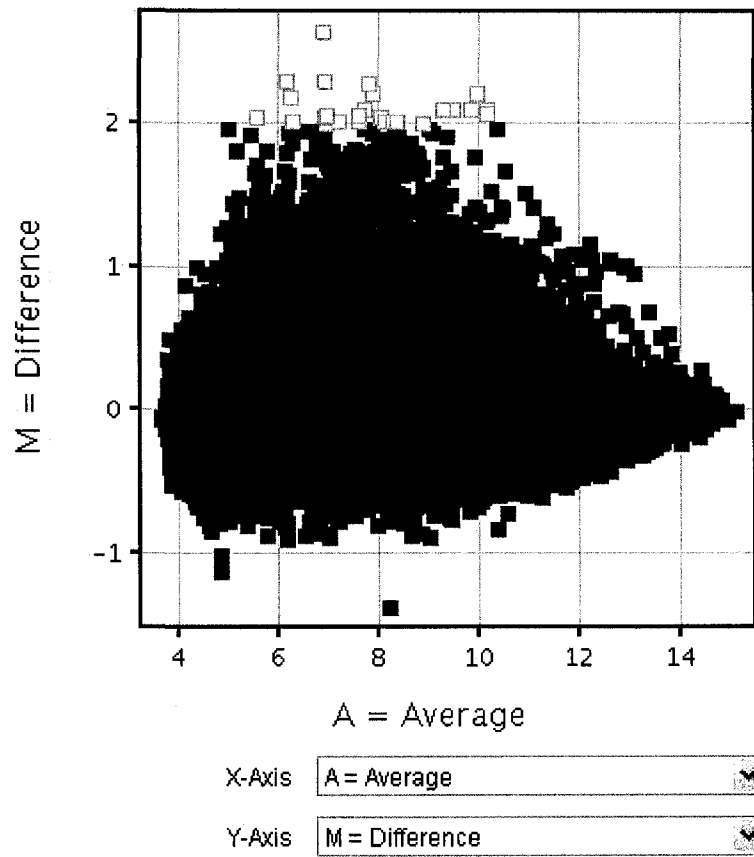


Figure AVII-1 - MVA scatterplot of differences in gene expression in mouse RAW 264.7 macrophages adherent to PCNU versus TCPS.

Table AVII-1 – List of genes that increased in expression in mouse RAW 264.7 macrophages adherent to PCNU versus TCPS

Probe Set ID	HDImedia	PSmedia	Unigene(Avadis)	Gene Title
1416732_at	7.6945	5.7545	Mm.130362	topoisomerase (DNA) II beta
1417770_s_at	7.9650	5.9601		
1418152_at	8.8626	6.9272	Mm.298443	nucleosome binding protein 1
1418429_at	7.3692	5.4276	Mm.223744	kinesin family member 5B
1419835_s_at	11.2342	9.1363	Mm.234912	plectin 1
1420171_s_at	11.3545	9.3924	Mm.29677	myosin
1420172_at	11.2058	9.1314	Mm.29677	Myosin
1420908_at	7.1804	5.2582	Mm.378895	CD2-associated protein
1421871_at	10.5242	8.4317	Mm.260760	SH3-binding domain glutamic acid-rich protein like
1423444_at	8.7586	6.6622	Mm.6710	Rho-associated coiled-coil forming kinase 1
1423445_at	8.2059	5.5577	Mm.6710	Rho-associated coiled-coil forming kinase 1
1423828_at	10.3370	8.4342	Mm.236443	fatty acid synthase
1427151_at	5.9943	4.0419	Mm.274314	RIKEN cDNA 4732486I23 gene
1427886_at	11.0584	8.8456	Mm.28399	nuclear pore membrane protein 121
1428107_at	10.3273	8.2314	Mm.260760	SH3-binding domain glutamic acid-rich protein like
1428410_at	8.2007	6.1938	Mm.278726	Mak3 homolog (S. cerevisiae)
1429660_s_at	7.3600	5.1809	Mm.2999	SMC2 structural maintenance of chromosomes 2-like 1
1433531_at	9.3118	7.3543	Mm.143689	acyl-CoA synthetase long-chain family member 4
1434061_at	7.9428	5.8999	Mm.288141	retinitis pigmentosa 2 homolog (human)
1434671_at	8.6373	6.6188		RIKEN cDNA B230337E12 gene
1434941_s_at	8.6986	6.7387	Mm.21228	RIKEN cDNA 2610101J03 gene
1435740_at	9.8681	7.8724	Mm.227484	retinoic acid induced 17
1436997_x_at	10.9009	8.7981	Mm.260760	SH3-binding domain glutamic acid-rich protein like
1437069_at	8.2379	6.2205	Mm.220204	oxysterol binding protein-like 8
1437442_at	6.5884	4.5440	Mm.332387	Transcribed locus
1438695_at	7.8875	5.9912	Mm.9002	Ubiquitin protein ligase E3A (Ube3a)
1440346_at	8.6203	6.5701	Mm.261201	Jumonji domain containing 3
1448402_at	9.9909	8.0353	Mm.208601	talin 1
1448635_at	10.0557	8.1221	Mm.2999	SMC2 structural maintenance of chromosomes 2-like 1
1450690_at	9.0925	7.0449	Mm.142730	RAN binding protein 2
1450896_at	6.4043	4.4833	Mm.35059	Rho GTPase activating protein 5
1450897_at	9.1346	7.1246	Mm.35059	Rho GTPase activating protein 5
1450994_at	8.9770	6.7705	Mm.6710	Rho-associated coiled-coil forming kinase 1
1452829_at	9.3728	7.4733	Mm.305535	carbamoyl-phosphate synthetase 2
1452830_s_at	9.2940	7.2793	Mm.305535	carbamoyl-phosphate synthetase 2
1453727_at	7.3079	5.0110	Mm.21228	RIKEN cDNA 2610101J03 gene
1455096_at	8.0883	5.8007		
1455476_a_at	7.2886	5.2746	Mm.334856	genetic suppressor element 1
1455556_at	9.3707	7.3609	Mm.254017	Notch gene homolog 2 (Drosophila)
1456651_a_at	7.9799	5.9225	Mm.174256	translocated promoter region
1458802_at	8.9325	6.6559	Mm.378956	human immunodeficiency virus type I enhancer binding protein 3

Curriculum Vitae

Donna Lee M. Dinnes

EDUCATION

Doctorate (PhD – Biochemistry; in progress)

Sept 2001 – Oct 2006

University of Ottawa (University of Ottawa Heart Institute – Ottawa, ON, Canada)

Supervisor: Dr. Rosalind S. Labow

Co-investigator: Dr. J. Paul Santerre (University of Toronto, Toronto, ON, Canada)

Thesis: Material surface chemistry influences the differentiation of human macrophages and their degradative and inflammatory responses.

The project focused on the role of phospholipase A₂ (PLA₂) enzymes in human macrophage-mediated degradation of polyurethane biomaterials (1). Since PLA₂ did not degrade the material directly, the project then focused on the contribution of inflammatory PLA₂ pathways to the foreign body reaction of macrophages to material surfaces (2). In order to identify additional proteins that contribute to macrophage-mediated polyurethane degradation, a comparative expression proteomics approach was applied to investigate differential proteome and secretome profile changes during cellular differentiation and material biodegradation (3). It was evident in these proteome and secretome studies and in a combined research project within the Labow research group (4) that *following* differentiation human macrophages were highly influenced in function and protein expression by slight differences in material chemistry (4). Collectively, this led to a study characterizing the structural and functional changes that occur *during* differentiation of monocytes to macrophages on polyurethane biomaterials (5).

Honours B.Sc. (Biomedical Biology Option)

1998 – 2001

Laurentian University (Sudbury, ON, Canada)

Supervisor: Dr. G. Ferroni

Graduated *Cum Laude*

Thesis: Cell yield as a function of electron donor for the bacterium *Acidithiobacillus ferrooxidans*

In this research project, the goal was to assess energy sources for the bacterium *A. ferrooxidans* that would have the electron donor generating the highest cell yield. This bacterium has an undesirable effect in the environment in that it contributes to acid mine drainage. However, a positive industrial application of this bacterium is that it is utilized in bioleaching techniques to extract precious metals from low-grade ores. In this application, maximizing the cell yield of *A. ferrooxidans* is critical for the bioleaching process as well as laboratory investigations with *A. ferrooxidans* that require high concentrations of macromolecules such as DNA, ribosomes and intracellular proteins.

Canadore College of Arts and Technology (North Bay, ON, Canada)
Graduated with Academic Honours

This program included a 2-year diploma program to gain the academic background for an 8 month clinical internship at the Sudbury Regional Hospital Laboratory. This was an intense course and clinical laboratory based program followed by national licensing examination procedures.

PUBLICATIONS

- (1) **Dinnes DLM**, Santerre, JP and Labow RS. Phospholipase A₂ pathway association with macrophage-mediated polycarbonate-urethane biodegradation. (2005) *Biomaterials* 26:3881-3889.
- (2) **Dinnes DLM**, Santerre JP and Labow RS. Intracellular phospholipase A₂ expression and location in human macrophages: Influence of synthetic material surface chemistry. *Submitted to Cellular Signalling* (2006).
- (3) **Dinnes DLM**, Marçal H, Mahler SM, Santerre JP, Labow RS. Material surfaces affect the protein expression patterns of human macrophages: a proteomics approach. (2006) *Journal of Biomedical Materials Research: Part A DOI:10.1002/jbm.a.30967*
- (4) Labow RS, Sa D, Matheson LA, **Dinnes DLM**, and Santerre JP. The human macrophage response during differentiation and biodegradation on polycarbonate-based polyurethanes: dependence on hard segment chemistry. (2005) *Biomaterials* 26:7357-7366.
- (5) **Dinnes DLM**, Santerre JP, Labow RS. The influence of biodegradable and non-biodegradable material surfaces on the differentiation of human monocytes to macrophages. *Submitted to Differentiation* (2006).

PRESENTATIONS

Peer-reviewed presentations:

- (1) **Dinnes DLM**, Santerre JP, Labow RS. Effect of material surface chemistry on the phospholipase A₂ pathways in human macrophages. Podium presentation - 22nd Annual Meeting of the Canadian Biomaterials Society (May 2002) Toronto, ON.
- (2) **Dinnes DLM**, Santerre JP, Labow RS. The role of phospholipase A₂ in the biodegradation of polyurethanes. Poster presentation - 9th Annual Ottawa Life Sciences Conference (Nov 2002) Ottawa, ON.
- (3) **Dinnes DLM**, Santerre JP, Labow RS. Effect of material surface chemistry on the phospholipase A₂ pathway in human macrophages. Podium presentation - 27th Annual Meeting of the Canadian Medical and Biological Engineering Society (Nov 2002) Ottawa, ON.

- (4) **Dinnes DLM**, Santerre JP, Labow RS. Phospholipase A₂ activation by material surface chemistry in U937-cell mediated biodegradation of polyurethanes. Podium presentation - 29th Annual Meeting of the Society for Biomaterials (April 2003) Reno, NV, USA.
- (5) **Dinnes DLM**, Santerre JP, Labow RS. The effect of phospholipase A₂ inhibition on U937 cell activation by polyurethanes. Poster presentation - 23rd Annual Meeting of the Canadian Biomaterials Society (May 2003) Montreal , QC.
- (6) **Dinnes DLM**, Santerre JP, Labow RS. The activation of phospholipase A₂ in the macrophage response to polyurethanes used in cardiovascular devices. Poster presentation - 26th Annual Meeting of the Canadian Federation of Biological Societies (Nov 2003) Ottawa , ON.
- (7) **Dinnes DLM**, Santerre JP, Labow RS. The effect of phospholipase A₂ inhibition on U937 cell activation by polyurethanes. Poster presentation - 10th Annual Ottawa Life Sciences Conference (BioNorth 2003) (Nov 2003) Ottawa, ON.
- (8) **Dinnes DLM**, Santerre JP, Labow RS. Macrophage-mediated degradation of polyurethanes: A role for phospholipase A₂. Podium presentation. 7th World Biomaterials Congress (May 17-21, 2004) Sydney, Australia.
- (9) **Dinnes DLM**, Sa D, Santerre JP, Labow RS. Methylenedianiline (MDA) affects monocyte-derived macrophage-mediated polycarbonate-urethane degradation. Podium presentation. 30th Annual Meeting of the Society for Biomaterials (Apr 27-30,2005) Memphis, TN, USA.
- (10) **Dinnes DLM**, Santerre JP, Labow RS. Material influence on phospholipase A₂ protein expression in human macrophages. Podium presentation. 24th Annual Meeting of the Canadian Biomaterials Society (May 26 - 28, 2005) Waterloo, ON.
- (11) Marçal H, **Dinnes DLM**, Raftery M, Labow RS, McFarland C, Santerre JP, Mahler SM. The effect of material chemistry on cellular morphology and membrane surface-interface proteins: an innovative explorative proteomics technique. Podium presentation. 8th Annual Tissue Engineering Society International Meeting (Oct 22-25, 2005) Shanghai, China.
- (12) **Dinnes DLM**, Santerre JP, Labow RS. Macrophage expression of phospholipase A₂ is altered by polycarbonate-urethane surfaces. Podium presentation. 31st Annual Meeting of the Society for Biomaterials (Apr 26-29, 2006) Pittsburgh, PA, USA.
- (13) **Dinnes DLM**, Marçal H, Mahler SM, Santerre JP, Labow RS. Material surfaces affect the protein expression patterns of human macrophages: a proteomic approach. Poster presentation. 31st Annual Meeting of the Society for Biomaterials (Apr 26-29, 2006) Pittsburgh, PA, USA.
- (14) **Dinnes DLM**, Matheson LA, Marçal H, Mahler SM, Santerre JP, Labow RS. Use of expression proteomics to define proteins secreted from monocyte-derived macrophages on polycarbonate-urethane surfaces. Poster presentation. 25th Annual Meeting of the Canadian Biomaterials Society (May 26-29, 2006) Calgary, AB.

Non-referreed presentations:

- (1) **Dinnes DLM**, Santerre JP, Labow RS. Effect of material surface chemistry on the phospholipase A₂ pathway in human macrophages. Poster presentation - University of Ottawa Graduate Department of Biochemistry Poster Day (Apr 2002).
- (2) **Dinnes DLM**, Santerre JP, Labow RS. Material surface chemistry affects the phospholipase A₂ pathway in human macrophages. Podium presentation - Heart Institute Research Day (Apr 2002).
- (3) **Dinnes DLM**, Santerre JP, Labow RS. The effect of material surface chemistry on the phospholipase A₂ pathway in human macrophages. Podium presentation - University of Ottawa Graduate Department of Biochemistry Student Seminar Series (Nov 2002)
- (4) **Dinnes DLM**, Santerre JP, Labow RS. The role of the phospholipase A₂ pathway in the biodegradation of polyurethanes. Podium presentation - Heart Institute Research Day (Apr 2003).
- (5) **Dinnes DLM**, Santerre JP, Labow RS. The effect of phospholipase A₂ inhibition on U937 cell activation by polyurethanes. Poster presentation - 2nd Annual Meeting - Cell Signaling in Mucosal Inflammation and Pain – CIHR Strategic Training Program (Apr 2004) Toronto, ON.
- (6) **Dinnes DLM**, Santerre JP, Labow RS. The effect of phospholipase A₂ inhibition on U937 cell activation by polyurethanes. University of Ottawa Graduate Department of Biochemistry Poster Day (April 2004).
- (7) **Dinnes DLM**, Santerre JP, Labow RS. Macrophage-mediated degradation of polyurethanes: The role of phospholipase A₂. Podium presentation - University of Ottawa Heart Institute Research Day (Apr 2004).
- (8) **Dinnes DLM**, Santerre JP, Labow RS. The road to biodegradation: Is it along the phospholipase A₂ pathway? Podium presentation - University of Ottawa Graduate Department of Biochemistry Student Seminar Series (Nov 2004).
- (9) **Dinnes DLM**. Material surface influence on the inflammatory, degradative and differentiation capacity of human macrophages. PhD Research Seminar. University of Ottawa Graduate Department of Biochemistry (Mar 2005).
- (10) **Dinnes DLM**, Marçal H, Mahler SM, Santerre JP, Labow RS. Macrophage-mediated polyurethane degradation: A proteomic approach to biomaterials research. Poster presentation. 3rd Annual Meeting – Cell Signaling in Inflammation and Pain – CIHR Strategic Training Program (Apr 2005) Toronto, ON.

ACADEMIC AWARDS

Endeavour Research Fellowship (\$22,500 AUS) Department of Education, Science and Training (Australian Government) - 5 month research visit to University of New South Wales, Sydney, AUS	May – Oct 2006
Short-Term Research Visit (\$11,000) Canadian Institutes of Health Research (Institute of Musculoskeletal Health and Arthritis) - 3 month research visit to University of New South Wales, Sydney, AUS	Jan – Apr 2005
CIHR Strategic Training Fellowship (\$53,000) CIHR Strategic Training Program Cell Signaling in Mucosal Inflammation and Pain	2003 – 2006
Reuben Fisher Scholarship (\$10,000) MSH Foundation (Montreal, QC)	2002
Biochemistry Graduate Program Entrance Award (\$4,000)	2001
Laurentian University Academic Achievement Award (\$3,000)	1999 - 2001

EMPLOYMENT

University of Ottawa Exam proctor - supervision of 2 nd year Biochemistry final exam	Apr 2004 Ottawa, ON, Canada
University of Ottawa Teaching Assistant - BCH 3346 (Biochemistry Laboratory II) - supervision of laboratory sessions and grading lab reports	Sept 2003 – Dec 2003 Ottawa, ON, Canada
Sudbury Regional Hospital Medical Laboratory Technologist	June 1999 – Aug 2001 Sudbury, ON, Canada
MDS Laboratories Medical Laboratory Technologist	May 1999 – June 1999 Sudbury, ON, Canada
Fort St. John Hospital and Health Centre Medical Laboratory Technologist	May 1997 – Aug 1998 Fort St. John, BC, Canada

DISTINCTIONS / CREDENTIALS

Poster Presentation Award 25 th Annual Meeting of the Canadian Biomaterials Society (Calgary, AB, Canada)	May 2006
Student Travel and Professional Development Award Honourable Mention 30 th Annual Meeting of the Society for Biomaterials (Memphis, TN, USA)	Apr 2005
Poster Presentation Award 23 rd Annual Meeting of the Canadian Biomaterials Society (Montreal, QC, Canada)	May 2003
Student Travel and Professional Development Award Honourable mention 29 th Annual Meeting of the Society for Biomaterials (Reno, NV, USA)	Apr 2003
Basic Science Oral Presentation Award Honourable Mention University of Ottawa Heart Institute Research Day	Apr 2002
Bayer Scholarship Award Canadore College (Proficiency in Clinical Chemistry)	1996
Don Harron Award Canadore College (Academic Achievement)	1995

LABORATORY TECHNIQUES

Phlebotomy	Thin Layer Chromatography
1D Gel Electrophoresis	2D Gel Electrophoresis
Western Blotting	MALDI-ToF MS
Cell Culture	ELISA
- cell lines	Immunofluorescence
- primary cells	Laser Scanning Confocal Microscopy
Bacterial Culture	Protein / DNA / RNA Isolation
Enzyme Assays	Anti-sense Oligonucleotide Techniques
Radioisotope Techniques	Quantitative RT-PCR

OTHER ACTIVITIES

Australian Proteomics Society Member	2005 – 2006
Canadian Biomaterials Society Student Member	2002 – 2006
Ottawa Sports and Social Club	2004
Girl Guides of Canada Sparks Leader	2002 – 2003
Canadian Medical and Biological Engineering Society Student Member	2002
French Language Training – University of Ottawa Heart Institute	2001 – 2002
French as a Second Language (College Boreal)	2000
Canadian Medical Laboratory Technologists of Ontario	1997 – 2004

REFEREES

Dr. Rosalind S. Labow PhD

Professor

Departments of Surgery and Biochemistry, Microbiology & Immunology

Director, Taichman Laboratory

University of Ottawa Heart Institute

Dr. Stephen M. Mahler PhD

Senior Lecturer

Bioengineering Center – S119

Department of Biotechnology and Biomedical Science

University of New South Wales

Dr. Odette Laneuville PhD

Associate Professor

Department of Biochemistry, Microbiology and Immunology

University of Ottawa – Faculty of Medicine

451 Smyth Road

Dr. J. Paul Santerre PhD

Professor

Associate Dean Research

Director, Dental Research Institute

Department of Biological and Diagnostic Sciences

University of Toronto – Faculty of Dentistry