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Pathogenesis of rheumatoid arthritis: Insights into mechanisms of synovial hyperplasia and of reactive nitrogen species mediated inflammatory damage

A THESIS SUBMITTED TO THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES
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THESIS ABSTRACT

Synovial hyperplasia is a hallmark of rheumatoid arthritis (RA). This pathological change may be due to an increase in cell proliferation and/or a decrease in cell death (apoptosis). However, the rates at which these opposing mechanisms occur in the arthritic and control synovium remain a subject of debate. Our objective was to address this debate using recently developed polyclonal antibodies to thymidylate synthase (TS, another potential cell proliferation marker), and activated caspase 3 and cleaved polyADPribose polymerase (PARP p85), two markers of apoptosis. PCNA and Ki-67, two well characterized proliferation markers were also used in this study. Germinal centers of human tonsil tissue were used as positive controls for proliferation and apoptosis. Using immunohistochemistry, I showed that monoclonal antibodies to PCNA and Ki-67, and polyclonal antibodies to TS detected proliferating B-lymphocytes in the germinal centers of tonsil tissue. Mitotic figures were also clearly observed in the germinal centers. When the synovial tissue of RA, OA and control patients were studied, no mitotic figures were seen. Antibodies directed against "proliferation markers" PCNA, Ki-67 and TS produced discordant results. TS and Ki-67 antibodies stained a small minority of synovial lining cells in RA, OA and control patients with a modest increase of TS and Ki-67 labeling observed in RA and OA compared to controls. By contrast, a high number of synovial lining cells in RA, OA and controls were stained with an antibody directed against PCNA. In agreement with my immunohistochemical observation, Western blot analysis of synovial homogenates in a minor fraction of RA and OA patients detected PCNA but no cases detected Ki-67 or TS. Immunohistochemistry of tonsil tissue showed that apoptosis could be detected in

germinal center lymphocytes by PARP p85 and activated caspase 3. Using identical conditions, apoptotic cells were rarely detected in the synovial lining RA, OA and control patients. The rate of apoptosis was similarly low in all three patients groups studied with less than 0.5 % of synovial lining cells staining with PARP p85 and activated caspase 3. On the basis of this evidence, I conclude that the increased number of cells in the synovial lining of RA patients is a consequence of a modest increase in proliferating synoviocytes associated with a normally low rate of cell death. Moreover, my study indicates that TS can be used as a proliferation marker and also questions the utility of PCNA for this purpose.

Another equally important factor in RA is the role of reactive nitrogen and oxygen species (RNOS) such as nitric oxide (NO) and superoxide (O_2^-) in inflammation mediated protein damage. Such damage is evidenced by the immunohistochemical detection of nitrotyrosine in a variety of animal models and human inflammatory disorders. This has lead to the hypothesis that nitrotyrosine may play a role in inflammatory disease progression. Therefore, I studied arthritic joint tissue for the presence of protein nitrotyrosine using immunohistochemical and immunoblotting techniques. Nitrated proteins were detected at varying degrees in the synovial tissue from most RA and OA patients. The extent of nitrotyrosine staining in RA synovium was significantly greater than that observed in OA synovium ($p<0.008$). Protein nitrotyrosine was detected throughout the synovium of most samples, including extracellular matrix, lymphocyte aggregates, macrophage and endothelial cells. Immunoblots revealed several high and low molecular mass proteins in synovial homogenates of some RA and OA patients, confirming the presence of in situ protein nitrotyrosine. Taken together, these

data suggest that protein nitrotyrosine is present in a variety of cell types in the inflamed joint and may play a role in RA disease progression. However, the consequence of protein nitration in the joint has yet to be determined.

The work presented in this thesis has addressed mechanisms involved in synovial hyperplasia and the consequence of RNOS in the rheumatoid synovium. Findings from both parts of my study contribute to our current knowledge of the pathogenesis of RA. The observation that a very modest increase in proliferation in RA and OA synovia coupled with a normally low rate of apoptosis may produce the observed synovial hyperplasia and may also be a significant contribution to the current knowledge of the pathogenesis of RA. The second part of my study examined the detection of protein nitrotyrosine in RA synovium and the fact that nitrated proteins are detected in a variety of cell types provides additional evidence for the role of RNOS in RA pathogenesis. Since this protein damage may contribute to RA pathology, my study suggests that inhibition of NO production may have therapeutic benefits in this disease.

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Finally, I wish to thank the members of the Birnboim and Goldstein laboratory for their technical assistance and encouragement. Thank you all very much.

DEDICATION

In the memory of my father,

and to

my mother and brother

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LIST OF ABBREVIATIONS

ACR	American College of Rheumatology
AR	antigen retrieval
BdrU	bromodeoxyuridine
bFGF	basic fibroblast growth factor
BSA	bovine serum albumin
CH_2THF	5, 10 methylenetetrahydrofolate
cNOS	constitutive nitric oxide synthase
CO_2	carbon dioxide
COX-1	constitutive cyclooxygenase
COX-2	inducible cyclooxygenase
dTDP	deoxythymidine diphosphate
dTMP	deoxythymidine monophosphate
dTTP	deoxythymidine triphosphate
dUMP	deoxyuridine monophosphate
EtOH	ethanol
FADD	Fas-associated death domain
FEN-1	flap endonuclease
FLS	fibroblast-like synoviocytes
GDP	guanosine diphosphate
GTP	guanosine triphosphate
HOCl	hypochlorous acid
H & E	hematoxylin & eosin
Hprt	hypoxanthine phosphoribosyltransferase
IAPs	inhibitors of apoptosis proteins
IL-1β	interleukin-1beta
iNOS	inducible nitric oxide synthase
ISNEL	*in situ* nick-end labeling
kDa	kilodalton
LI	labeling index
$LiCO_3$	lithium carbonate
MAbs	monoclonal antibodies
MLS	macrophage-like synoviocytes
MMPs	matrix metalloproteinases
MPO	myeloperoxidase
N-BSA	nitrated bovine serum albumin
NF-κB	nuclear factor-kappaB
NO	nitric oxide
NOS	nitric oxide synthase
NSAIDs	nonsteroidal antiinflammatory drugs
O_2^-	superoxide radical
OA	osteoarthritis
$ONOO^-$	peroxynitrite
PARP p85	cleaved 85 kDa fragment of polyADPribose polymerase
PARP	polyADPribose polymerase

PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PDGF	platelet derived growth factor
PGH-2	prostaglandin H-2
PMSF	phenylmethylsulfonyl fluoride
RA	rheumatoid arthritis
RFC	replication factor C
RNOS	reactive nitrogen and oxygen species
SCID	severe combined immunodeficient mouse
SF	synovial fluid
ST	synovial tissue
TBS	Tris buffered saline
TNF-α	tumor necrosis factor-alpha
TNFRI	tumor necrosis factor receptor I
TRAMPs	tyrosine rich acidic matrix proteins
TS	thymidylate synthase
UDPGD	uridine diphosphoglucose dehydrogenase
VCAM-1	vascular cell adhesion molecule-1

CHAPTER 1: GENERAL INTRODUCTION

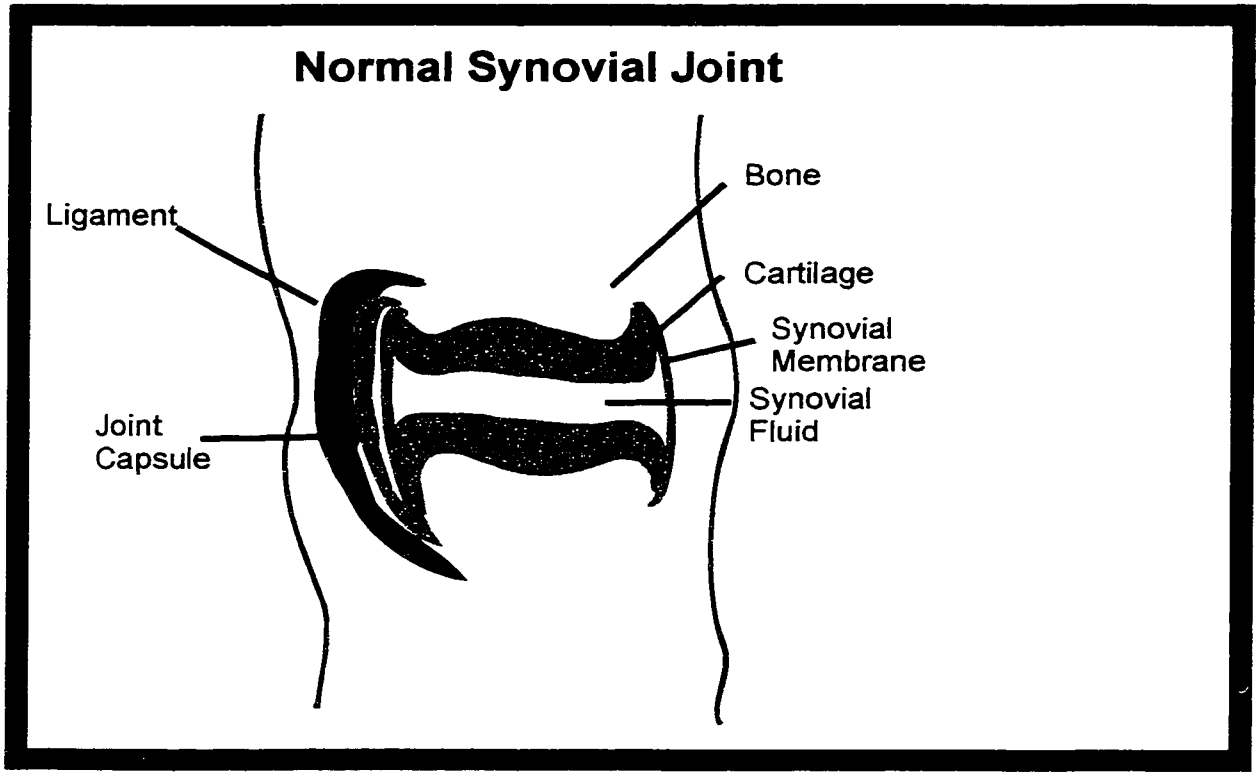
1.1 Arthritis overview

Arthritis ("arthr" meaning joint and "itis" meaning inflammation) is a disease of the synovial joints. There are over 150 different types of arthritis affecting approximately 4 million people in Canada alone. The two major types, rheumatoid arthritis (RA) and osteoarthritis (OA), affect 2.9 million people and 300,000, respectively, according to 1994 statistics (Arthritis Canada, 1996). OA is a degenerative and primarily non-inflammatory disease, whereas RA involves joint swelling and synovitis. Both diseases impair the structure and function of the joint.

1.2 Joint architecture

Understanding synovial joint anatomy will help in demonstrating how arthritis affects the synovial joint. The joint is where two or more bones meet and also referred to as an articulation. Cartilage caps the ends of the bones providing a smooth surface for movement. A 1-3 layer synovial membrane surrounds the articulation nourishing the joint and secreting lubricants to permit mobility. A tough capsule encloses the whole joint and ligaments join bones promoting joint stability (Figure 1.1).

Figure 1.1: Model of a normal synovial joint


Normal Synovial Joint
Ligament
Bone
Cartilage
Synovial Membrane
Synovial Fluid
Joint Capsule

1.3 Osteoarthritis

Osteoarthritis (OA) is a degenerative disease characterized by loss of articular cartilage. While OA is not generally considered an inflammatory disease, mild to moderate inflammatory changes have been reported (Haraoui et al., 1991). Typically these changes have been hypothesized to occur secondary to joint damage. Possible pro-inflammatory mediators of OA include interleukin-1 (IL-1), tumor necrosis factor (TNF) and reactive nitrogen and oxygen species (RNOS) such as nitric oxide (Pelletier et al., 1999; Venn et al., 1993; Studer et al., 1999; Evans and Robbins, 1999). OA often strikes joints of the hands and large weight bearing joints such as the hip or knees, and can present itself as a monoarthritis or polyarthritis. In severe cases joint deformation occurs and surgery is required to reduce pain and ameliorate the condition.

1.4 Rheumatoid arthritis

Rheumatoid arthritis (RA) is a debilitating, inflammatory autoimmune disease of unknown etiology. Landre Beauvais first medically described RA at the dawn of the 19th century. He termed the disease "la goutte asthenique primitive," in other words "debilitating gout". However, unlike gout, this new disease presented itself as a polyarthritis versus a monoarthritis and was predominantly observed in women.

Epidemiologically, RA has a worldwide distribution affecting approximately 1% of Caucasian adults and 5% of Native American populations (Maini and Zvaifler N.J., 1994); genetic and environmental aspects probably accounting for these differences. Like many other autoimmune diseases, RA is two to three times more frequent in women than in men, suggesting a role for sex hormones (Cutolo et al., 1996). Hallmarks of the

disease are chronic inflammation of synovial joints and thickening of the synovial membrane. In most cases, this is followed by progressive loss of joint structure and function resulting in severe deformity.

Although the cause of RA is unknown, the progression of the disease is better characterized. After initiation, immune cells such as T lymphocytes, B-lymphocytes, macrophage, neutrophils and mast cells infiltrate the synovium and synovial fluid of the affected joint. Cytokines such as TNF-α, IL-1, and reactive nitrogen and oxygen species (RNOS) are secreted by both infiltrating and resident cells. A sustained inflammatory response ensues, causing widespread damage. Furthermore, the synovial membrane becomes hyperplastic, increasing in thickness from 1-3 cell layers to greater than 10 cell layers. The synovial lining cells become attached to the articular cartilage, releasing degradative enzymes that contribute to joint destruction. This results in major deformity and often a loss in joint mobility. Although joint involvement is a hallmark of the disease, the process is systemic and involves vital organs where the disease presents itself as inflammatory lesions. The process is associated with increased morbidity and mortality (Symmons et al., 1986; Vandenbroucke et al., 1984; Gabriel et al., 1999; Guedes et al., 1999).

1.5 Proliferation and apoptosis in the RA synovium

Considerable information has been gathered regarding the histology and immunohistology of both RA and OA synovium. Immunohistochemistry has helped identify subsets of T cell and mononuclear nuclear cell populations in the synovium (Haraoui et al., 1991). Monoclonal antibodies such as proliferating cell nuclear antigen

(PCNA) and Ki-67 have been used to investigate the extent of synovial lining proliferation. However, both antibodies have produced opposite results (Lalor et al., 1987; Kinne et al., 1995a; Kinne et al., 1995b). This discrepancy has led to ambiguity about whether synovial thickening is due to an increase in synoviocyte proliferation and/or a decrease in apoptosis. The quantitative extent of synoviocyte proliferation is not clear in RA or normal tissue. The availability of a new polyclonal antibody against thymidylate synthase (TS 7.4), a potentially useful proliferation marker may resolve the issue (Haqqani et al., 1999).

As indicated, a decrease in synovial cell death in RA could contribute to synovial hyperplasia. Apoptosis of synoviocytes does occur in the synovium; however the rate remains somewhat ambiguous. Activated caspase 3 and cleaved polyADPribose polymerase are recently established hallmarks of apoptosis that may be helpful in determining the *in situ* rate of apoptosis in the synovial lining. *In vitro* studies on cultured RA synoviocytes showed that apoptosis occurred via a fas mediated pathway including the activation of caspase 3 and subsequent cleavage of PARP (Kobayashi et al., 1999a). Neither marker has been studied *in situ* in the synovium of RA, OA or control patients. Understanding the main processes involved in synovial hyperplasia could serve in the focused development of new treatment for those affected with this crippling disease. For example, if apoptosis is defective in RA as compared to controls, local induction of apoptosis using gene-based therapies could be devised to counter synovial proliferation.

1.6 RA and reactive nitrogen and oxygen species

Nitric oxide (NO) is a small, diffusible molecule that is synthesized from L-arginine by the nitric oxide synthase family (NOS). NO performs many functions in the body. Physiological functions include participation in endothelial relaxation, neurotransmission, and immune defense. However, accumulating evidence implicates NO as a mediator of pathophysiology, for example, in inflammation and autoimmune-mediated tissue destruction (Kolb and Kolb-Bachofen, 1992; Stichtenoth and Frolich, 1998).

Several lines of evidence suggest that NO may play a part in the pathogenesis of RA. First, increased nitrite/nitrate levels have been detected in the serum and synovial fluid (SF) of RA patients compared to controls (Farrell et al., 1992). Second, primary cultures of OA and RA synoviocytes produced NO spontaneously and at higher concentrations in response to certain cytokines (Grabowski et al., 1996a). Third, iNOS is expressed by resident and infiltrating cells in the inflamed joint of RA patients (Sakurai et al., 1995; McInnes et al., 1996). Finally, inhibition of iNOS ameliorates the symptoms of experimental RA-like arthritis in several animal models while supplementation with L-arginine results in exacerbation of inflammation typical of RA (McCartney-Francis et al., 1993; Stefanovic-Racic et al., 1994; Miesel et al., 1996).

NO mediated damage may occur via several pathways (Patel et al., 1999). One pathway involves the generation of peroxynitrite ($ONOO^-$), a reaction product of NO and superoxide (O_2^-). $ONOO^-$ is negatively charged and does not readily cross membranes, compared to NO. However, it is potent oxidizing molecule capable of nitrating tyrosine residues resulting in the formation of nitrotyrosine. Macrophages, foam cells (Buttery et

al., 1996), chondrocytes (Hashimoto et al., 1999), and endothelial cells (Kooy and Royall, 1994) have all been demonstrated to produce $ONOO^-$. As assessed by immunohistochemical detection of nitrotyrosine, recent data show evidence for the *in vivo* generation of $ONOO^-$ in disorders such as inflammatory bowl disease (Dijkstra et al., 1998), acute respiratory distress syndrome (Haddad et al., 1994), atherosclerosis (Beckmann et al., 1994), multiple sclerosis (Bagasra et al., 1995), Alzheimer's disease (Good et al., 1996), canine model of osteoarthritis (Pelletier et al., 1999) and mouse model of collagen induced-arthritis (Szabo et al., 1998). High levels of 3-nitrotyrosine have also been detected by HPLC in the serum and synovial fluid of RA patients compared to OA and control patients (Kaur and Halliwell, 1994). Thus far, the presence of nitrotyrosine-modified proteins has not been determined immunohistochmically in the synovial joints of patients with RA. This has led us to investigate the localization of protein nitrotyrosine in the synovial tissue of RA and OA patients. Since the etiology of RA remains unclear, understanding the link between RNOS, and inflammation and disease progression could better serve in the development of new drugs that target members of the NO pathway.

1.7 Hypothesis

a) We hypothesize that altered rates of proliferation and/or apoptosis are mechanisms that can lead to synovial hyperplasia. The rates at which both processes occur remain unclear in RA as compared to controls. The development of new markers, which can examine proliferation and apoptosis, could help resolve the rates at which these opposing processes occur. Such markers include the cytosolic TS enzyme (elevated in the S phase

of the cell cycle) and activated caspase 3 and cleaved PARP (markers of apoptosis). If proliferation is a major contributor to synovial hyperplasia then the labeling index (LI) will be higher in RA as compared to controls. However, if the proliferation LI is low in RA and controls then a decreased apoptosis rate compared to controls could account for the synovial thickening.

b) Chronic inflammation of the synovial joint is an important feature of RA. A form of damage acquired during chronic inflammation can be measured at the protein level. The arthritic joint accumulates many inflammatory immune cells important in the pathogenesis of RA, such as T-cells, B-cells, macrophage and neutrophils. Upon activation, resident cells, macrophage and neutrophils can generate relatively high levels of radical species such as NO and $O_2^{\cdot-}$. NO and $O_2^{\cdot-}$ can react to form peroxynitrite ($ONOO^-$) a molecule that can nitrate tyrosine residues forming nitrotyrosine. We hypothesize that NO is important for the damage incurred during the pathogenesis of RA. If NO is responsible for protein damage thought to occur within the inflamed joints then protein nitrotyrosine should be detectable *in situ* in the RA synovium and to a lesser extent in OA.

1.8 Objectives

a) To understand the balance of proliferation and apoptosis in synovial tissue, both in normal controls and in patients with OA and RA. A major outcome of this objective is to understand the mechanism of synovial hyperplasia in RA, one of the hallmarks of this disease.

b) To determine the presence and localization of protein nitrotyrosine (a marker of peroxynitrite generation) in the synovium of RA and OA patients.

1.9 Significance

This research is significant in that it attempts to address two aspects that are involved in the pathogenesis of RA. The first aspect is examining synovial hyperplasia, using several new markers to study proliferation and apoptosis in RA. Determining the process involved in synovial hyperplasia in RA will lead to a better understanding of the disease and lead to new therapies. The second part of this study represents the first attempt to determine whether RNOS contributes to protein modification preferentially in the inflamed joints of RA patients. RNOS can potentially modify many proteins altering their structure and function. Determining where NO mediated protein modification is taking place will help us better understand the effects of inflammation and NO in this disease. For example, protein modification such as tyrosine nitration may contribute to the pathology of RA by serving as a neoantigen that propagates the inflammatory cycle until the joint is destroyed. If identified as a key player, then new drugs could be developed to target the enzymes or molecules in the nitric oxide pathway.

CHAPTER 2: PROLIFERATION AND APOPTOSIS IN ARTHRITIS

2.1 Abstract

Synovial hyperplasia is a morphologic feature of rheumatoid arthritis (RA). However, whether this is due to an increase in the rate at which the synovial lining cells proliferate remains a subject of controversy. To clarify this, mitotic counting and, immunohistochemistry and Western blot analysis using antibodies directed against PCNA, Ki-67 and thymidylate synthase (TS 7.4) were performed on synovial tissue from 11 RA, 13 osteoarthritis (OA) and 4 control subjects. The apoptosis index was determined using polyclonal antibodies directed against a cleaved form of polyADPribose polymerase PARP p85 and activated caspase-3. The germinal center of adult tonsil tissue was used as a positive control for proliferation and apoptosis, since it is a well-defined site of B cell proliferation and cell death. Mitotic figures were frequently observed in the tonsil tissue. Immunoreactivity was found in the proliferating germinal centers using antibodies against PCNA, Ki-67 and TS 7.4. Using identical conditions, the synovial tissue of RA, OA and control patients were examined. In contrast to germinal centers of tonsillar tissues, mitotic figures were rarely observed in the hyperplastic synovium of RA, OA and control patients. Antibodies to both TS and Ki-67 stained a similar, but relatively low number of synovial lining cells in RA and OA, only modestly higher than in the synovium of control patients. By contrast, the antibody to PCNA stained a high number of synovial lining cells in RA, OA, and control patients, statistically significantly higher than that stained with Ki-67 ($p<0.001\%$) and TS ($p < 0.001\%$). In agreement with immunohistochemistry, Western blot analysis of RA and OA synovial homogenates detected PCNA, but not Ki-67 or TS. Germinal centers in

adult tonsillar tissues stained positively for PARP p85 and activated caspase 3, both of which are established markers of apoptosis. Identical conditions were used to stain synovial tissue of arthritic patients. Both antibodies stained an equivalently very low number of synovial lining cells in RA, OA and control patients. I conclude that the increased number of cells in the synovial lining is a consequence of a slow accumulation of modestly proliferating synoviocytes associated with normal low levels of apoptosis. Moreover, this study confirms the utility of anti-thymidylate synthase antibody, TS 7.4, as a useful proliferation marker and also questions the validity of PCNA in proliferation studies.

2.2 Introduction

2.2.1 Role of the synovial lining in joint destruction

Among the many cell types in the synovium, there has been increasing attention directed to non-immune elements, such as the synoviocytes, that make-up the synovial membrane. The synovial membrane, (also referred to as the intimal lining, synovial lining, synovial surface cells or synovial layer), is the interface between the synovium and the intraarticular space. It is typically 1-2 cell layers deep and composed of type A macrophage-like synoviocytes (MLS) and type B fibroblast-like synoviocytes (FLS). MLS express various macrophage markers such as CD11b, CD14, CD33 and CD68. The FLS can be distinguished from other fibroblasts by their surface expression of the cell adhesion molecule, vascular cell adhesion molecule-1 (VCAM-1) and intracellular expression of uridine diphosphoglucose dehydrogenase (UDPGD), a marker for the production of proteogylcans (Firestein, 1996). The intimal lining cells primarily function

to lubricate articular surfaces with glycosaminoglycans to permit joint mobility, as well as providing oxygen and nutrients to chondrocytes.

In a disease such as RA, the synovial layer can thicken to greater than 10 cell layers and become one of the mediators of joint destruction. MLS can produce proinflammatory cytokines such as IL-1 and TNF-α, both of which are known to induce the production of matrix degrading enzymes from FLS and articular chondrocytes (Tetlow and Woolley, 1995). Several classes of enzymes are involved in the degradation of joint tissue, including protein degrading enzymes such as cathepsins and matrix metalloproteinases (MMP), stromelysisn-1 and collagenase. These enzymes have been primarily localized to FLS by in situ hybridization studies (McCachren et al., 1990; Firestein et al., 1991; Keyszer et al., 1995).

Although FLS can react to their microenvironment, they are also able to act as independent aggressors. The most convincing evidence for the latter has been demonstrated in a severe combined immunodeficient (SCID) mouse model (Geiler et al., 1994). SCID mice lack a humoral and cell mediated immune response and thus can sustain xenografts for prolonged periods of time. FLS from synovial tissue of RA patients was co-implanted into SCID mice with normal human cartilage. After several weeks, the RA derived FLS invaded and destroyed the cartilage. Long term cultures of RA FLS showed the same phenomenon. Interestingly, OA FLS or normal skin fibroblasts did not invade the co-implanted cartilage (Geiler et al., 1994).

2.2.2 Gene mutations and hyperplasia in the RA synovium

Although the mechanism by which RA FLS become invasive remains elusive, mutations in the genes of p53 and c-Ras in RA FLS have been suggested to play a role in synovial expansion that leads to joint destruction (Firestein et al., 1997; Roivainen et al., 1997; Reme et al., 1998)

The tumor suppressor p53, a central player in proliferation and apoptosis, blocks the progression of the cell cycle from G1 to S phase upon UV or oxidant damage to DNA. Extensive DNA damage can trigger p53-mediated apoptosis. However, when dysfunctional, p53 is associated with unregulated cell proliferation. In RA, earlier studies showed a discrepancy between excessive strand breaks and morphological characteristics of apoptosis in the synovial lining of RA patients. Subsequently, marked p53 overexpression was observed in regions of DNA fragmentation in RA patients compared to controls (Firestein et al., 1996). Since high p53 expression has been associated with point mutations the role of p53 in synovial expansion was pursued (Davidoff et al., 1991; Marks et al., 1991) . The revelation of somatic point mutations in the p53 gene in RA synovium and the fact that the growth rate of E6 (inactivates endogenous p53 protein) transfected FLS was significantly higher than compared to controls and more invasive into cartilage extracts strengthened the role of p53 in synovial hyperplasia (Firestein et al., 1997; Aupperle et al., 1998).

The ras proto-oncogene may also play a role in synovial expansion. Ras is a small guanine nucleotide binding protein that transduces biological information from the cell surface to cytoplasmic components within cells. It cycles between an active guanosine triphosphate (GTP) bound form and inactive guanosine diphosphate (GDP) bound form.

A single point mutation in the ras oncogene can lead to the locking of ras protein in the active form and sustained signaling to downstream effectors (Zwartkruis and Bos, 1999). Consequently, this induces cell transformation and up regulation in proliferation. The fact that RA synovial cells express elevated levels of several proto-oncogenes including c-Myc, c-Fos and c-Jun led to the examination of the mutational activation of ras in arthritic synovium (Kinne et al., 1995c; Roivainen et al., 1995; Roivainen et al., 1996). Recently, point mutations in H-ras were detected in the synovial tissue of RA patients and potentially involved in synovial hyperplasia (Roivainen et al., 1997). To this end, it is conceivable that mutant p53 and mutant ras could participate in the observed synovial expansion in RA.

2.2.3 Studies of proliferation in the synovial tissue of arthritic patients

Cellular proliferation is a fundamental feature of human neoplasms and possibly of synovial hyperplasia. Several methods and techniques have been used to identify cell proliferation in histological material. A traditional approach is to simply assess the mitotic phase of the cell cycle by microscopic examination of tissue sections and has been quantified in various tumors (Baak and Fleege, 1990). Another approach to assessing cell proliferation is identifying cells in the S phase, i.e., the period of DNA synthesis. This can be measured by incubating biopsies or cells for a period of time with [^{3}H] thymidine or bromodeoxyuridine prior to fixation (Quinn and Wright, 1990). More conveniently, antibodies such as PCNA and Ki-67 have become an important means of assessing the cell growth fraction (Yu and Filipe, 1993).

Synovial hyperplasia and chronic inflammation are hallmarks of RA. Expansion in the number of both type A and type B synoviocytes causes the formation of villous projections that invade and destroy the joint architecture. The mechanism and rate of synovial hyperplasia still remains a subject of debate. Active proliferation is one explanation for the notable increase in the number of synoviocytes in the intimal lining of the joints of RA patients, a previous study using Ki-67 as a marker of proliferation showed that less than 0.1% of the synovial lining cells in RA synovia were proliferating (Lalor et al., 1987). Furthermore, older studies have demonstrated negligible incorporation of [^{3}H] thymidine, and a lack of mitotic figures (Mohr et al., 1975; Nykanen et al., 1978; Coulton et al., 1980; Dreher, 1982) in the synovium of RA patients. This would suggest that local rapid proliferation of synoviocytes does not contribute to the increased number of cells observed in the intimal lining in RA synovium.

More recent studies, using proliferating cell nuclear antigen (PCNA) and c-myc as markers of proliferation, demonstrated that up to 70% of the synovial lining cells were locally proliferating (Qu et al., 1994a). PCNA positive synoviocytes displayed type-I procollagen positivity, indicating that the proliferating cells were predominantly type B fibroblasts and not of macrophage origin (Qu et al., 1994a; Kinne et al., 1995a). This suggested that synovial hyperplasia is attributed to local rapidly proliferating FLS.

Our objective was to resolve the conflicting evidence concerning the rate at which the intimal lining cells proliferate. Therefore, to identify the proliferative activity of the synovial lining, we studied synovial tissue from RA, OA and control patients using mitotic counting, and immunohistochemical staining of Ki-67, PCNA, and a newly

suggested marker of cell proliferation, thymidylate synthase (TS 7.4) (Haqqani et al., 1999)

2.2.4 Cell proliferation marker Ki-67

Ki-67 is a large nuclear protein of unknown function (Endl and Gerdes, 2000) that exists in two main isoforms with calculated molecular masses of 320 kDa and 359 kDa; alternate splicing generates the smaller fragment. Ki-67 is known to be strictly associated with cell proliferation. Immunizing mice with nuclear extracts from a Hodgkin lymphoma cell line L428 (Gerdes et al., 1983) originally generated the monoclonal antibody directed against the Ki-67 protein. The name was derived from the city of origin Kiel, Germany and the number of the original clone in a 96-well plate. Upon characterization, it was determined that Ki-67 antigen was present during all phases of the cell cycle except in resting cells (G_0). Ki-67 was localized in the nucleoli and nucleoplasm throughout G1, S and G2 phase of the cell cycle and increased in intensity upon entering the M phase of the cell cycle (Yu and Filipe, 1993). Furthermore, the Ki-67 antigen was found to be tightly associated with chromatin in S and G2, and co-localized with chromatids during mitosis (van Dierendonck et al., 1989). In comparison to mitotic counting, Ki-67 detection is a more sensitive technique for studying proliferation in that it recognizes longer phases of the cell cycle. Thus the use of Ki-67 has greatly expanded the opportunities for measuring cell proliferation.

Numerous studies have been performed to assess the usefulness of Ki-67 as a prognostic molecular marker in a variety of cancers (Scholzen and Gerdes, 2000). A relatively high Ki-67 labeling index appears to be associated with a poor prognosis in

patients with lung carcinoma (Mehdi et al., 1998), multiple myeloma (Drach et al., 1992), non-Hodgkin's lymphoma (Gerdes, 1990), melanoma (Hernberg et al., 1998), hepatocellular carcinoma (Tannapfel et al., 1999; King et al., 1998), soft tissue tumors (Heslin et al., 1998; Huuhtanen et al., 1999), pituitary tumors (Zhao et al., 1999), breast (Rudolph et al., 1999) and prostate cancer (Borre et al., 1998). The prognostic value for survival and recurrence has been repeatedly proven for non-Hodgkin's lymphoma, breast and prostate cancer (Scholzen and Gerdes, 2000). Conversely, some diseases, such as colorectal carcinomas, show no correlation with Ki-67 positivity and prognostic parameters (Jansson and Sun, 1997; Allal et al., 1998).

A practical disadvantage of the original Ki-67 antibody was that it required fresh frozen tissue samples and could not be used in formalin-fixed paraffin embedded sections that are commonly used in histopathology. This setback was overcome upon the development of the monoclonal Ki-67 antibody MIB-1 clone and the use of new antigen retrieval (AR) techniques (Cattoretti et al., 1992). Recently a comparative study of MIB-1 on paraffin sections and Ki-67 on frozen sections revealed that MIB-1 represented a more valuable tool in measuring clinical outcome of breast carcinoma (Veronese et al., 1995).

"Antigen retrieval" (AR) significantly aided the field of immunohistochemistry, especially with respect to formalin fixed paraffin embedded tissue antigens. Typically, formalin fixed tissues were digested with pepsin, trypsin, proteinase K or pronase for the recovery of antigenicity. However, a practical disadvantage of enzymatic treatment was the destruction of tissue architecture. In addition, this technique was only effective in

improving detection for a minority of commercially available antibodies (Brown and Chirala, 1995).

The antigen retrieval technique provided a non-enzymatic antigen unmasking method based on microwave heating of formalin fixed paraffin embedded tissues in a sodium citrate buffer. The exact mechanism behind microwave AR remains unknown, but it is hypothesized that the high temperature breaks some of the cross-links induced by formalin fixation, thereby unmasking the antigen. Moreover, chelation of calcium ions complexed to proteins during microwave heating in citrate buffer is also thought to play a role (Morgan et al., 1994).

Seventeen years after its discovery, the biological function of Ki-67 remains unknown, despite its extensive molecular characterization and routine use as a marker of proliferation. Ki-67 has been hypothesized to be DNA structural protein whose primary role could be to maintain a higher DNA order during mitosis where histone proteins are in a state of flux (Sawhney and Hall, 1992). Several studies microinjecting nuclei with antibodies against Ki-67 and the use of anti-sense oligonucleotides demonstrated an inhibition of thymidine incorporation into cultured cells suggesting that Ki-67 is essential for cell proliferation (Schluter et al., 1993; Starborg et al., 1996).

2.2.5 Proliferating cell nuclear antigen

Prior to the availability of AR and MIB-1, PCNA was the marker of choice for proliferation studies because of its usefulness on formalin fixed paraffin embedded tissues. However, the recent finding that PCNA plays a role in cell processes other than cell replication has questioned its use as a proliferation marker (Tsurimoto, 1998).

Proliferating cell nuclear antigen (PCNA) is a 36 kDa nuclear protein that is mainly expressed during G1 and S phase while declining progressively during G2 and M phase. It functions as an auxiliary protein for DNA polymerase delta and epsilon whose function is to encircle DNA and load the polymerase onto the DNA template (Prosperi, 1997). Antibodies against PCNA were originally detected in serum from patients with systemic lupus erythematous (Miyachi et al., 1978).

Several studies have been performed assessing the value of PCNA as a proliferation and prognostic marker in some cancers (Yu and Filipe, 1993). Typically, a higher PCNA index was associated with poorer survival in cancers such as breast carcinomas (Aaltomaa et al., 1992), prostatic carcinomas (Harper et al., 1992), ovarian carcinoma (Hartmann et al., 1992), gastrointestinal lymphomas (Woods et al., 1991), lung carcinomas (Ishiwata et al., 2000) and hepatocellular carcinomas (Ng et al., 1995).

Despite the numerous studies that have lead to the wide acceptance of PCNA as a proliferation marker, recent studies have cast doubt on this. There are ealier reports that show that PCNA does not correlate with other indices of cell proliferation such as Ki-67 labeling, and bromodeoxyuridine (BdrU) incorporation (Yu et al., 1991; Rosa et al., 1992). Recently, in diseases such as undifferentiated nasopharyngeal carcinomas and breast carcinomas, PCNA counts showed no correlation with Ki-67 counts (Markiewski and Domagala, 1996; Genc et al., 2000). These reports could be supported by the multiple roles of PCNA in the cell such as in cell cycle control, DNA replication, nucleotide excision repair, post-replication mismatch repair, base excision repair, role in maintaining chromosome structure, chromatin remodeling and assembly, its possible role in apoptosis and by 24 hour half-life (Sanchez and Elledge, 1995; Jonsson and Hubscher,

1997; Prosperi, 1997). Furthermore, PCNA can be transiently activated by the presence of superoxide, hydrogen peroxide and certain growth factors such as platelet-derived growth factor (PDGF) (Savio et al., 1998; Balajee et al., 1999). Therefore, one must always consider the biology of PCNA when interpreting and drawing conclusions from such studies.

2.2.6 Thymidylate synthase as a new marker of proliferation

Thymidylate synthase (TS) catalyzes the reductive methylation of deoxyuridine 5'-monophosphate (dUMP) to thymidine 5'-monophosphate (dTMP) using the reduced folate cofactor 5,10-methylenetetrahydrofolate (CH2THF) (Chu and Allegra, 1996). Once synthesized, dTMP is phosphorylated to dTDP and then to dTTP, which is one of the four deoxynucleoside triphosphates essential for DNA biosynthesis. dTMP can also be formed through a salvage pathway involving the phosphorylation of thymidine by thymidine kinase. Given the essential role that TS plays in the biosynthesis of an essential DNA precursor and given that inhibition of this reaction leads to the prompt cessation of cellular growth (Powell et al., 2000), and its known absence in G0 cells TS appears to represent a useful new marker for cellular proliferation.

Similar to Ki-67 and PCNA, the usefulness of TS as a prognostic marker has been examined in a variety of cancers. Studies have found that patients with TS positive tumor cells generally demonstrate poorer survival. The best characterized examples in this regard are colorectal, gastric, ovarian carcinomas and breast carcinomas (Yamachika et al., 1998; Yeh et al., 1998; Fujiwaki et al., 2000; Nishimura et al., 1999). Specifically in breast cancer TS levels reflected proliferation (significantly correlated with S phase) and

malignancy (Nishimura et al., 1999). However, it must be noted that TS is elevated in some tumors regardless of cell proliferation, which is believed to be due to some sort of dysregulation of the TS gene.

The 36 kDa TS protein forms a functional homo-dimer found in the cytosol. Not surprisingly, it is predominantly found at higher levels in the S-phase, and at lower concentrations in G2/M phase G1 phases (Cadman and Heimer, 1986). In our laboratory, Haqqani and coworkers (1999) developed a rabbit anti-human TS polyclonal antibody (TS 7.4). TS 7.4 stained proliferating fibroblast cultures while not staining confluent ones. Moreover, quiescent confluent monolayers of fibroblasts were stimulated to grow by scratching the monolayer and assessed for TS 7.4 immunoreactivity. Cells near the "wound" entered the cell cycle and became reactive to TS 7.4 whereas the confluent areas were devoid of staining. Western blot analysis was consistent with the immunocytochemistry. Finally, TS 7.4 stained germinal centers (known regions of proliferating B lymphocytes) of formalin fixed paraffin embedded tonsil tissue. A commercially available monoclonal antibody to TS (TS 106) stained tonsil tissue as well (Haqqani et al., 1999). Haqqani and coworkers (1999) suggested that TS 7.4 could be used as a new marker of proliferation marker.

2.2.7 Apoptosis in the synovium

In normal tissues, the balance between a variety of cell parameters including cell immigration, cell emigration, cell proliferation and apoptosis controls cell growth. Apoptosis is a highly regulated process of cell death. It is the main mechanism of cell deletion during vertebrate development (e.g., immune system and nervous system),

normal cell turnover (e.g., skin and gastrointestinal tract) and can be triggered by a myriad of signals including irreparable cell damage. Morphologically, apoptotic cells can be distinguished from normal cells based on cell shrinkage, nuclear condensation and membrane blebbing and DNA fragmentation (Nishioka et al., 1998). Following these morphologic changes, cells undergoing apoptosis shed membrane-bound apoptotic bodies that are quickly engulfed by macrophages ensuring that a localized inflammatory response does not occur (Firestein et al., 1995). This differs markedly from necrosis where an injured cell swells and explodes, releasing intracellular contents, which leads to an inflammatory response (Kuby, 1997).

Defective apoptosis is associated with autoimmune diseases such as systemic lupus erythematous, scleroderma and RA (Mountz et al., 1994). Clearly, a decreased rate of apoptosis could, in conjunction with modest proliferation, lead to synovial hyperplasia. The extent of apoptosis in the rheumatoid synovium has been examined. Recent studies suggest that proteins such as NF-κB and sentrin 1 are involved in decreased synovial apoptosis (Franz et al., 2000; Zhang et al., 2000). Some earlier studies showed that genomic DNA extracted from RA and OA tissue demonstrated a DNA laddering formation typical of apoptosis. Furthermore, in situ nick end labeling (ISNEL), which labels cells with fragmented DNA (indicative of apoptosis), localized positively staining cells in the sublining and intimal lining regions of RA synovia (Firestein et al., 1995; Nakajima et al., 1995) indicating that cell turnover is occurring. However, the rate of in situ apoptosis in the intimal lining of RA patients remains unclear. Using ISNEL, Nakajima and coworkers (1995) determined that approximately 30% of RA synovial lining cells were undergoing apoptosis while another study using the same technique

determined that less than 1% of RA synoviocytes were undergoing apoptosis (Sugiyama et al., 1996). A third study using the same technique but on frozen synovial tissue showed that between 5-50% of cells were positive for apoptosis in the RA synovium (Firestein et al., 1995). Recently, it has become evident that ISNEL is prone to false positive and false negative results dependant on tissue fixation and concentration of reagents (Saraste, 1999). To complicate things further, ISNEL positivity can also reflect a wide range of cellular conditions; viable cells undergoing DNA repair, apoptosis, and necrosis (Saraste and Pulkki, 2000; Yaoita et al., 2000). Given that ISNEL can label both apoptotic and necrotic cells, a more specific marker of apoptosis was required for *in situ* examination. Recently, it has been shown that fas ligation of cultured RA synoviocytes induced the activation of caspase 8, caspase 3, with the subsequent cleavage of human polyADPribose polymerase (PARP) a substrate of activated caspase-3 (Kobayashi et al., 1999a; Kobayashi et al., 2000a). Caspase-3 and cleaved PARP have repeatedly been shown to be key players associated with apoptosis in cultured synoviocytes but they have not been measured *in situ* (Kobayashi et al., 2000a; Okamoto et al., 2000). Therefore, to improve the specificity of apoptosis measurements in the synovial lining we used antibodies against cleaved PARP p85 and activated caspase 3. Presumably, a decreased rate of cell death in the intimal lining could lead to hyperplasia over a long period of time even with nominal levels of proliferation.

2.2.8 Hypothesis

Synovial hyperplasia is a common feature during the course of RA. Understanding both the rates of proliferation and of apoptosis in synovial tissue, in

controls and in RA patients, is necessary in order to understand the mechanism(s) leading to synovial thickening/ hyperplasia. We hypothesize that an altered rate of proliferation and/or apoptosis would lead to synovial expansion.

2.2.9 Specific objectives

a) To determine if TS can be used as a marker of proliferation as compared to other known markers of proliferation by staining germinal centers of adult tonsil tissue.

b) To determine the proliferation rate of the synovial lining in RA patients compared to the synovial lining of OA patients and normal controls by using mitotic figure counting, and by immunohistochemical staining with antibodies directed against PCNA, Ki-67 and TS.

c) To determine the *in situ* rate of apoptosis of the synovial lining in RA patients by using PARP p85 and activated caspase 3, compared to synovium of OA patients and normal controls.

2.2.10 Significance

This aspect of my research is of significance as it represents a first attempt to resolve and clarify the existing controversy concerning the *in situ* rates of proliferation and apoptosis in both the normal and arthritic synovium. It will use the promising new marker of cell proliferation, TS and apoptosis markers PARP p85 and activated caspase 3 for the first time in RA synovium to study these mechanisms. Determining which process

is involved in synovial hyperplasia in RA would lead to better understanding of the disease with the potential development of new drugs that could be useful in therapy.

2.3 Materials and Methods

2.3.1 Description of patients and control tissue samples

Synovial tissue was obtained from a patient group that consisted of 11 RA, 13 OA and 4 control patients. The patients were followed at the Ottawa Hospital, General Campus and underwent joint replacement surgery. RA patients met the criteria of the American College of Rheumatology (ACR, formerly the American Rheumatism Association) (Arnett et al., 1988). The site of surgery, disease, age, duration, gender, degree of synovial hyperplasia, and medication was obtained from the patient's history and medical chart (RG). Hyperplasia of the synovial lining was assessed on a semi-quantitative, scale developed with the help of Dr. Susan Robertson, a pathologist at the Ottawa Hospital, General Campus as follows: 1+ = 1-2 cells thick, 2+ = 3-5 cells thick, and 3+ = 5-9 cells thick. The control tissue was obtained from cadaver subjects with no known history of arthritis. The protocol for obtaining synovial tissue samples from patients and controls was approved by the institutional review board.

2.3.2 Synovial tissue processing

Synovial tissue (ST) was obtained during joint replacement surgery from patients with RA (n = 11) and OA (n = 13). Synovial samples from RA and OA patients were obtained from metacarpal-phalangial, wrist, hip, knee, toe or shoulder joints. Synovial tissue from the left shoulder was utilized from cadavers. Briefly, each synovial specimen was cut with a sterile blade into 3 sections. Two sections were placed into cryovials,

snap frozen in liquid nitrogen and placed into a –100°C freezer. The remaining synovial section was placed into 10% neutral buffered formalin and fixed overnight. The following day the fixed tissue was placed into 70% ethanol and routinely paraffin embedded in the Department of Pathology at the University of Ottawa.

2.3.3 Hematoxylin and eosin staining for the grading of synovial hyperplasia and mitotic figure counting

Paraffin embedded synovial tissues were cut into 4 μm serial sections and laid onto superfrost plus slides (BWR Scientific). The sections were left to dry overnight at room temperature. The following day, the sections were deparaffinized in toluene, dehydrated in 100 % ethanol and placed into running H_20 for 5 min. The slides were counterstained in Mayer's hematoxylin for 1 min and washed in running water for 2 min. Excess hematoxylin was removed after rinsing 10 times in acid alcohol (0.2% of 12 N HCl in 70% EtOH) followed by rinsing for 2 min in running water and by a 1 dip in saturated lithium carbonate ($LiCO_3$). After a final 2 min wash in running water, the slides were counterstained with eosin (10 g eosin Y, 5 g potassium dichromate, 10% picric acid, 10 % ethanol and 70% H_2O) for 30 sec and dehydrated 6 times in 100% ethanol, cleared 4 times in toluene and mounted with permount (Fisher Scientific).

2.3.4 Immunohistochemistry for PCNA, Ki-67, TS 7.4, PARP p85, activated caspase 3, Factor 8 and CD68.

Deparaffinize sections. After routine paraffin embedding, the tissues were cut into 4 μm serial sections and laid onto superfrost plus slides (BWR Scientific). The

sections were left to dry overnight at room temperature. The following day, the sections were deparaffinized in toluene, rehydrated in 100 % ethanol and placed into running H_20 for 5 min.

Microwave method for antigen retrieval. The slides were placed into antigen retrieval solution (10 mM sodium citrate, pH 6.0) that was warmed to 37°C. This was followed by two incubations of 7 and 5 min each in a 700 W household microwave oven with a 5 min resting time following the first incubation. The sections were kept immersed in antigen retrieval solution, throughout the microwave processing. After microwave irradiation the slides were allowed to cool for 40 min, then immersed for 5 min in running H_20.

Blocking endogenous peroxidase activity. To prevent non-specific background, endogenous peroxidase activity was quenched in 3% H_2O_2 for 15 min followed by washing in 1X TBS (50 mM Tris, 150 mM NaCl, pH 7.6).

Blocking endogenous immunoglobulins. General suppressor (1% normal horse serum or 1% normal goat serum, 4% bovine serum albumin in 1X TBS) was added for 20 min to block endogenous immunoglobulins in order to prevent non-specific antibody binding.

Application of the primary antibody. After 20 min the excess suppressor was gently shaken off and replaced with antibodies to PCNA (DAKO), Ki-67 (Immunotech, Beckman Coulter), TS 7.4 (Haqqani et al., 1999), PARP p85 (Promega), Activated Caspase-3 (Cell Signaling Technology), Factor 8 (DAKO) and CD68 (DAKO) at dilutions of 1:1000, 1:10, 1:75, 1:50, 1:50, 1:100 and 1:100 respectively. After 1hr incubation, the slides were washed extensively in 1X TBS.

Application of secondary antibody and avidin-biotin complex. A 1:100 dilution of biotinylated goat anti-rabbit antibody (Vector Laboratories) was applied to sections incubated with the TS 7.4, PARP p85 and Activated Caspase-3 antibody for 40 min. A 1:100 dilution of biotinylated horse anti-mouse antibody (Vector Laboratories) was applied to sections incubated with Ki-67 and PCNA for 40 min. After 3 washes in 1X TBS, the avidin-biotin peroxidase complex (Vector Laboratories) was applied to all sections at a 1:200 dilution for 30 min. Factor 8 and CD68 were detected using undiluted peroxidase labeled polymer conjugated to anti-mouse and anti-rabbit immunoglobulins (Dako Envision System) for 30 min at room temperature followed by rinsing in 1X TBS.

Development with DAB chromogen. After 3 washes in TBS, the antigen-antibody complex was visualized using a freshly prepared solution of diaminobenzidine (0.02%), H_2O_2 (0.06%) in TBS for 10 minutes in the dark.

Counterstaining, clearing and mounting. The slides were then washed in running H_2O and counterstained in Mayer's hematoxylin (Fischer Scientific) for 1 min. To remove excess hematoxylin the sections were rinsed 10 times into acid alcohol (0.2% HCl in 70% EtOH) followed by a rinse in saturated lithium carbonate ($LiCO_3$). The slides were then dehydrated twice in 100% ethanol, cleared 4 times in toluene and mounted with permount (Fisher Scientific). All RA, OA and control (cadaver) samples were stained on the same day. Immunohistochemistry was repeated twice for some patients to ensure consistency in staining. Negative controls were performed on each occasion by omitting the primary antibody. Formalin fixed paraffin embedded adult tonsil tissue was used as a positive control for the demonstration of proliferating and apoptotic cells in lymphoid tissue.

2.3.5 Western blot analysis

Western blot analysis was performed on a small group of RA and OA synovial tissue samples. Snap frozen synovial tissue stored at –100°C was thawed, weighed and homogenized on ice in 2 volumes of PBS (2.7 mM KCl, 1.5 mM KH_2PO_4, 140 mM NaCl, and 8 mM NA_2HPO_4, pH 7.4) containing PMSF (1 mM). Homogenates were diluted with SDS sample buffer (2% SDS, 10% glycerol, 1% 2-mercaptoethanol, 0.0005% bromophenol blue, 125 mM MOPs, pH 6.8) and heated at 100°C for 5 min. Protein was then quantified by using a fluorescamine assay (Udenfriend et al., 1972), an amine reactive fluorophore, as described elsewhere (De Vouge et al., 1994). Protein samples (30 μg/lane) were resolved on a 12% SDS-PAGE for 1hr at 200V and transferred to a 0.45 μm PVDF membrane at 15V for 18 h in Dunn buffer (3 mM Na_2CO_3, 10 mM $NaHCO_3$ and 10 % methanol). After transferring the membranes were washed in 100% methanol for 5 minutes, followed by 1 wash in TBST (10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20, pH-8). The membranes were stained with Ponceau S to reveal the markers, washed twice in TBST followed by blocking in 5% skim milk powder for 1hr at room temperature. The membranes were incubated with antibodies to PCNA, Ki-67 and TS 7.4 at dilutions of 1:1000, 1:250 and 1:500 respectively in 5% milk for 1hr at room temperature. The membranes were washed 4 times in TBST and then incubated with a 1:100 dilution of peroxidase labeled polymer conjugated to anti-mouse and anti-rabbit immunoglobulins (Dako Envision System) in 5% skim milk powder for 1 h at room temperature. The membranes were washed 4 times in TBST and the antigen-antibody complex was visualized using LumiGLO Chemoluminescent Substrate Kit (Kirkegaard & Perry Laboratories). HeLa cell extracts (10 μg) were used as a positive control.

2.3.6 Determination of the proliferation and apoptosis labeling index

Three groups of 300 synovial lining cells were counted per section under a high power field (total magnification 600X). Therefore, a total of 900 synovial cells were counted for each tissue section with the exception of patients 26, 51a, 3, 4, 229 and 141 where only 600 hundred synovial lining cells were counted due to sample size. The total number of mitotic figures, PCNA, Ki-67, TS 7.4, PARP p85 and activated caspase-3 positively staining synovial cells was counted in each group of 600 or 900 cells to estimate the proportion of proliferating and dying synoviocytes. The final results are expressed as the percentage of synovial lining cells staining positive for an antigen, with the maximum score being 100%. Proliferation or apoptosis labeling index = [total positive cells/900]*100

2.3.7 Statistical analysis

The proliferation and apoptosis labeling indices were expressed as a percent value. Mean ± standard deviation of the mean was calculated for each group. To compare 3 or more unpaired groups one-way analysis of variance was used. A value of $p < 0.05$ was considered to be statistically significant.

2.4 Results

2.4.1 Comparison of patient sample groups

Synovial tissue was obtained from a patient group that consisted of 11 RA, and 13 OA patients undergoing joint replacement surgery at the General Campus of the Ottawa

Hospital. Control synovial tissue was obtained from 4 cadaver subjects at the time of autopsy. The site of surgery, disease, average age, average duration, gender, and medication currently used are summarized in Table 2.1. The average age of the RA patients (60.4 ± 12.0 years) did not vary significantly from the average age of OA (68.9 ± 8.4 years) or control patients (68.0 ± 10.6 years). The RA patient group consisted mostly of females with an average disease duration of 14.5 ± 8.0 years while the OA group consisted mainly of male patients with an average disease duration of 6.9 ± 6.8 years. As indicated earlier control patients had no medical history of arthritis. Medication usage was more frequent in RA patients than in OA and controls.

2.4.2 Characteristics of RA, OA and control synovium

Of the 11 RA synovium samples examined for mitotic activity, and PCNA, Ki-67 and TS 7.4 immunoreactivity, 7 exhibited moderate to strong increase in hyperplasia of the synovial lining, characterized by an increase of 3-8 layers (Figure 2.1a). The RA synovial sections showed heterogeneous histological changes in terms of inflammatory infiltration (Figure 2.1b and 2.1c), increased vascularity (Figure 2.1b) and fibrosis in the sublining regions. Four samples showed little to no hyperplasia, with 1-2 layers of lining synoviocytes (Table 2.2). Most OA synovia (8 out of 11) exhibited some increase in the thickness of the synovial lining with 3 to 5 layers (Figure 2.2a). The remaining OA patients' synovium showed no signs of hyperplasia. Typically, OA synovia showed a spectrum similar to RA, but there was somewhat lesser degree of inflammation and hyperplasia(Figure2.2a)

Table 2-1: Characteristics of the rheumatoid arthritis (RA) patients, osteoarthritis (OA) patients and normal controls.

Age and duration are given as the mean in years ± standard deviation. Gender, joint distribution and medication are reported as well. Some RA patients were on more than one type of drug.

	RA (n=11)	OA (n=13)	Control (n=4)
Age (years) ± SD	60.4 ± 12.0	68.9 ± 8.4	68.0 ± 10.6
Disease Duration (years) ± SD	14.5 ± 8.0	6.9 ± 6.8	NA
Gender	F = 8 M = 3	F = 5 M = 8	F = 1 M = 3
Site of Surgery	8 Knee 1 Toe 1 Hip 1 Nodule	12 knee 1 toe	4 Shoulder
Current Medication (Number of Patients)			
imuran	1	1	0
methotrexate	5	0	0
folate	4	2	0
plaquenil	5	0	0
steroid	5	2	0

Figure 2.1: Pathological characteristics of the RA synovium from different patients.

(A) Hematoxylin and eosin (H & E) stain of the thickened synovial lining (SL) as indicated by the black bracket. (B) Lymphocyte aggregation and Factor 8 (an endothelial cell marker) stain showing angiogenesis. (C) CD68 staining of infiltrating macrophage. A, x200 total magnification; B, x100 total magnification; C, x600 total magnification. (LA) = lymphocyte aggregate, (E) = endothelium, (M) = macrophage

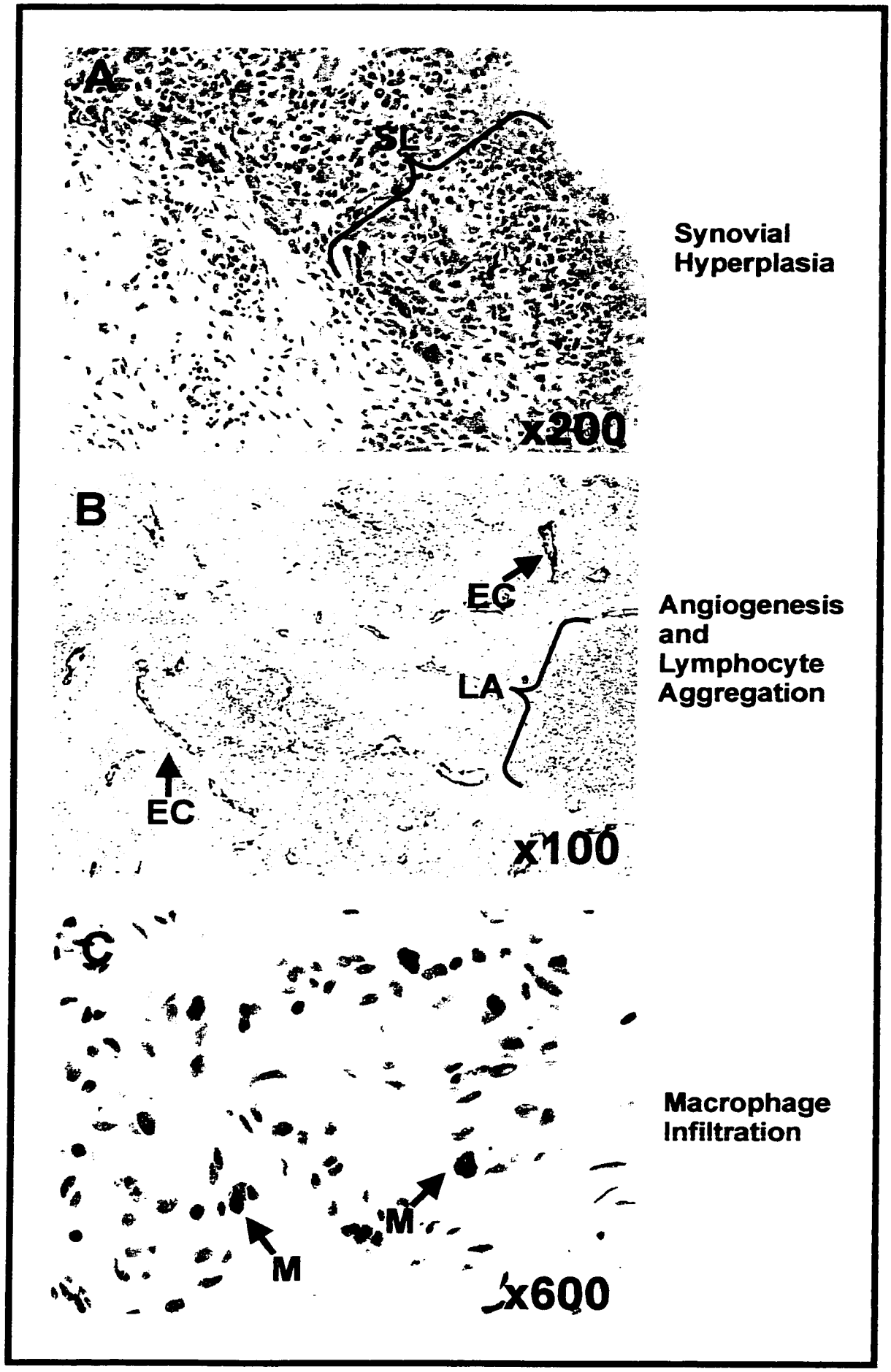
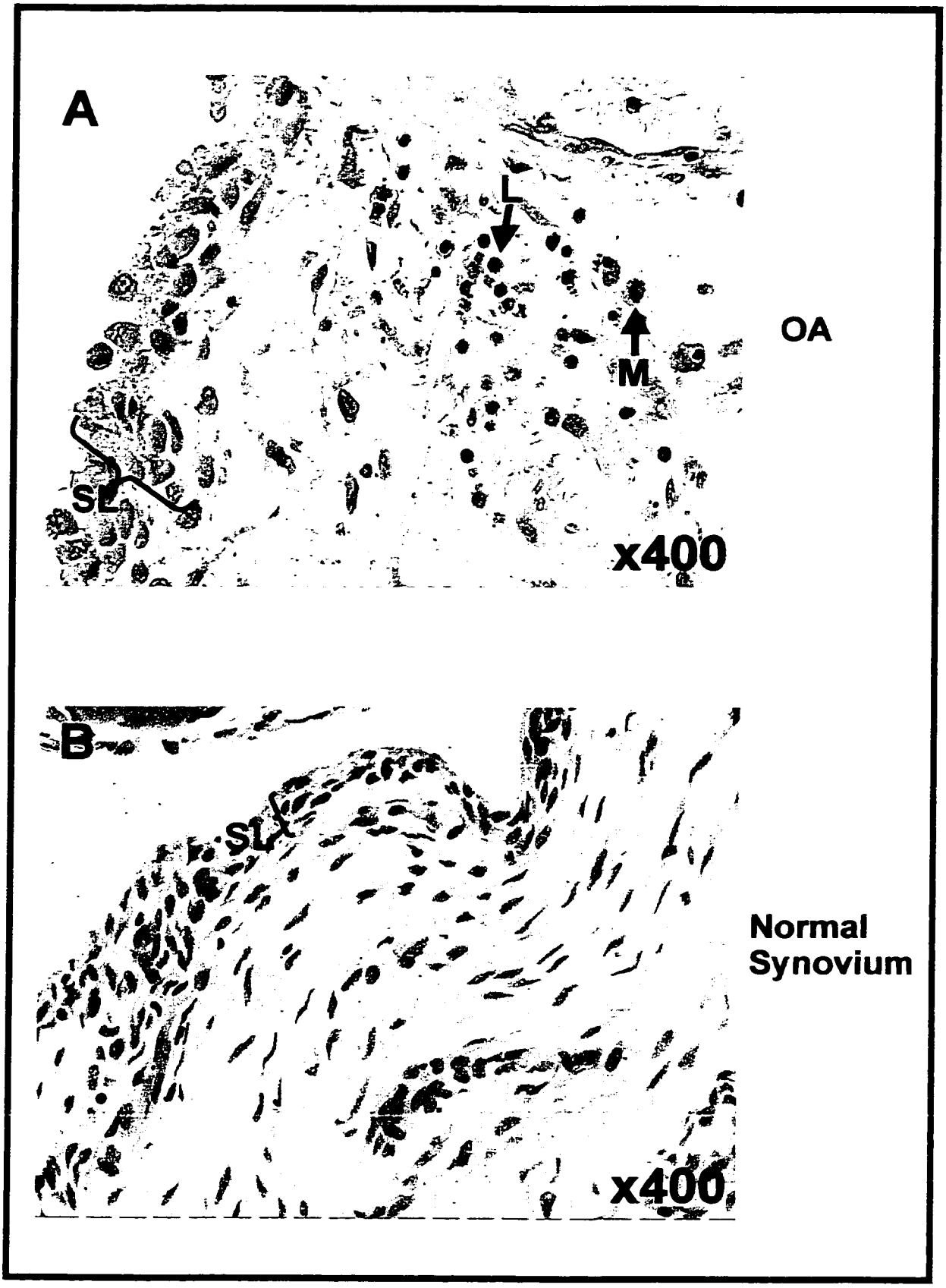

A
SL
x200
Synovial Hyperplasia
B
EC
LA
EC
x100
Angiogenesis and Lymphocyte Aggregation
C
M
M
x600
Macrophage Infiltration

Figure 2.2: Pathological characteristics of OA and normal synovium.

H & E stained osteoarthritic and control synovial sections. (A) The OA sample has a much lower degree of inflammation with a very mild proliferative change in the synovial lining (SL) compared to RA. (B) The normal synovium is free of immune cell infiltration and of any proliferative changes in the SL. A and B, x400 total magnification. (L) = lymphocytes, (M) = macrophage.


A
L
M
SL
x400
OA
B
SL
x400
Normal
Synovium

Thickening of the intimal layer and inflammatory cells were absent in control samples (Figure 2.2b). In all samples, the synovial layer contained no basement membrane (a characteristic feature of synovial lining) and was separated from the sublining by either loose connective tissue or by infiltrated lymphocytes.

2.4.3 Immunohistochemical staining of adult tonsil tissue for proliferation and apoptosis markers

Tonsils are peripheral lymphoid organs that are composed of lymphoid follicles consisting of various cells and lymphatic capillaries. A primary follicle is composed of small resting B cells, macrophage and dendritic cells compose a primary follicle. Upon antigen activation, B cells are induced to proliferate and differentiate into plasma cells and memory cells. A secondary follicle is formed consisting of proliferating B lymphocytes (germinal center), plasma cells, and memory B cells interspersed with antigen presenting cells (Kuby, 1997). In the midst of the proliferating germinal center some B cells die by programmed cell death. This death may occur to remove inactivated and self-reactive B cells (Kuby, 1997).

Since lymphoid follicles are known to consist of proliferating B lymphocytes, formalin-fixed paraffin embedded adult tonsil tissue were utilized as a positive control for proliferation by observing for the presence of mitotic figures and the proliferation markers PCNA, Ki-67 and TS 7.4. Tonsil tissue staining was carried out on serial sections on the day of synovial tissue staining for PCNA, Ki-67 and TS 7.4. Mitotic figures were regularly observed in the germinal centers of tonsil tissue (Figure 2.3a). Strong immunoreactivity was found in the proliferating germinal centers for PCNA, Ki-

67 and TS 7.4 (Figure 2.3 b-d). Staining for Ki-67 and TS 7.4 was primarily restricted to the germinal centers with a few labeled cells scattered throughout the paracortex (Figure 2.4 b and c). By contrast, PCNA labeled more cells in the germinal center and a much higher number of cells in the paracortex of the lymphoid tissue (Figure 2.4a). As expected, omission of the primary antibodies gave no staining in the tonsil specimens showing that non of the immunostaining was due to our secondary antibody. Our results confirm that TS 7.4 can be used as a proliferation marker.

Apoptosis is known to occur in the germinal center of tonsil tissue. Therefore, germinal centers of tonsil tissue could serve as a positive control for apoptosis. Both activated caspase 3 and PARP p85, which are specific markers of apoptosis, showed prominent nuclear staining in the germinal centers of tonsil tissue (Figure 2.5a-d). Furthermore, neutrophils in tonsil tissue stained for activated caspase 3 but not PARP (data not shown), which correlates with recent evidence that PARP is absent in neutrophils all together (Sanghavi et al., 1998). The latter demonstrates the specificity of the new antibodies. Some cells showed staining for both markers in the paracortex as well. This is the first evidence that PARP p85 can be used in the immunohistochemical detection of apoptosis in formalin fixed paraffin embedded sections. These results are consistent with the fact that apoptosis occurs in germinal centers (Tabe et al., 1996; Krajewski et al., 1997). Again, the control with secondary alone gave no staining in our tonsil tissue.

Figure 2.3: High power view of a germinal center from adult tonsil tissue stained with proliferation markers.

Briefly, consecutive tonsil sections were antigen retrieved and incubated with PCNA, Ki-67 or TS 7.4 for 1 h at 25°C. Biotinylated secondary antibodies were used to detect our primary antibodies followed by the addition of the peroxidase labeled avidin-biotin complex for 40 min. DAB chromogen was used to visualize the antigen-antibody complex in the tonsil tissue. (A) Mitotic figures are shown on a control tonsil section (primary antibody omitted) that was counterstained with hematoxylin. Consecutive sections of a germinal center stained for (B) PCNA, (C) Ki-67 and (D) thymidylate synthase (TS). Positive immunoreactivity is located in the nuclei of synovia immunolabeled with PCNA and Ki-67. Cytoplasmic immunoreactivity is observed for TS staining. A, x600 total magnification; B-D, x400 total magnification.

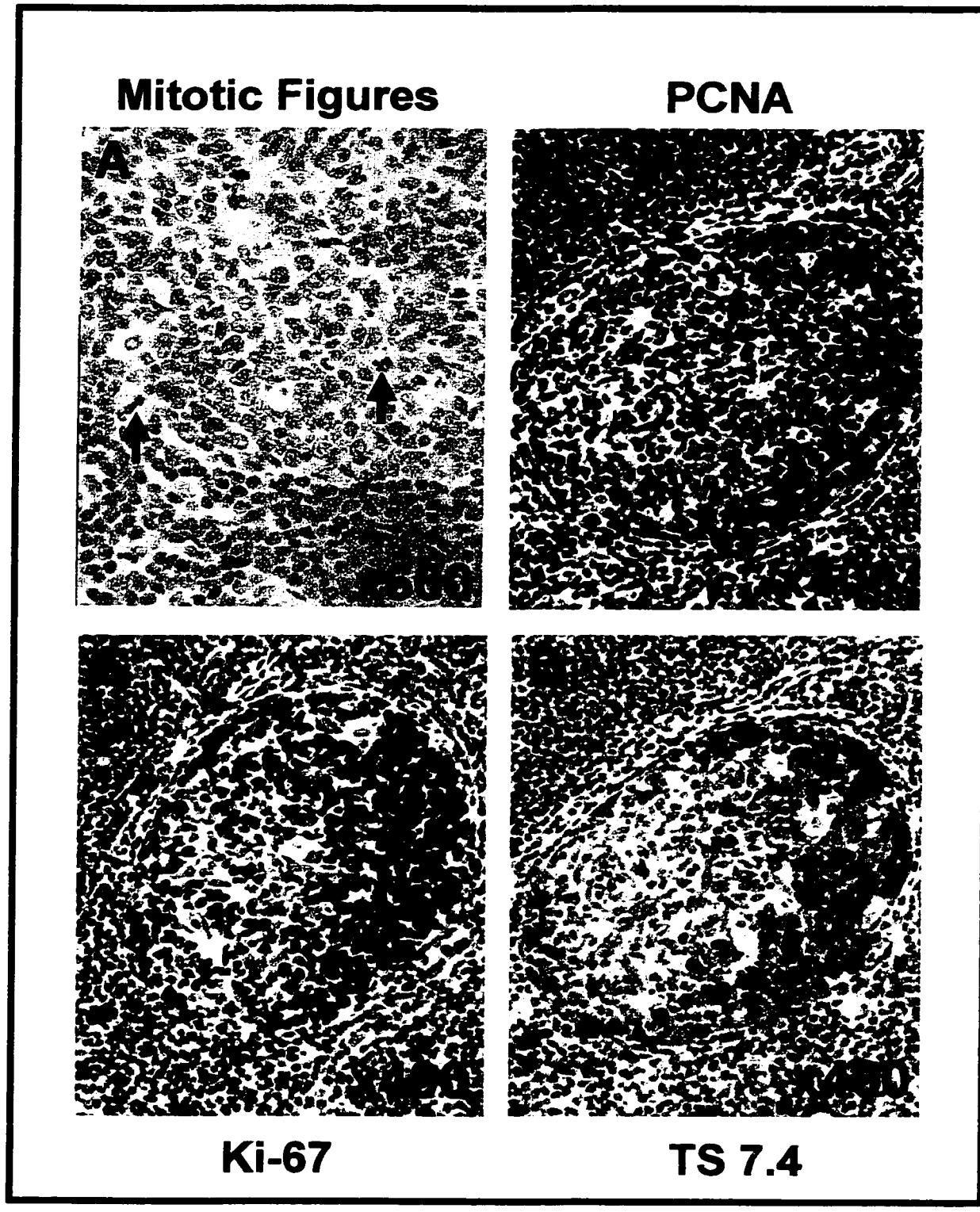
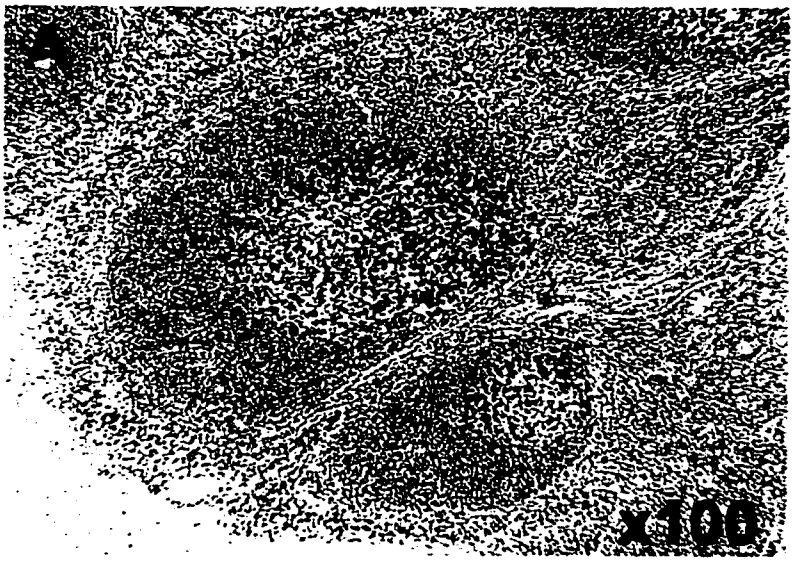
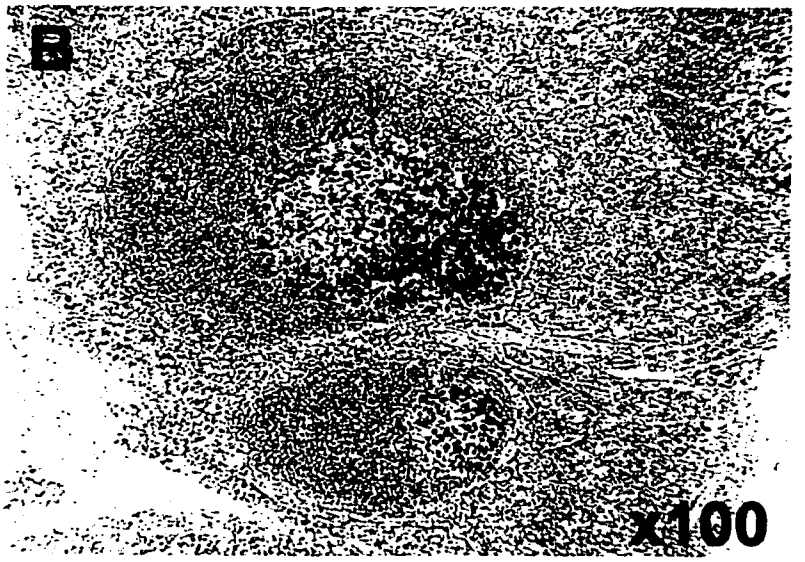

Mitotic Figures
PCNA
A
Ki-67
TS 7.4

Figure 2.4: Low power view of serial sections in adult tonsil tissue immunolabeled by PCNA, Ki-67 and TS 7.4.

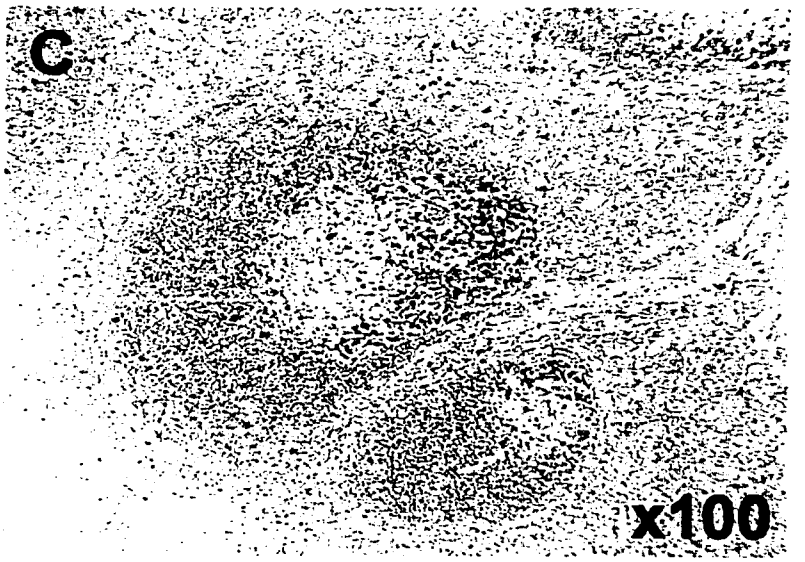
Briefly, consecutive tonsil sections were antigen retrieved and incubated with PCNA, Ki-67 or TS 7.4 for 1 h at 25°C. Biotinylated secondary antibodies were used to detect our primary antibodies followed by the addition of the peroxidase labeled avidin-biotin complex. DAB Chromogen was used to visualize the antigen-antibody complex in the tonsil tissue. (A) Extensive PCNA staining inside and outside the germinal center. (B) Ki-67 and (C) TS staining is restricted to the germinal centers, with only few cells staining in the paracortex. A-C, x100 total magnification.



PCNA



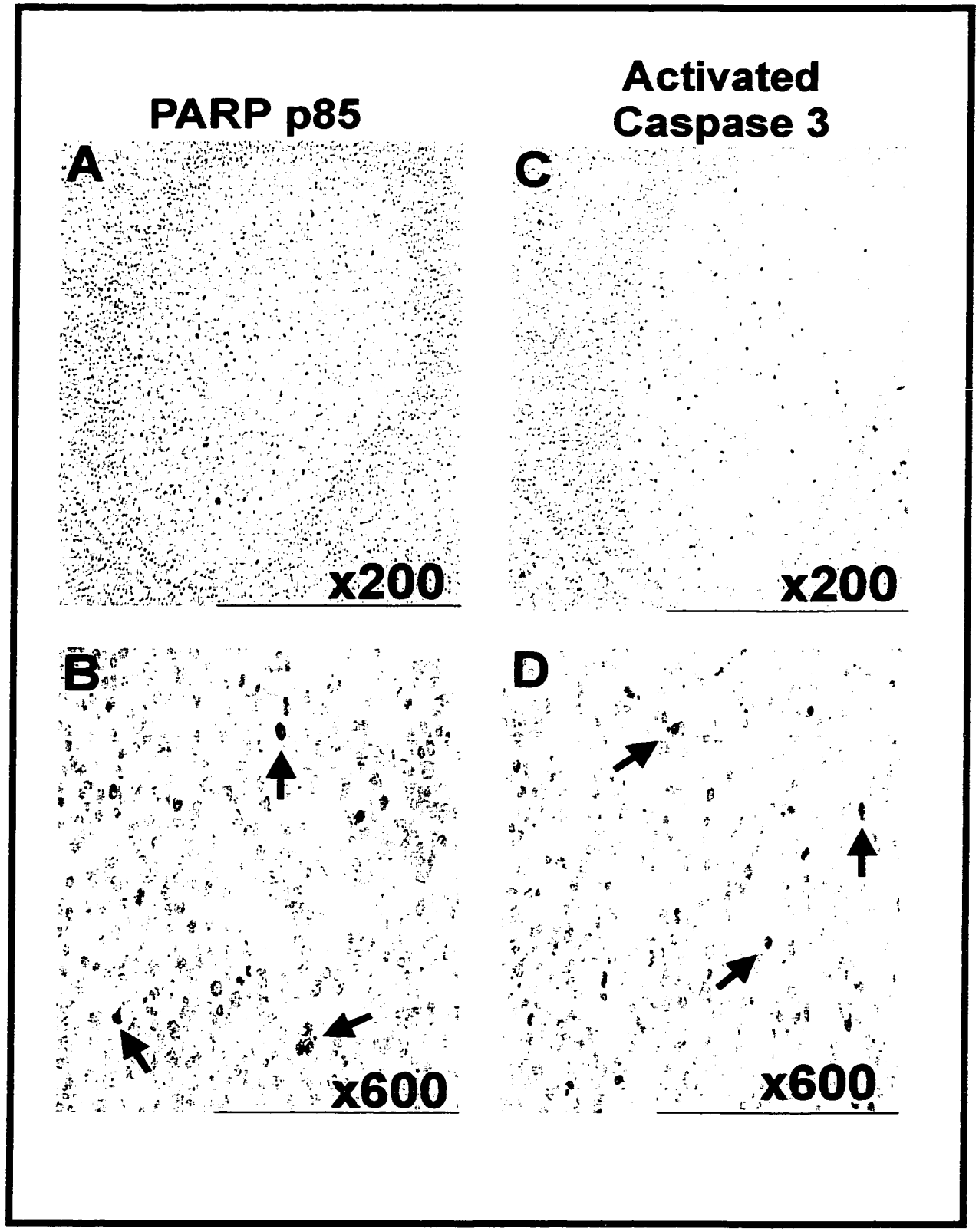
Ki-67



TS 7.4

Figure 2.5: Detection of apoptosis by PARP p85 and activated caspase-3 in the germinal center of tonsil tissue.

Briefly, consecutive tonsil sections were antigen retrieved and incubated with PARP p85 and activated caspase 3 for 1 h at 25°C. Biotinylated secondary antibodies were used to detect our primary antibodies followed by the addition of the peroxidase labeled avidin-biotin complex for 40 min. DAB Chromogen was used to visualize the antigen-antibody complex in the tonsil tissue. (A, B) Strong PARP p85 and (C, D) strong activated caspase 3 staining is detected in serial sections of germinal centers in tonsil tissue. Positively stained cells are indicated by black arrows. A and C, x200 total magnification; B and D, x600 total magnification.


PARP p85
Activated Caspase 3
A
x200
C
x200
B
x600
D
x600

2.4.4 PCNA, Ki-67 and TS 7.4 staining of lymphoid aggregates in RA patient 52b

Germinal centers were also evident throughout the synovial tissue of RA patient 52b and so this patient served as another positive internal control. PCNA, Ki-67 and TS 7.4 stained the same lymphoid follicles in serial sections of this tissue (Figure 2.6a-c). This data again demonstrate that TS 7.4 is staining proliferating cells in synovial tissue, further strengthening its use as a proliferation marker.

2.4.5 Mitotic figures not observed in the synovial tissue of RA, OA and control patients

Mitotic figure counting is a well-established and simple method for measuring proliferation of cells in the M phase of the cell cycle. Mitotic figures were not observed in hematoxylin & eosin (H & E) stained synovial tissues of RA, OA or control patients with the single exception of RA patient 26 where only one mitotic figure was observed.

2.4.6 Immunohistochemical staining of synovial tissue for PCNA in RA, OA and control patients

Rheumatoid synovia with marked hyperplasia exhibited extensive and strong PCNA immunoreactivity in the lining synoviocytes (Figure 2.7a, Table 2.2). The labeling index (LI) of positively staining cells ranged from 65 to 92% and varied little from region to region and from patient to patient (Table 2.3). Staining was observed in the nuclei of synoviocytes throughout the lining layers. Moreover, PCNA-positive synoviocytes either showed round nuclei or typical fibroblast like-morphology with

Figure 2.6: Detection of proliferating germinal centers in the RA synovium.

Briefly, consecutive sections of RA tissue were antigen retrieved and incubated with PCNA, Ki-67 or TS 7.4 for 1 h at 25°C. Biotinylated secondary antibodies were used to detect our primary antibodies. Biotinylated secondary antibodies were detected with peroxidase labeled avidin-biotin complex. DAB Chromogen was used to visualize the antigen-antibody complex in the RA tissue. Serial sections of germinal centers in RA patient 52b staining for (A) PNCA, (B) Ki-67 and (C) TS 7.4. A-C, x200 total magnification.

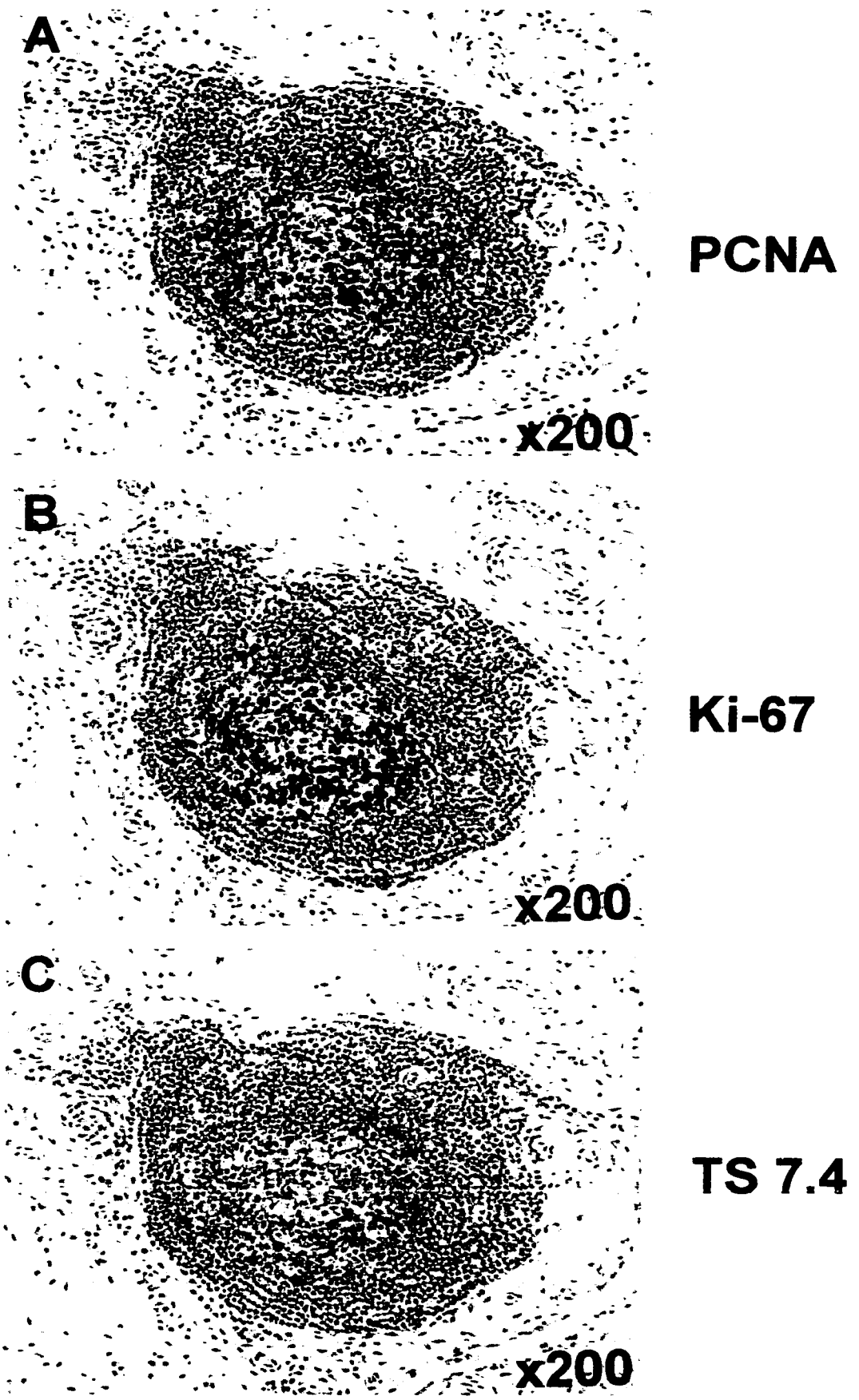

A
PCNA
x200
B
Ki-67
x200
C
TS 7.4
x200

Figure 2.7: PCNA, Ki-67 and TS 7.4 staining in the synovial tissue of RA patient 55b.

Briefly, consecutive sections of RA tissue were antigen retrieved and incubated with PCNA, Ki-67 or TS 7.4 for 1 h at 25°C. Biotinylated secondary antibodies were used to detect our primary antibodies. Biotinylated secondary antibodies were detected with peroxidase labeled avidin-biotin complex. DAB Chromogen was used to visualize the antigen-antibody complex in the RA tissue. (A) Strong and abundant PCNA staining in the lining synoviocytes of RA patient 55b. Consecutively stained sections with (B) Ki-67 and (C) TS label far fewer lining synoviocytes. Positively stained cells are indicated with black arrows. A-C, x600 total magnification. SL = synovial lining.

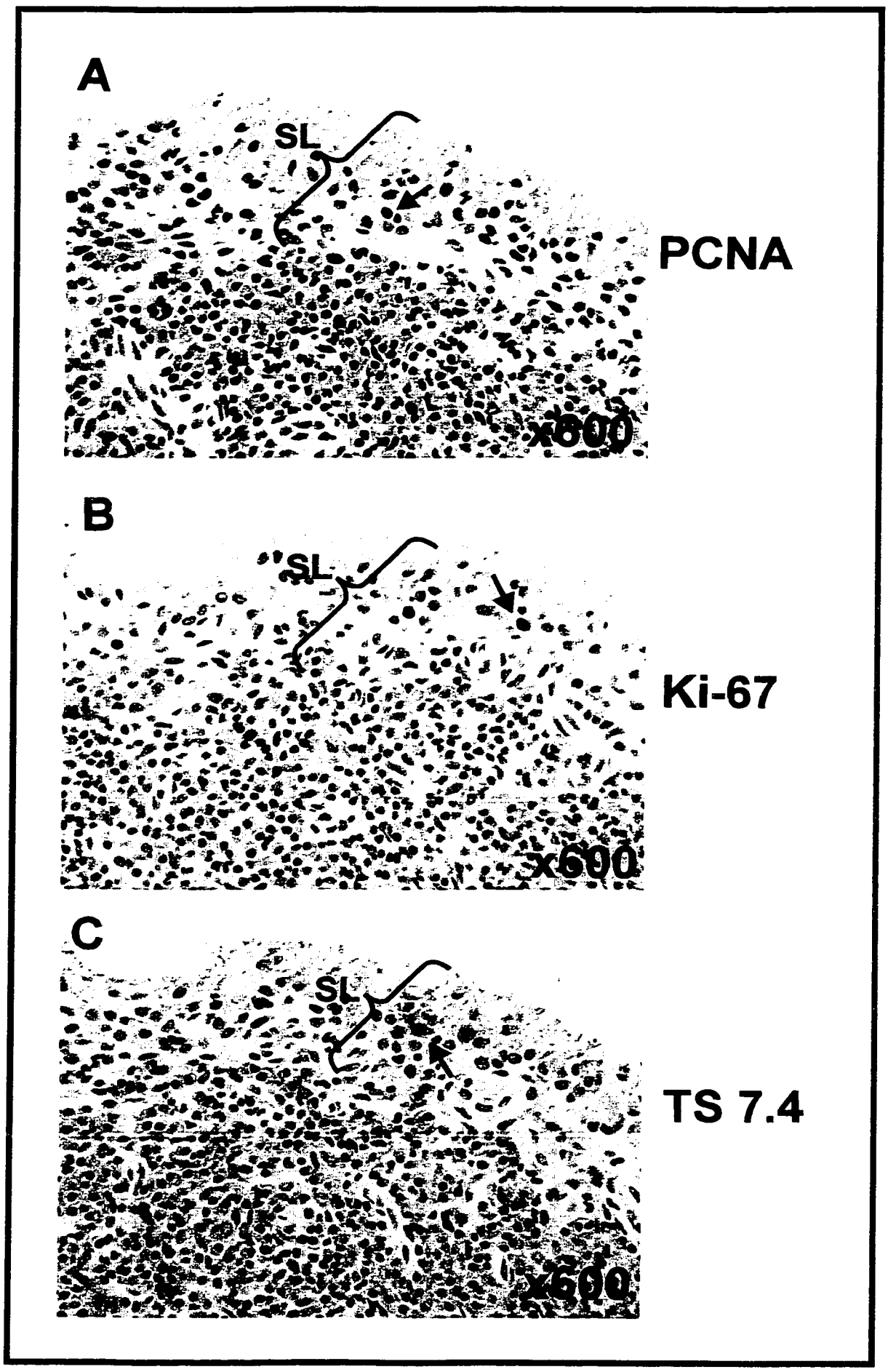

A
SL
PCNA
x600
B
SL
Ki-67
x600
C
SL
TS 7.4
x600

Table 2-2: Degree of Hyperplasia, Proliferation Labeling Index and Apoptosis Labeling Index for RA, OA and Control Patients.

The RA, OA and control patients are aligned with respect to their degree of hyperplasia, proliferation labeling index (LI), and apoptosis LI. The degree of hyperplasia was assessed for RA, OA and control patients according to the number of cell layers of synoviocytes: 1-2 layers = 1+, 3-5 layers = 2+ and 5-9 layers = 3+. The proliferation LI was assessed by mitotic figure counting and with the use of proliferation markers, PCNA, Ki-67 and TS 7.4. The apoptosis LI was assessed using activated caspase 3 (C-3) and the cleaved polyADPribose polymerase (p85). Both proliferation and apoptosis LI are expressed as a percentage.

	Patient ID #	Degree of Hyperplasia	Proliferation Index (%)				Apoptosis Index (%)	
			PCNA	Ki-67	TS 7.4	Mitosis	P85	C-3
RA	1	2+	77.33	5.33	1.33	0.00	0.00	0.44
	11	2+	91.55	5.77	1.67	0.00	0.00	0.44
	26	3+	79.16	8.16	4.33	0.11	0.00	0.00
	37	1+	83.77	1.44	0.55	0.00	0.00	0.00
	43	2+	89.33	2.33	1.55	0.00	0.00	0.11
	50c	1+	84.22	2.88	0.55	0.00	0.00	0.11
	51a	2+	65.83	0.22	0.00	0.00	0.00	0.00
	52b	1+	76.55	0.88	0.11	0.00	0.00	0.00
	55b	2+	79.33	3.55	2.00	0.00	0.00	0.11
	65p2	1+	56.33	1.00	0.44	0.00	0.00	0.00
	68	1+	88.66	0.33	0.00	0.00	0.00	0.00
OA	3	2+	80.16	2.00	1.16	0.00	0.22	0.00
	4	2+	42.12	2.16	1.33	0.00	0.22	0.00
	5	1+	73.22	0.00	0.22	0.00	0.00	0.00
	10	2+	79.66	2.66	1.00	0.00	0.00	0.00
	47	2+	88.00	4.88	1.55	0.00	0.00	0.11
	53b	1+	49.44	0.00	0.00	0.00	0.00	0.00
	54b	1+	55.77	0.11	0.00	0.00	0.11	0.00
	66	1+	73.11	0.77	1.44	0.00	0.00	0.00
	71	1+	82.00	2.77	1.11	0.00	0.00	0.00
	72	2+	87.55	1.66	0.88	0.00	0.00	0.22
	74	2+	46.55	0.55	1.00	0.00	0.00	0.22
	91fb	2+	87.33	4.77	4.33	0.00	N/A	0.00
	100	2+	86.22	1.55	1.11	0.00	N/A	0.00
Control	229	1+	56.00	0.00	0.00	0.00	N/A	0.00
	215	1+	48.66	0.11	0.00	0.00	0.00	0.00
	141	1+	60.16	0.16	0.00	0.00	N/A	0.00
	269	1+	83.33	0.66	0.00	0.00	0.00	0.00

Table 2-3: Proliferation and apoptosis markers in RA and OA patients and controls.

The range, mean, standard deviation, and median were determined for each marker of proliferation and apoptosis from RA, OA and controls. They are expressed as a percentage.

		RA (n=11)	OA (n=13)	Control (n=4)
PCNA	Range	56 - 92	42 - 88	49 - 83
	Mean	**79**	**72**	**62**
	Std. Deviation	11	17	15
	Median	79	80	58
Ki-67	Range	0.2 - 8.2	0.0 - 4.9	0.0 - 0.7
	Mean	**2.9**	**1.8**	**0.2**
	Std. Deviation	2.6	1.6	0.3
	Median	2.3	1.7	0.1
TS 7.4	Range	0.0 - 4.3	0.0 - 4.3	0.0 - 0.0
	Mean	**1.1**	**1.2**	**0.0**
	Std. Deviation	1.3	1.1	0.0
	Median	0.6	1.1	0.0
M	Range	0.0 - 0.10	0.0 - 0.0	0.0 - 0.0
	Mean	**0.01**	**0.00**	**0.00**
	Std. Deviation	0.03	0.00	0.00
	Median	0.00	0.00	0.00
p85	Range	0.0 - 0.0	0.0 - 0.22	0.0 - 0.0
	Mean	**0.00**	**0.05**	**0.00**
	Std. Deviation	0.00	0.09	0.00
	Median	0.00	0.00	0.00
C3	Range	0.0 - 0.44	0.0 - 0.22	0.0 - 0.0
	Mean	**0.11**	**0.04**	**0.00**
	Std. Deviation	0.17	0.08	0.00
	Median	0.00	0.00	0.00

ellipsoid or ovoid shaped nuclei. Notably, the synovium of RA patients with little to no hyperplasia also showed extensive and strong PCNA positive cells with an LI range of 56 to 89%. The mean PCNA LI of all RA samples was 79.3 ± 10.5% (Table 2.3 and Figure 2.10). Along with the extensively labeled cells in the synovial lining, abundant PCNA labeling was found throughout the RA synovia including endothelial, sublining cells, multinucleated giant cells, lymphocytes and fibroblast-like cells scattered throughout the extracellular matrix.

Abundant and strong PCNA immunoreactivity was also detected in all of the 13 OA cases examined with a LI ranging from 42 to 88%. The mean PCNA LI for OA synovia was 71.6 ± 17.0% which was similar to the mean LI of RA patients (Table 2.3, Figure 2.10). Staining was similar from region to region and from patient to patient. Again a similar staining pattern to the one seen in RA synovia was noted (Figure 2.8a).

In control non-arthritic synovium, abundant intimal lining staining for PCNA was detected (Figure 2.9a) with an LI range of 48 to 83% and a mean LI of 62.0 ± 15.0% (Table 2.3 and figure 2.10). One patient sample exhibited weak to moderate PCNA immunoreactivity whereas 3 out of 4 patients showed moderate to strong staining of synovial lining cells. PCNA labeling was seen throughout the extracellular matrix in fibroblast-like cells and endothelial cells. The negative control of secondary antibody alone gave no staining in all the specimens studied. Our control samples lack any signs of synovial hyperplasia and are hypothesized to not be proliferating extensively. The fact that PCNA labels such a high percentage of intimal lining cells in the arthritic and control subjects suggests it may be involved in other processes than DNA replication.

Figure 2.8: PCNA, Ki-67 and TS 7.4 staining in the synovial tissue of OA patient 91fb.

Briefly, consecutive sections of OA tissue were antigen retrieved and incubated with PCNA, Ki-67 or TS 7.4 for 1 h at 25°C. Biotinylated secondary antibodies were used to detect our primary antibodies. Biotinylated secondary antibodies were detected with peroxidase labeled avidin-biotin complex. DAB Chromogen was used to visualize the antigen-antibody complex in the OA tissue. (A) Strong and abundant PCNA staining in the synovial lining cells of OA patient 91fb. In comparison to PCNA, (B) Ki-67 and (C) TS label significantly fewer lining synoviocytes. Positively staining cells are indicated with a black arrow. A-C, x400 total magnification. SL = synovial lining.

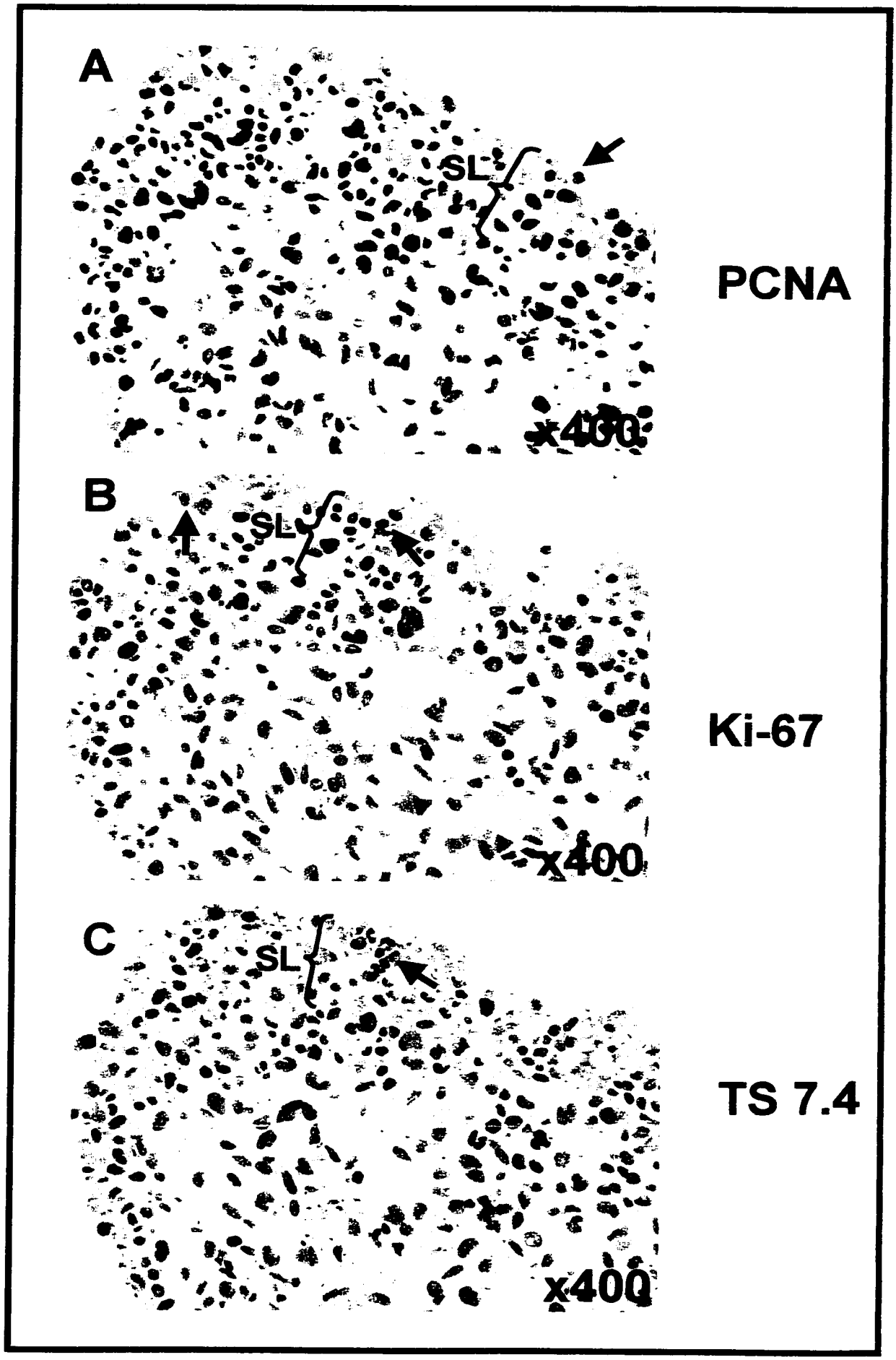

A
SL
x400
PCNA
B
SL
x400
Ki-67
C
SL
x400
TS 7.4

Figure 2.9: Representative PCNA, Ki-67 and TS 7.4 staining of the synovial lining in a control patient.

Briefly, consecutive sections of control tissue were antigen retrieved and incubated with PCNA, Ki-67 or TS 7.4 for 1 h at 25°C. Biotinylated secondary antibodies were used to detect our primary antibodies. Biotinylated secondary antibodies were detected with peroxidase labeled avidin-biotin complex. DAB Chromogen was used to visualize the antigen-antibody complex in the control tissue (A) Abundant but moderate to strong PCNA staining in the synovial lining (SL) of control patients. (B) Ki-67 rarely stained lining synoviocytes and (C) TS was predominantly absent in the SL. Black arrows indicate positively staining cells. A-C, x600 total magnification. SL = synovial lining.

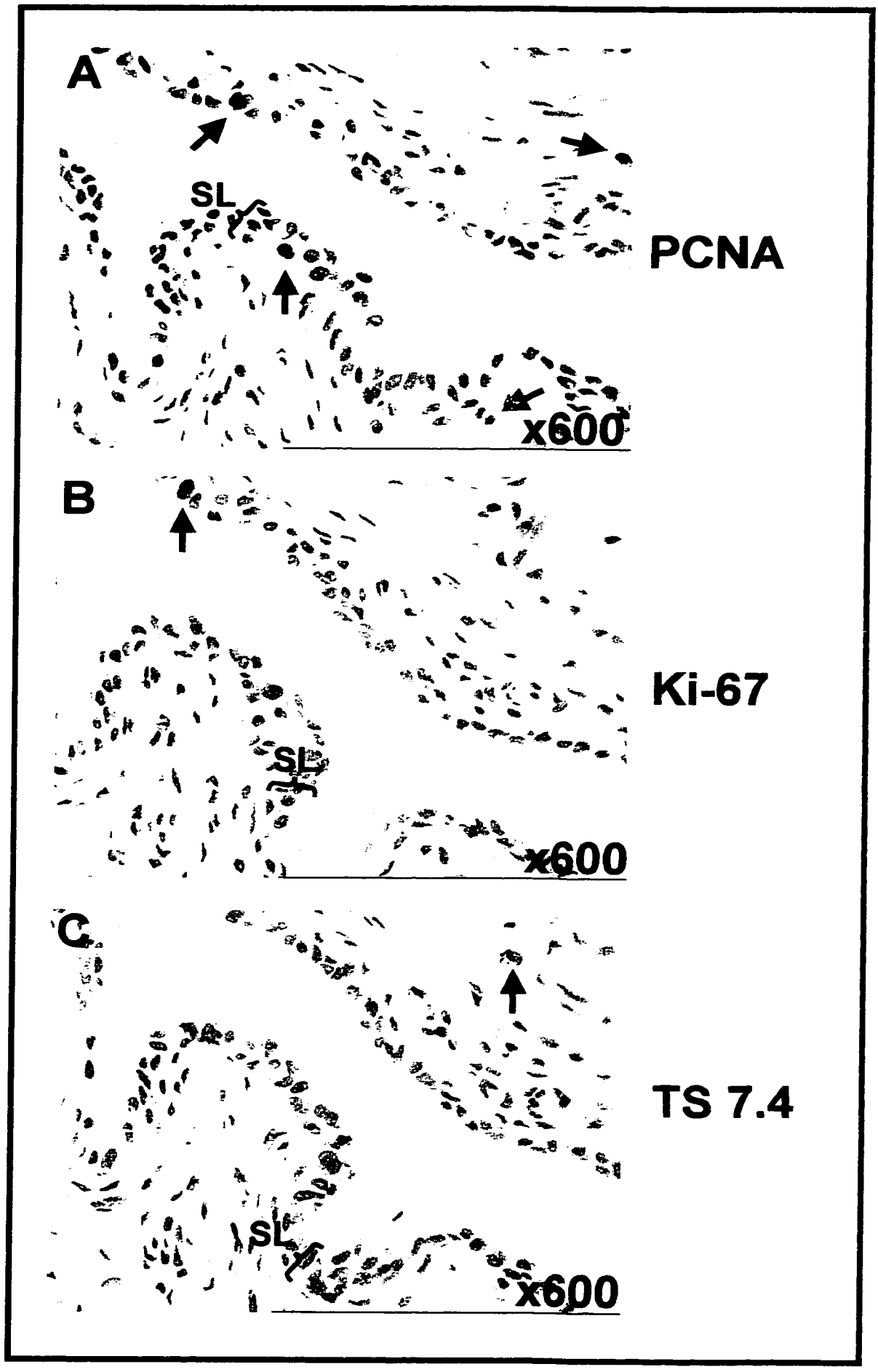

A
SL
PCNA
x600
B
SL
Ki-67
x600
C
SL
TS 7.4
x600

Figure 2.10: Summary graph of proliferation and apoptosis in the synovial lining of RA, OA and controls.

The proliferation and apoptosis index of RA, OA and control synovial tissue from Table 2.2 is summarized in a scattergram. Analysis of stained section was carried out as described in Materials and Methods. Each symbol represents tissues from a single patient: the bar represents the mean for each disease group. The statistical analysis for this figure is found in Table 2.4

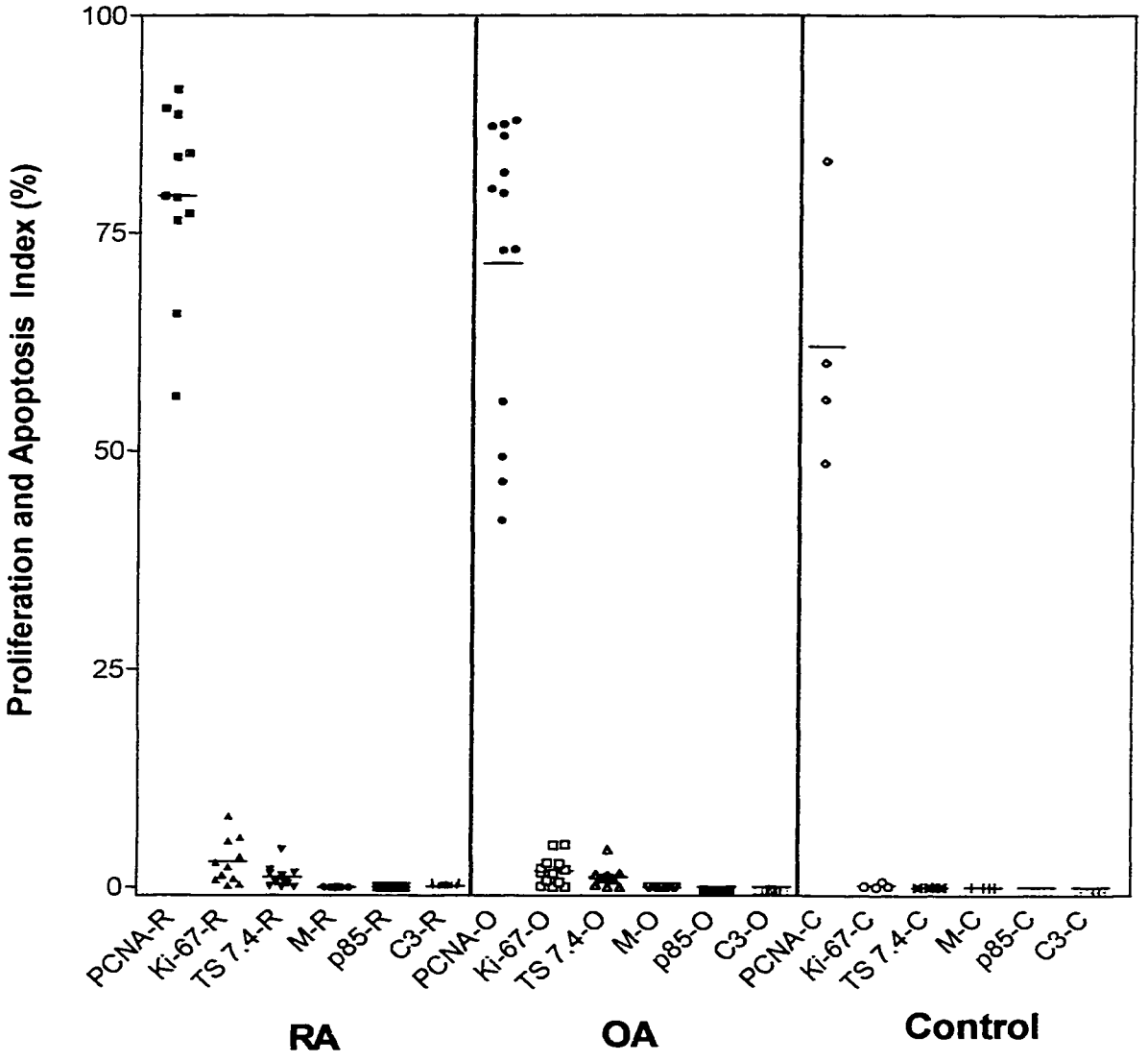

Proliferation and Apoptosis Index (%)
100
75
50
25
0
PCNA-R
Ki-67-R
TS 7.4-R
M-R
p85-R
C3-R
PCNA-O
Ki-67-O
TS 7.4-O
M-O
p85-O
C3-O
PCNA-C
Ki-67-C
TS 7.4-C
M-C
p85-C
C3-C
RA
OA
Control

Table 2-4: Statistical analysis of proliferation and apoptosis results in RA, OA and controls.

The proliferation and apoptosis indices of RA, OA and controls (from Figure 2.10) using the various markers were analyzed using Graphpad Prism. Statistical analysis was performed using a one way ANOVA (nonparametric) followed by Bonferroni's Multiple Comparison Test. TS and Ki-67 indices for RA, OA and controls were not statistically different with respect to each other but significantly lower than the PCNA indices for the same patient group ($p<0.001$). The apoptosis indices as indicated by PARP p85 and activated caspase 3 were low (<0.5%) and did not differ statistically.

Bonferroni's Multiple Comparison Test	P value
PCNA-R vs Ki-67-R	P < 0.001
PCNA-R vs TS 7.4-R	P < 0.001
PCNA-R vs M-R	P < 0.001
PCNA-R vs PCNA-O	P > 0.05
PCNA-R vs PCNA-C	P < 0.001
PCNA-O vs Ki-67-O	P < 0.001
PCNA-O vs TS 7.4-O	P < 0.001
PCNA-O vs M-O	P < 0.001
PCNA-O vs PCNA-C	P > 0.05
PCNA-C vs Ki-67-C	P < 0.001
PCNA-C vs TS 7.4-C	P < 0.001
PCNA-C vs M-C	P < 0.001
Ki-67-R vs TS 7.4-R	P > 0.05
Ki-67-R vs M-R	P > 0.05
Ki-67-R vs Ki-67-O	P > 0.05
Ki-67-R vs Ki-67-C	P > 0.05
Ki-67-O vs TS 7.4-O	P > 0.05
Ki-67-O vs M-O	P > 0.05
Ki-67-O vs Ki-67-C	P > 0.05
Ki-67-C vs TS 7.4-C	P > 0.05
Ki-67-C vs M-C	P > 0.05
TS 7.4-R vs M-R	P > 0.05
TS 7.4-R vs TS 7.4-O	P > 0.05
TS 7.4-R vs TS 7.4-C	P > 0.05
TS 7.4-O vs M-O	P > 0.05
TS 7.4-O vs TS 7.4-C	P > 0.05
TS 7.4-C vs M-C	P > 0.05
M-R vs M-O	P > 0.05
M-R vs M-C	P > 0.05
M-O vs M-C	P > 0.05
p85-R vs p85-O	P > 0.05
p85-R vs p85-C	P > 0.05
p85-R vs C3-R	P > 0.05
p85-O vs C3-O	P > 0.05
p85-O vs p85-C	P > 0.05
p85-C vs C3-C	P > 0.05
C3-R vs C3-O	P > 0.05
C3-R vs C3-C	P > 0.05
C3-O vs C3-C	P > 0.05

2.4.7 Immunohistochemical staining for Ki-67 in RA, OA and control tissue

All 11 RA synovia exhibited nuclear staining of some cells for Ki-67 with a LI mean and range of 2.9 ± 2.57% and 0.2 to 8.0% respectively (Table 2.3). The mean LI was 26 fold lower than that of consecutive sections stained with PCNA ($p < 0.001$), which was statistically significant (Table 2.4 and Figure 2.10). A maximum Ki-67 index of 8 % was seen in one RA tissue with marked thickening of the synovial lining (Table 2.2). A representative picture is shown in figure 2.7b.

Eleven out of the 13 OA patients stained positively for Ki-67 (Figure 2.8b). The LI range was 0.0 to 4.9% with a mean LI of 1.8 ± 1.6% (Table 2.3). These values were lower than those observed in RA patients but consistent with the fact that some proliferative changes occur in the OA synovium. In brief, the Ki-67 LI of the OA synovial lining was significantly lower ($p<0.001$) than that observed in consecutive sections stained with PCNA (Table 2.4 and Figure 2.10).

In cadaver control samples, 3 out of 4 cases demonstrated infrequent Ki-67 positive cells as compared to PCNA labeling in consecutive section of the same tissue (Figure 2.9b). In one case no labeling was observed at all. The LI ranged from 0.0 to 0.6% with a mean LI of 0.23 ± 0.29%, which was significantly less ($p<0.001$) than that seen in RA and OA samples (Table 2.3, Table 2.4 and Figure 2.10). This was an expected result since there are no signs of synovial hyperplasia in the control subjects.

Ki-67 positive cells were present in some lymphocytes, endothelial cells and cells scattered throughout the extracellular matrix in the RA and OA patient samples. Synovia from cadaver specimens showed rare to no Ki-67 staining in the synovial lining, which

differed significantly from RA ($p < 0.001$) and OA ($p < 0.001$, Table 2.4). Again, omission of the primary antibody gave no staining in any of the specimens studied.

As Ki-67 is a nuclear antigen that stains all phases of the cell cycle except G_0, it should theoretically stain more cells than PCNA (S phase). However, the results are opposite of that expected, which is in keeping with our conclusion that PCNA may not be labeling only proliferating cells.

2.4.8 Immunostaining of TS 7.4 in the synovial tissue of RA, OA and controls

To address the apparent discrepancies between PCNA and Ki-67 staining in the synovial tissue of RA patients, TS 7.4 (an antibody that detects cytosolic thymidylate synthase elevated in S phase and absent in G_0 cells) was used in the staining of consecutive sections of the same RA, OA and control tissue used for PCNA and Ki-67 immunostaining. Nine out of 11 RA specimens exhibited cytosolic staining of TS 7.4 in the synovial lining cells with a LI range of 0.0 - 4.33% and mean LI of 1.14 ± 1.27 % (Table 2.3). TS 7.4 immunoreactivity was absent in patient 51a and 68f. Both of these patients also had a low Ki-67 LI of 0.22% and 0.33% respectively (Table 2.2). Similar to Ki-67, the mean TS 7.4 LI for RA patients was significantly lower than that for consecutively stained section with PCNA ($p<0.001$, Table 2.4 and Figure 2.10). A representative picture is shown in figure 2.7c.

Cytosolic staining for TS 7.4 was present in 11 out of 13 OA patients with a LI range of 0.0 to 4.33%, and a mean LI of 1.16 ± 1.08%, which was similar to the one observed in RA patients (Table 2.3). Staining was absent in patient 53b and 54b. Both these patients also had low Ki-67 indices of 0.0 and 0.11% respectively (Table 2.2). The

TS LI was similar to that of Ki-67, but it was significantly lower than that observed in PCNA ($p<0.001$, Table 2.4 and Figure 2.10). A representative picture is shown in figure 2.8c.

TS 7.4 positive cells were also observed in some lymphocytes, endothelial cells and some cells scattered throughout the extracellular matrix of RA, OA and controls. None of the control samples showed staining for TS in the synovial lining, which was in marked contrast to that of PCNA staining (Figure 2.9c).

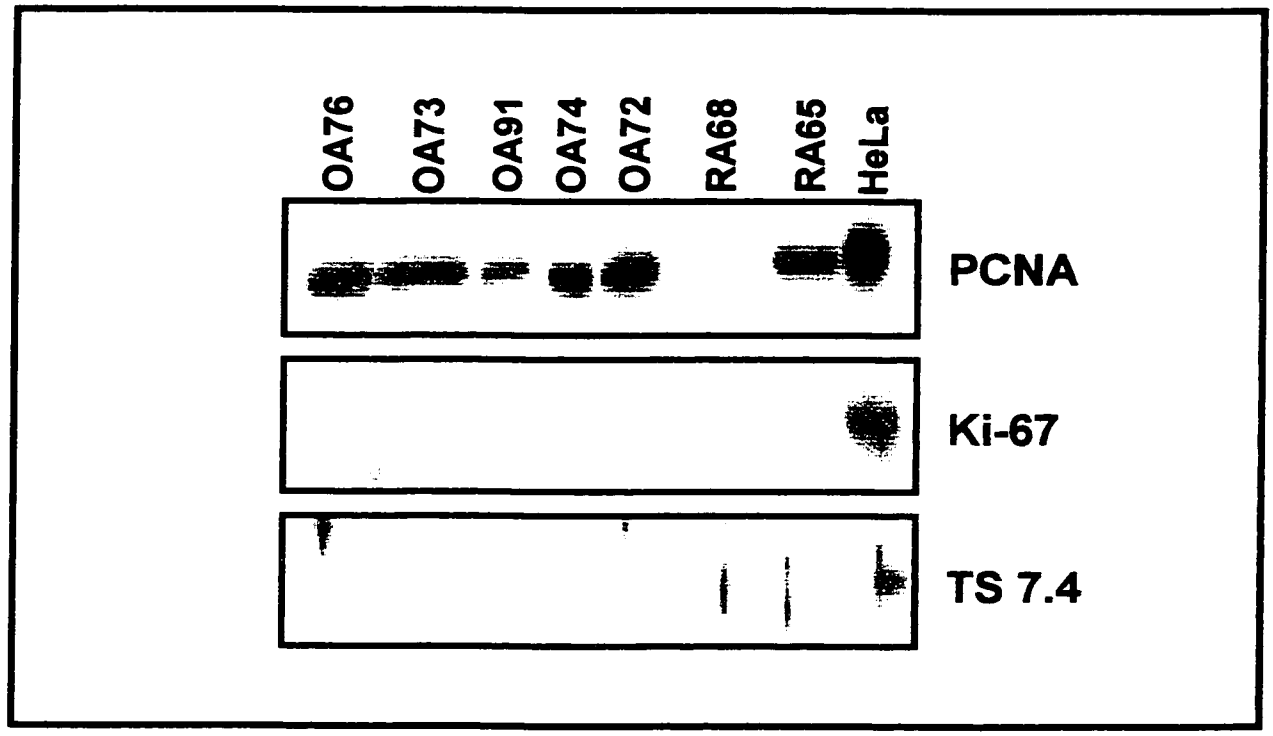
The Ki-67 and TS 7.4 proliferation indices for RA, OA and controls were significantly lower than those seen with PCNA staining. Overall the staining pattern for TS was similar to that of Ki-67. However, the means and ranges were a little lower for TS 7.4 as compared to Ki-67. A reason for this could be the staining of predominantly S phase cells by TS 7.4 compared to Ki-67, which stains all phases of the cell cycle relatively strongly.

2.4.9 PCNA but not Ki-67 or TS is detected in Western blot analysis of RA and OA synovial homogenates

To confirm our immunohistochemistry results, Western blot analysis was performed on some RA and OA synovial homogenates using antibodies directed against PCNA, Ki-67 and TS (PC 10, MIB-1 and TS 7.4). Seven out of 8 synovial homogenates showed a single strong positive 36 kDa band for PCNA (Figure 2.11). A strong PCNA band was detected in the homogenates for RA patient 65 and OA patient 72, 74 and 91 which corresponded with the high PCNA LI observed *in situ* for those samples (Table

Figure 2.11: Western blot analysis of a minor fraction of RA and OA homogenates for PCNA, Ki-67, TS.

A total of 30 μg of protein for each RA and OA homogenate was migrated on a 12% SDS-PAGE and transferred to PVDF membrane except for RA patient 68 where 15μg was migrated and transferred due to availability. Synovial homogenates from RA patient 65, 68 and OA patient 72, 74 and 91 had high in situ PCNA labeling indexes, but low Ki-67 and TS labeling indexes (Table 2-2). In support of the immunohistochemical staining, a single PCNA band was detected in all RA and OA homogenates. Ki-67 and TS were not detected in the same homogenates. Ki-67 and TS were clearly detected in our positive control lane that contained 10 μg of a total HeLa cell extract, indicating no technical problem with detection of TS or Ki-67 by Western blot analysis.


OA76
OA73
OA91
OA74
OA72
RA68
RA65
HeLa
PCNA
Ki-67
TS 7.4

2.2). A weaker PCNA signal was detected for RA patient 68 compared to its high percentage of staining *in situ* because only 15 μg of protein extract was loaded due to available sample size. PCNA immunohistochemistry was not performed on the remaining three OA samples (76, 73 and 90), though these homogenates showed a strong single PCNA band.

By contrast, TS and Ki-67 were not detected in the homogenates (65, 68, 72, 74, 91) confirming our *in situ* staining with these antibodies in RA patient 65, 68 and OA patient 72, 74, and 91 (Table 2.2). Moreover, Ki-67 and TS was clearly detected in our positive control lane that contained a total HeLa cell extract. These results suggest that the absence of a TS or Ki-67 band was not a consequence of technical problems with detection.

2.4.10 Activated caspase 3 and cleaved PARP (PARP p85) demonstrate a low rate of apoptosis in the intimal lining of arthritis and control patients

The rate of apoptosis was studied using activated caspase 3 and its substrate cleaved PARP in the synovium of RA, OA and control patients. Activated caspase 3 and PARP 85 staining was primarily seen in occasional sublining cells, infrequently in lymphocytes, some scattered cells through the synovium and rarely in the intimal lining of RA samples (Figure 2.12a and e). The activated caspase 3 LI range of the intimal lining was 0.0 - 0.44% with a mean LI of 0.11 ± 0.17% (Table 2.3). A similar LI range and mean was determined for PARP p85 staining.

Figure 2.12: PARP p85 and activated caspase 3 staining in RA synovium.

Representative samples of RA synovia stained for PARP p85 and activated caspase 3. (A, D) Consecutive sections of RA patient 1 showing absence of staining by PARP p85 and activated caspase 3 in the synovial lining (SL); (D) sublining cells staining for activated caspase 3. (B, E) Consecutive sections showing absence of staining for (B) PARP p85 and some staining for (E) activated caspase 3 in the SL of RA patient 26. In the synovium of patient 52b scattered lymphocytes stain for (C) PARP p85 and (F) activated caspase 3. Stained cells are indicated with a black arrow. A-D, x600 total magnification; E-F, x400 total magnification. SL = synovial lining.

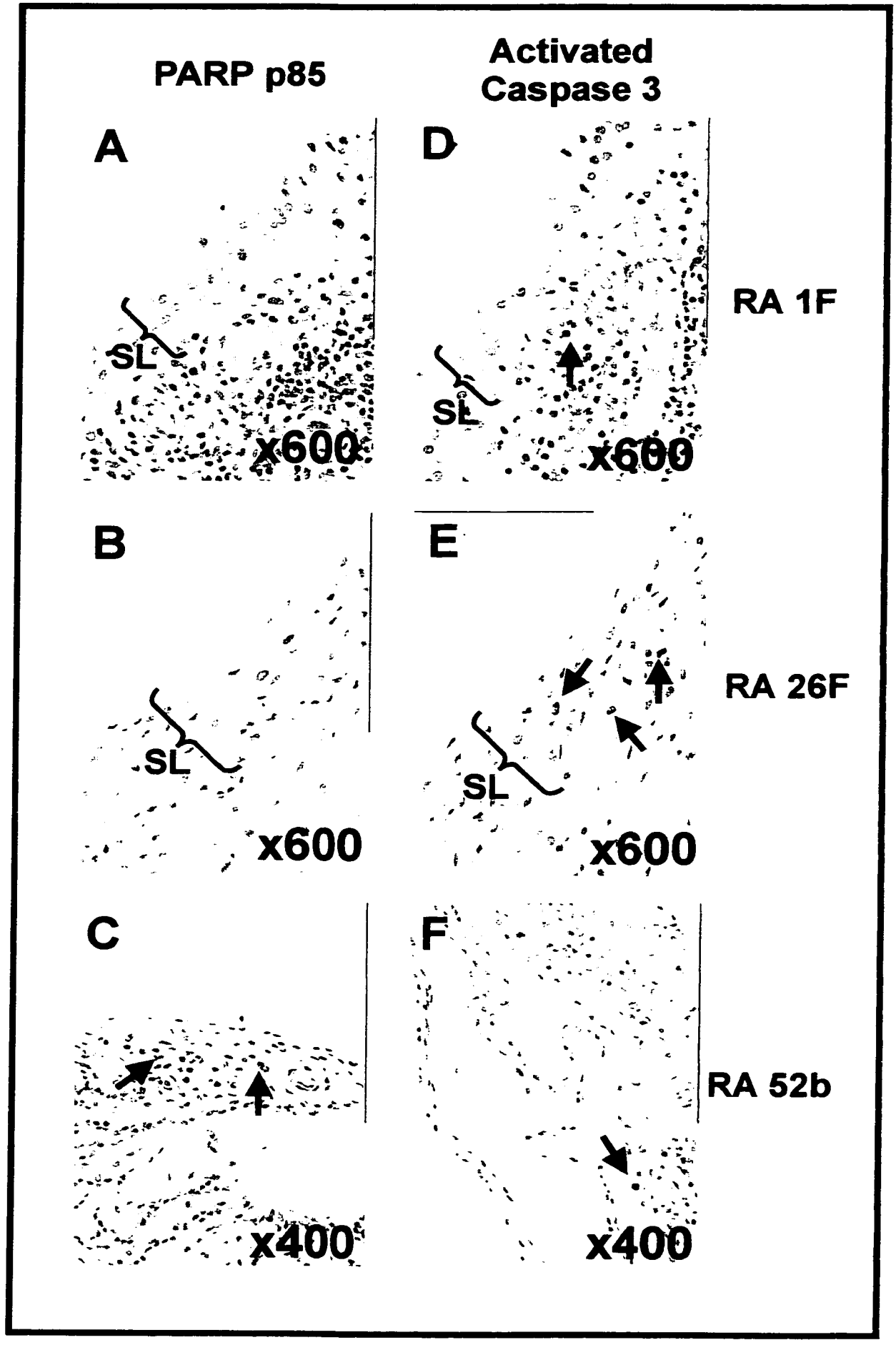
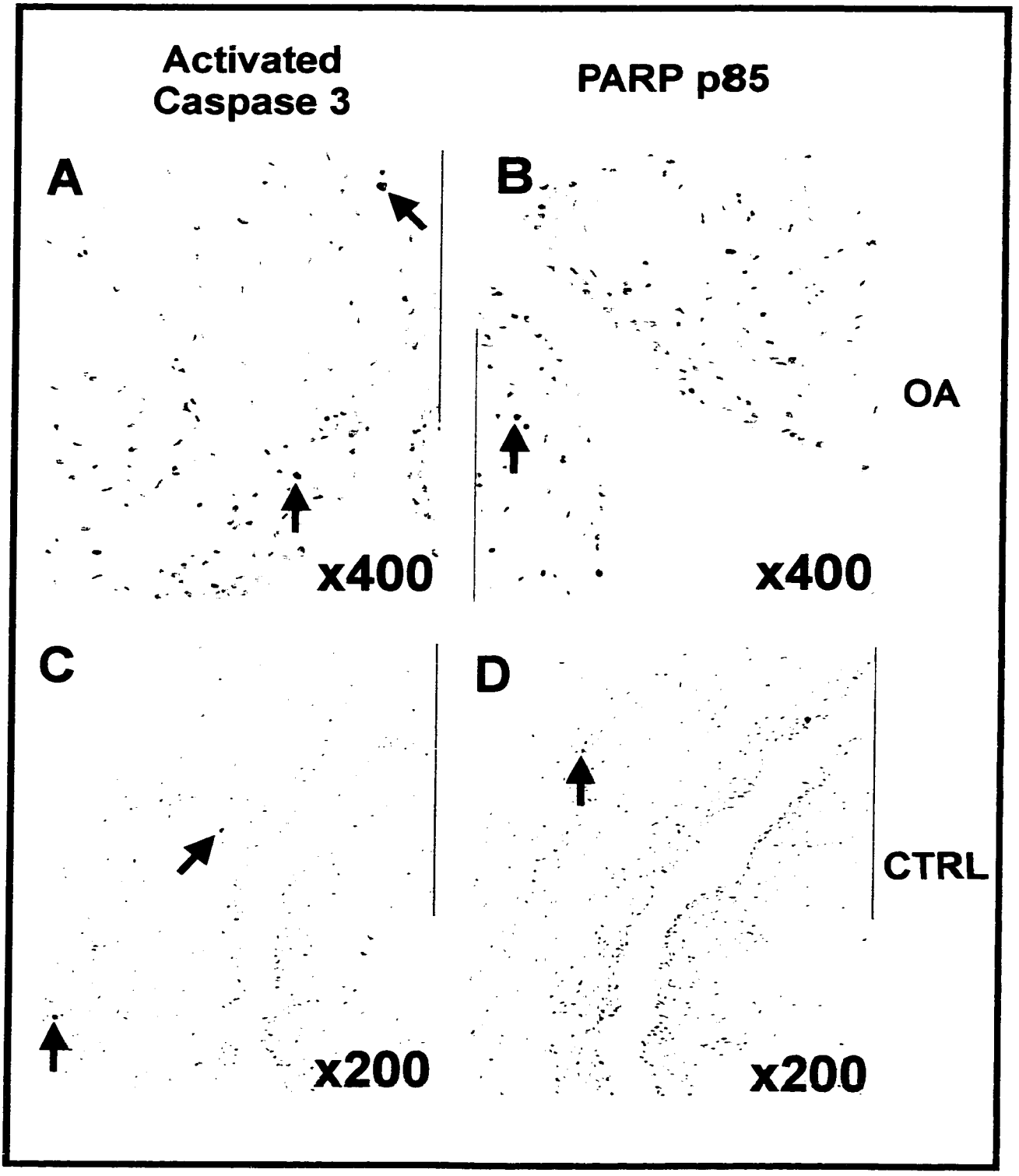

PARP p85
Activated Caspase 3
A
D
SL
SL
x600
x600
RA 1F
B
E
SL
SL
x600
x600
RA 26F
C
F
x400
x400
RA 52b

Figure 2.13: PARP p85 and activated caspase 3 immunostaining in OA and control synovium.

Representative sample of OA and control synovium stained for PARP p85 and activated caspase 3 (A-D). (A,B) OA synovium showing staining of sublining cells for (A) activated caspase 3 and (B) PARP p85. Sections of control synovium show some cells within blood vessels staining for (C) activated caspase 3 and some scattered cells for (D) PARP 85. Staining of the synovial lining for both apoptosis markers is absent in all control and in most OA samples. A-B, x400 total magnification; C-D, x200 total magnification.. Stained cells are indicated with a black arrow.


Activated
Caspase 3
PARP p85
A
B
OA
x400
x400
C
D
CTRL
x200
x200

Some of the 13 OA synovial tissue samples stained for activated caspase 3 and cleaved PARP (Figure 2.13a and d). The LI range and mean for both Activated caspase 3 and PARP p85 in the OA synovial lining was not statistically different with respect to each other, and to the LI observed in RA samples ($p > 0.05$).

Staining for activated caspase 3 and PARP p85 was not observed in the synovial lining of cadaver samples (Table 2.3). Rare staining cells were detected for caspase 3 and PARP p85 in the synovium. These results indicate that the rate of apoptosis in RA OA and control patients is low and that a moderate increase in proliferation could indeed lead to synovial hyperplasia. Once again, omission of the primary showed no staining in the synovial tissue samples of RA, OA and control patients.

2.5 Discussion

Defining the mechanisms that control the rate of cell growth is fundamental to normal immunity and the pathogenesis of autoimmune diseases such as RA. An important feature of normal tissue homeostasis is the balance between proliferation and cell death, including apoptosis. In diseased RA tissue with hyperplastic synovium, this balance could be altered either by increased proliferation and/or by a decrease in apoptosis. These fundamental issues related to the development of RA are an area of much interest and speculation. A better understanding of the roles of these processes will provide more knowledge of the pathology of RA and could better serve in the development of new therapeutic agents or contribute to the improvement of current therapies.

The present study was designed to examine whether synovial thickening is a result of increased cell proliferation and/or decreased apoptosis. Studies have tried to use PCNA and Ki-67 to clarify this matter (Qu et al., 1994a). The work presented in this chapter replicates an earlier report that a high proportion of intimal lining cells from RA, OA and control patients stained for PCNA in synovial tissues with hyperplastic and non-hyperplastic intimal linings. However, no correlation was found between the PCNA values and other indices of proliferation obtained with Ki-67, TS and mitotic counting. Though PCNA stained lymphoid follicles in tonsil tissue, which are considered to be regions of high B cell proliferation, large numbers of cells also stained in the paracortex, which are not known to contain proliferating cells.

Several possible reasons could account for the abundantly high PCNA levels found in the intimal lining. First, unlike Ki-67, (which has a half-life of 20 minutes throughout the cell cycle, with major catabolism occurring upon completion of mitosis), PCNA has a half-life of 24 hours. Thus, PCNA could be a fingerprint of cells that have recently undergone proliferation. On the other hand it could demonstrate staining of quiescent cells.

Second, PCNA is involved in DNA repair such as nucleotide excision repair, post-replication mismatch repair and base excision repair. DNA damage is known to occur in the oxidatively stressed synovium as indicated by DNA damage at the hypoxanthine phosphoribosyltransferase (hprt) gene locus in T-cells, and point mutations in the p53 gene and H-ras gene of type-B synoviocytes (Firestein et al., 1997; Roivainen et al., 1997; Cannons et al., 1998). Therefore, PCNA could be upregulated in response to DNA damage in the synovium. Moreover, PCNA can be transiently activated by the

presence of superoxide, hydrogen peroxide (Savio et al., 1998; Balajee et al., 1999) and platelet derived growth factor (Udenfriend et al., 1972), all of which have been demonstrated in the RA synovium (De Vouge et al., 1994; Sharma et al., 1999; Qu et al., 1994b).

Third, evidence exists for the involvement of PCNA in cell cycle control including cellular growth arrest, terminal differentiation, and apoptosis (Sanchez and Elledge, 1995; Prosperi, 1997). This is based on the fact that PCNA interacts with cyclin D (Matsuoka et al., 1994), cyclin-dependant kinases (cdks), GADD45 (Chen et al., 1995), MyD118 (Vairapandi et al., 2000) and cdk-inhibitor p21/Waf1/Cip1 protein (Oku et al., 1998).

Fourth, some of the PCNA staining can in part be due to its role in DNA replication evidenced by its interaction with DNA polymerase δ and ε, and with replication factor C (RFC), a PCNA clamp loader (Tsurimoto, 1998).

Finally, it has been suggested that two populations of PCNA exist, one that is associated with chromatin and the other form that is unbound. The bound form is typically associated with the S phase. Both forms are detected in formalin and stained by the monoclonal antibody unless the free form is extracted by detergent from both paraffin embedded sections and frozen section (Sasaki et al., 1995). Accumulating evidence suggests that PCNA is a key player in a variety of important cellular processes and thus may not be a reliable proliferation marker.

Unlike PCNA, the expression of Ki-67 is strictly associated with the cell cycle. The present study showed that the number of proliferating cells labeled with Ki-67 in the intimal lining of RA and OA synovia is low and nearly non-existent in control synovia.

This correlates well to previous studies using [^{3}H] thymidine and Ki-67 (Mohr et al., 1975; Nykanen et al., 1978; Dreher, 1982; Lalor et al., 1987). Our Ki-67 labeling index of the synovial lining was higher than that of the previous thymidine incorporation studies. This is an expected result since the latter studies label cells strictly in the S phase and the former label all phases of the cell cycle except G_0. Furthermore, our Ki-67 index was somewhat higher than that observed by Lalor and coworkers of < 0.1% (1987). This is also not surprising since we are using new antigen retrieval techniques and the new more sensitive Ki-67 (MIB-1) clone that recognizes a formalin fixed epitope. The low number of Ki-67 positive cells in the intimal lining was not due to technical reasons since our tonsil tissue revealed ample Ki-67 staining as did lymphoid follicles found in some patients with RA. This was further supported by Western blot analysis.

To resolve the discrepancy between the Ki-67 and PCNA data, polyclonal antibody TS 7.4 was used. TS protein is a cytosolic enzyme present throughout the cell cycle but elevated in the S phase and absent in G_0 cells (Haqqani et al., 1999). TS staining revealed low but specific staining of the intimal lining in both RA and OA synovia and no staining in control synovia. The staining pattern was similar to that seen with Ki-67. As expected, the Ki-67 index was slightly higher than that of TS since the latter is mainly associated with the S phase and the former is associated with all phases of the cell cycle except G_0. The low levels of staining in the intimal lining were not due to technical difficulties since lymphoid follicles in both tonsil tissue and some RA tissue stained strongly for TS. These results support earlier conclusions that TS 7.4 could be used as a marker of proliferation.

Ki-67, TS and mitotic indices support only a low level of increase in proliferation of synovial cells in RA, and even lesser in OA, compared to controls. Earlier studies of the rate of apoptosis *in situ* in the synovial lining of RA and OA patients have not produced clear conclusions. Studies have mainly used morphologic characteristics and ISNEL techniques to identify cell death with varying results. Since ISNEL could label apoptotic cells, necrotic and viable cells undergoing DNA repair more specific markers were sought. Fas-mediated apoptosis is observed in cultured synoviocytes and *in situ* in the synovial lining from RA patients (Asahara et al., 1997; Kobayashi et al., 1999a). In cultured RA synoviocytes, Fas ligand leads to the activation and high expression levels of Caspase 8, Caspase 3, FADD (Fas-associated death domain protein) and the cleavage of PARP corresponding to apoptosis (Okamoto et al., 2000). In vitro, synoviocyte apoptosis is suppressed in a dose and time dependant manner when inhibitors of caspase 8 or caspase 3 are used (Kobayashi et al., 2000a). Thus, the FADD /Caspase 8/Caspase 3/ PARP pathway is implicated as the major signaling pathway required for fas-mediated apoptosis in RA synoviocytes. The c-jun amino- terminal kinase (JNK)/activator protein –1 (AP-1) pathway is also activated during Fas mediated synoviocyte apoptosis but it appears to play a less important role (Kobayashi et al., 1999b). Interestingly, TNF-α sensitizes RA and even OA synoviocytes (normally insensitive to Fas mediated apoptosis in spite of Fas antigen expression) to Fas-mediated apoptosis and leads to activation of the FADD/caspase 8/caspase 3/PARP pathway (Kobayashi et al., 1999a). In sum, activated caspase 3 is intimately involved in the synoviocyte apoptosis being responsible for the proteolytic cleavage of key proteins such as the nuclear enzyme PARP.

In the present study, recently available polyclonal antibodies to activated caspase 3 and cleaved PARP were used to study the *in situ* apoptosis of the synovial lining in RA, OA and control patients. Activated caspase 3 and PARP p85 are established hallmarks of apoptosis (Fernandes-Alnemri et al., 1994; Duriez and Shah, 1997). Our results indicate that a low but similar number of synovial lining cells were undergoing apoptosis in RA, OA and control patients. This leads us to conclude that synovial hyperplasia is a result of a modest increase in synoviocyte proliferation associated with normally low rates of apoptosis. If one considers our report of decreased apoptosis and the high proportion of PCNA positive synovial lining cells as proliferating, RA synovial hyperplasia would be predicted to be much greater than actually observed. Furthermore, RA would have a labeling index as high as malignant non-Hodgkin's lymphomas (Gerdes et al., 1984).

Based on findings presented in this chapter, we propose that a modest increase in the rate of intimal lining proliferation associated with a normally low rate of apoptosis leads to the development of synovial hyperplasia. We demonstrate that the proliferation of lining synoviocytes is moderately increased in RA and OA as compared to controls. Furthermore, we examined the rate of apoptosis using specific polyclonal antibodies to PARP p85 and activated caspase 3. Very little staining was observed in the intimal lining using these specific apoptosis markers in RA, OA and control patients. This study establishes TS as a reliable proliferation marker and questions the use of PCNA in proliferation studies of the synovial lining in arthritic patients. Furthermore, we established that PARP p85 could be used in formalin fixed paraffin embedded sections. Our findings provide more insight into the pathology of RA and could help introduce new pharmacological approaches in addition to current therapies.

Such an approach could use drugs to decrease synovial proliferation (Hui et al., 1997). However this probably would not be very effective as the proliferation rates of the synovial lining are not very dramatic. Alternatively, adopting a strategy to locally increase synovial lining apoptosis could be potentially beneficial by reducing the number of synoviovytes involved in joint destruction. This strategy could be achieved with a novel gene therapy approach that transfects the FADD gene into synoviocytes via an adenoviral vector. As indicated earlier FADD plays a key role in Fas-mediated apoptosis of cultured RA synoviocytes. Recently, transfection of the FADD gene into cultured RA synoviocytes and local injection of FADD adenovirus into SCID mice engrafted with proliferating RA synovium induced up-regulation of FADD and apoptosis (Kobayashi et al., 2000b). Furthermore, this study showed that apoptosis was specific to synovial cells and not chondrocytes (Kobayashi et al., 2000b). The latter is an important feature since the cartilage will be protected from further degradation. Though promising, adenoviral therapies are not perfect. First, adenoviruses are episomal and have a limited life-span in the host cell. Second they can induce a host antiviral immune responses which reduces the duration of vector persistence and precludes long-term transgene expression by repeated injection of the vector. In sum, my research strengthens the argument for the use of novel therapies that induce apoptosis locally.

2.6 Future Direction

In future work, we will need to confirm our data in a larger series of RA, OA and control patients. For example, Western blots analysis will be performed on synovial homogenates for both markers of apoptosis to confirm our immunohistochemistry results.

It will be also crucial to compare these new apoptosis markers to the older standards of measuring apoptosis such as TUNEL and DNA laddering to see whether or not the old and new standards correlate.

It is evident that the balance of cell proliferation and apoptosis play a critical role in a variety of diseases such as RA. We determined that TS 7.4 and Ki-67 stain similarly in the synovial lining of consecutive sections. This result is interesting and warrants further investigation. Co-localization of TS and Ki-67 to the same cell type would strengthen the argument for the use of TS 7.4 as a proliferation marker in the synovium. Alternatively, determining an inverse relationship between TS LI and statin LI would be further convincing evidence of the pertinence of TS as a proliferation marker. Statin is a 57 kDa nuclear protein that is highly expressed in non-cycling cells (G0/early G1), but disappears when proliferation resumes or is induced by growth factors (Tsanaclis et al., 1991). Typically, low statin LI is inversely related to Ki-67 and is associated with poor prognosis in human brain tumors and childhood acute lymphoblastic leukemia (Tsanaclis et al., 1991; Volm et al., 1995). In summary, future data in relation to statin LI and co-localization of Ki-67 and TS will provide a stronger insight into cell growth in the synovial lining.

CHAPTER 3: WIDESPREAD DISTRIBUTION OF PROTEIN NITROTYROSINE IN THE SYNOVIAL TISSUE OF PATIENTS WITH RHEUMATOID ARTHRITIS AND OSTEOARTHRITIS

3.1 Abstract

There is increasing evidence that reactive nitrogen and oxygen species (RNOS) are present in the inflamed joints of patients with RA and to a lesser extent in OA. It has been postulated that these molecules may contribute to disease progression. RNOS can be cytotoxic, genotoxic and can modify tyrosine residues in proteins to form nitrotyrosine. The present study was undertaken to determine whether protein nitrotyrosine could be detected in the synovial joint tissue of patients with RA and osteoarthritis (OA). The presence and tissue distribution of nitrotyrosine was determined by using a rabbit polyclonal anti-nitrotyrosine antibody. Synovial tissue from 9 RA and 9 OA patients was used. Western blot analysis was also performed on homogenates of synovial tissue in some cases. Nitrotyrosine staining was detected to varying degrees in synovial tissue of most patients. Diffuse staining of the extracellular matrix was observed in most cases. Intense staining was observed in macrophages and endothelial cells in RA patients compared with the OA patients; both cytoplasmic and nuclear staining was observed. Nitrotyrosine staining was also detected in some lymphocytes but staining was absent in neutrophils. Antibody specificity was proven by showing that tissue staining could be blocked with nitrated bovine serum albumin (N-BSA), nitrotyrosine amino acid or reduction with sodium hydrosulphite. A number of high and low molecular mass proteins were seen on Western blots. This study provides the first *in situ* evidence that nitrated proteins are present in the synovial tissue of patients with RA and OA. These

findings also provide additional evidence that RNOS are present in the inflamed joints of these patients. The precise consequence of protein nitrotyrosine in the RA joint and its importance in disease progression remains to be determined.

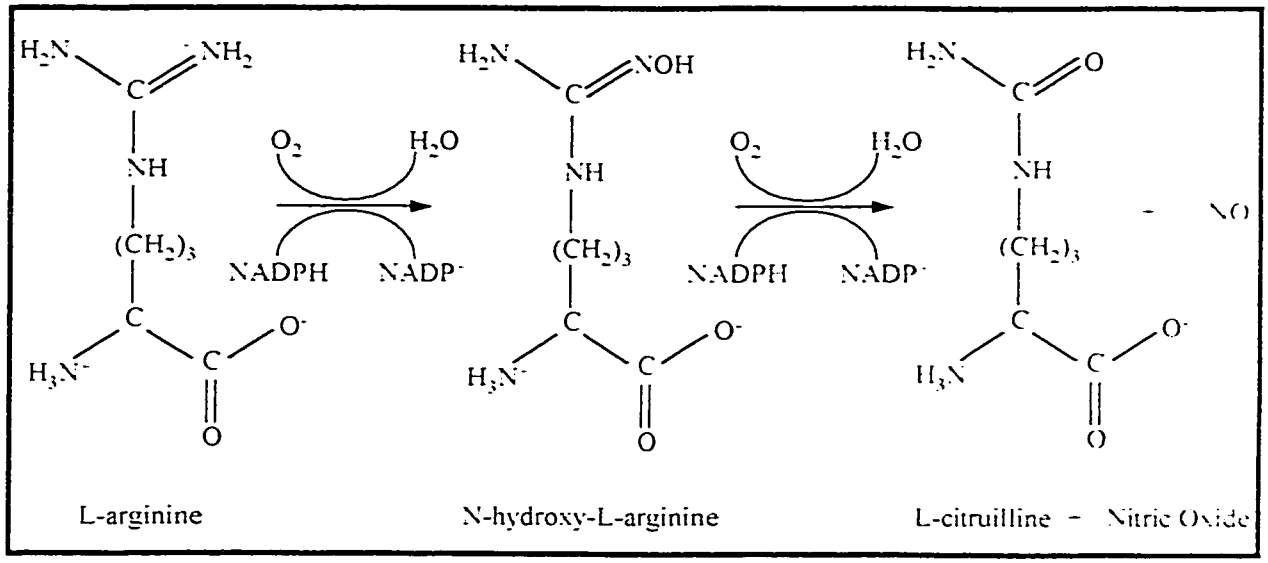
3.2 Introduction

3.2.1 Nitric oxide

Nitric oxide (NO), production of which is enhanced during inflammation, is a short lived, diffusible, pleiotropic messenger that is endogenously produced from L-arginine by the enzyme nitric oxide synthase (NOS) (Figure 3.1). At constitutively expressed levels (cNOS) NO has several important physiological functions that include vasodilation, neurotransmission, and intracellular signalling. However, when produced in excess by inducible nitric oxide synthase (iNOS), NO may have undesirable genotoxic and cytotoxic effects.

Since NO is a short lived radical it is detected using indirect methods. This includes the detection of stable NO metabolites such as nitrite and nitrate. Increased nitrite levels have been detected in the serum and synovial fluid of RA patients versus OA and controls (Farrell et al., 1992; Grabowski et al., 1996b). In both disease groups, the synovial nitrite levels were significantly higher than those detected in the serum suggesting that NO is being produced in the synovium.

Figure 3.1: Nitric oxide synthase mediated conversion of L-arginine to nitric oxide


L-arginine
N-hydroxy-L-arginine
L-citruilline + Nitric Oxide
NADPH
NADP+

Immunohistochemical detection of iNOS have revealed that NO is produced by many cell types found in the inflamed RA synovium including macrophages, fibroblasts, and endothelial cells (Grabowski et al., 1997). Furthermore, increased levels of N^G-hydroxy-L-arginine, an intermediate metabolite released by NOS, have been detected by HPLC in the serum of RA patients compared to controls suggesting NOS activity *in vivo* (Wigand et al., 1997). Finally, cultured RA synoviocytes produce NO spontaneously. Increased NO production follows the addition of pro-inflammatory cytokines and bacterial endotoxin lipopolysaccharide (LPS) suggesting that NO is present *in vivo* (Grabowski et al., 1996a; McInnes et al., 1996). Therefore, substantial amounts of NO are produced in the arthritic joint.

3.2.2 NO and RA

Pathophysiologically, NO may contribute to joint destruction by activating matrix-degrading metalloproteinases (MMPs) (stromelysin-1 and collagenase) from various cell types including type B synoviocytes and articular chondrocytes (Tetlow and Woolley, 1995; Tamura et al., 1996). For example, *in vitro* experiments performed on bovine and human cartilage explants showed that inflammatory mediators and a bacterial endotoxin induced NO activity and MMP activity while, N^G-monomethyl-L-arginine an inhibitor of NO synthesis inhibited both nitrite production and MMP activity; thus demonstrating a role for NO in the RA joint (Murrell et al., 1995).

NO may also contribute to the inflammatory process seen in RA. NO can activate enzymes such as cyclooxygenases (COX) in both macrophage and articular cartilage (Salvemini et al., 1993; Manfield et al., 1996). COX exists in both constitutive (COX1)

and inducible (COX2) isoforms. Similar to iNOS, COX2 expression is upregulated in inflammatory joint disease when compared to controls (Kang et al., 1996). Briefly, COX enzymes are involved in the synthesis of prostaglandin-H2 (PGH-2) from arachidonic acid. Subsequently, PGH-2 is converted to several prostaglandin isoforms and thromboxane-A2. Prostaglandins, such as PGH-2 are potent pro-inflammatory molecules involved in vasodilation, increasing vascular permeability, chemotaxis and sensitizing pain fibers to stimulatory molecules such as bradykinin.

NO may also be involved in DNA damage, which may contribute to RA pathology. Previous work from our laboratory demonstrated an elevated mutation frequency at the hypoxanthine guanine phosphoribosyltransferase (hprt) locus in T cells derived from the peripheral blood and synovial tissue of RA compared to OA and control patients (Cannons et al., 1998). RA patients had a 5 fold higher T cell mutation frequency in the peripheral blood compared to controls. In addition, a further 10 fold increase in mutant T cell frequency was observed in synovial tissue as compared to the peripheral blood. This suggests that T cells and other cell types are exposed to genotoxic species *in vivo* such as RNOS. Furthermore, *in vitro* evidence suggests that NO is capable of inducing large-scale deletion mutations (> 826 Kb and > 1.2Mb) in WIL2-NS cells treated with NO donors (Grant et al., submitted). In keeping with the latter observations, type B synoviocytes from RA patients have been reported to have a higher mutation frequency in the tumor suppressor gene p53 (Firestein et al., 1997). Such mutations may play a role in synovial hyperplasia and the increased risk of hematopoietic cancers in patients with RA (Laakso et al., 1986).

Finally, NO is further implicated in the pathogenesis of RA with recent evidence that existing medications act by interfering with iNOS activity and expression. Presently used medications such as dexamethasone and cyclosporin work by inhibiting the activation and expression of iNOS at the level of transcription (De Vera et al., 1997; Attur et al., 2000). Other medications such as methotrexate also inhibit iNOS activity by interfering with the synthesis of an iNOS cofactor, tetrahydrobiopterin (Saura et al., 1996; Robbins et al., 1998). Even nonsteroidal antiinflammatory drugs (NSAIDs) interfere with iNOS expression. For example, aspirin has been shown to inhibit iNOS activity in murine macrophage and cell free extracts suggesting direct acetylation of the enzyme (Amin et al., 1995). Recently, tetracycline (including family members doxycycline and minocycline) was shown to inhibit NO production in murine macrophage and human chondrocytes at the level of mRNA and protein expression (Amin et al., 1995). Tetracycline may also scavenge peroxynitrite at sites of inflammation (Whiteman and Halliwell, 1997). Interestingly, tetracyclines have a chondroprotective effect in inflammatory arthridites in animal models (Yu, Jr. et al., 1992). Furthermore, minocycline treated RA patients showed superior improvement in joint swelling, joint tenderness and radiographic measurements compared to placebo controls (Tilley et al., 1995; Bluhm et al., 1997).

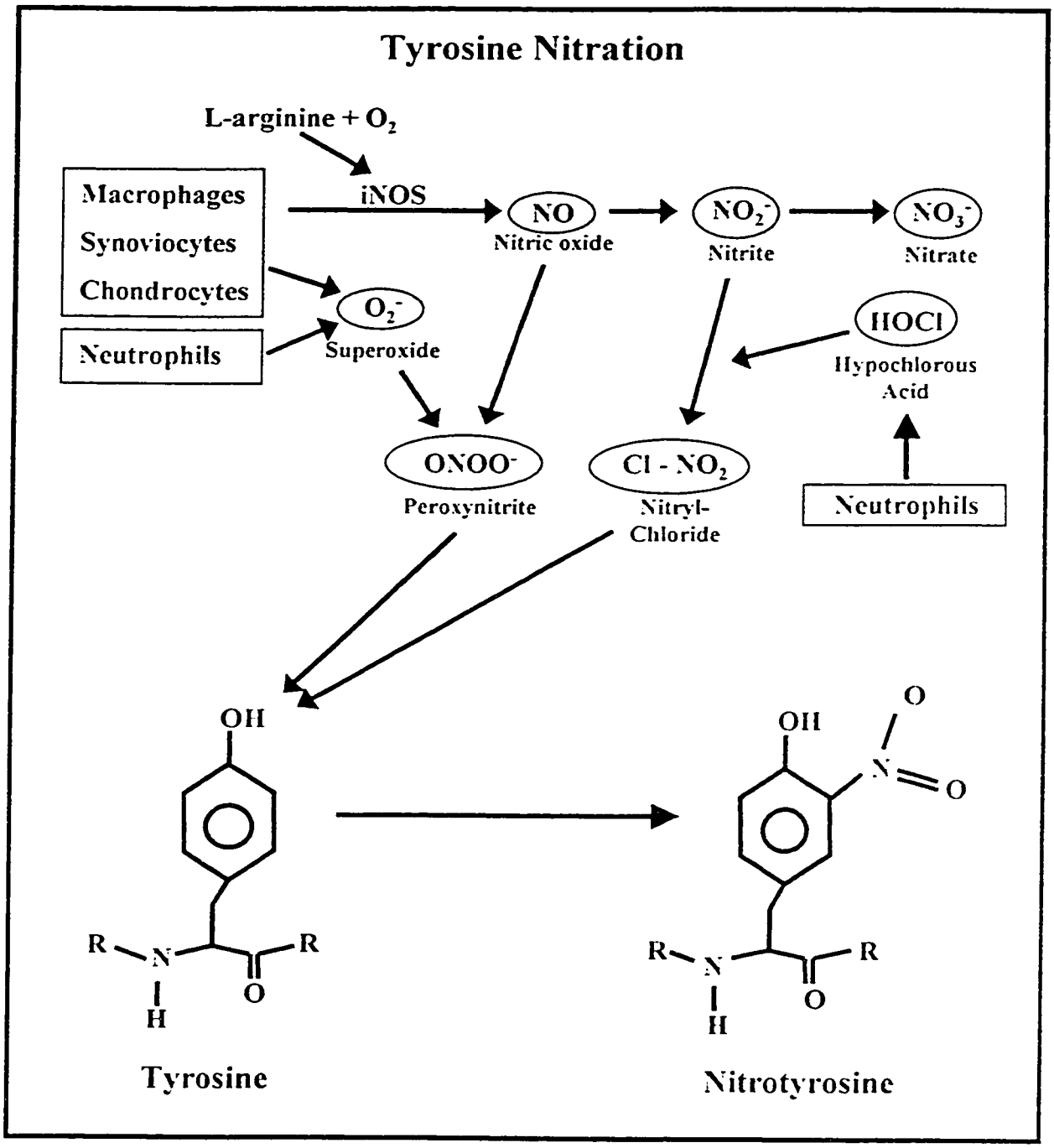
Potential therapies in RA include using a chimeric monoclonal antibody against tumor necrosis factor alpha (TNF-α; cA2). Clinical trials have shown that patients treated with cA2 had a significant reduction in iNOS antigen expression and iNOS activity (Perkins et al., 1998). This reduction correlated with marked improvement in disease measure. In sum, accumulating evidence suggests that NO is major mediator of

RA pathogenesis and represents a potential target for the development and improvement of new and current anti-arthritic arsenals. The fact that certain medications, used in the treatment of RA including NSAIDs, methotrexate, dexamethasone, and tetracyclines may mediate their effects at least in part through the inhibition of iNOS further strengthen the importance of NO in RA pathogenesis.

3.2.3 NO, protein nitrotyrosine and RA

Rheumatoid arthritis is a chronic, progressive and debilitating disease characterized by joint inflammation and eventual destruction of the joint. Osteoarthritis is not generally considered an inflammatory disease, however recent studies have shown evidence of mild to moderate inflammatory changes in OA synovium (Haraoui et al., 1991). Recently, it has been suggested that high levels of nitric oxide (NO) may be produced at sites of inflammation in the joints of patients with RA and to a lesser extent in OA by the activation of the inducible nitric oxide synthase (iNOS) pathway (Ishiuchi et al., 1999). Increased serum and urinary levels of nitrate/nitrite have been found in patients with RA, and synovial fluid nitrite levels are higher than those found in serum, suggesting that NO may be derived from within the joint (Farrell et al., 1992; Grabowski et al., 1996b). NO can react with superoxide (an oxygen radical also found at sites of inflammation) and lead to the formation of $ONOO^-$, which can react with proteins resulting in the formation of nitrotyrosine (Figure 3.2). Though nitrotyrosine formation is generally considered a specific marker of peroxynitrite, recent studies have proposed additional pathways of tyrosine nitration, such as the myeloperoxidase-dependant

Figure 3.2: Schematic of protein tyrosine nitration


Tyrosine Nitration
L-arginine + O_2
Macrophages
Synoviocytes
Chondrocytes
iNOS
NO
Nitric oxide
NO_2^-
Nitrite
NO_3^-
Nitrate
Neutrophils
O_2^-
Superoxide
HOCl
Hypochlorous Acid
$ONOO^-$
Peroxynitrite
$Cl - NO_2$
Nitryl-Chloride
Neutrophils
OH
R
N
H
R
O
Tyrosine
OH
O
N
O
R
N
H
R
O
Nitrotyrosine

conversion of nitrite to nitryl chloride and nitrogen dioxide (Eiserich et al., 1998). This type of protein damage is evidenced by the immunohistochemical detection of nitrotyrosine, in pro-inflammatory disorders such as inflammatory bowl disease (Dijkstra et al., 1998), acute respiratory distress syndrome (Haddad et al., 1994), atherosclerosis (Beckmann et al., 1994), multiple sclerosis (Bagasra et al., 1995), Alzheimer's disease (Good et al., 1996) and canine and rabbit models of osteoarthritis (Hashimoto et al., 1999; Pelletier et al., 1999). Since nitrotyrosine is a stable end product of nitration, it may serve as a collective indicator of reactive nitrogen and oxygen species (RNOS) in these inflammatory diseases.

In RA, high concentrations of nitrotyrosine have been detected in the serum and synovial fluid as compared to OA and control patients (Kaur and Halliwell, 1994). Furthermore, widespread *in situ* nitrotyrosine staining has been observed in a mouse and rodent model of collagen induced arthritis while no staining was observed in controls (Szabo et al., 1998; Cuzzocrea et al., 2000). Western blot analysis of paw extracts from the mouse model showed several high and low molecular weight bands indicating the presence of nitrated proteins *in vivo*. Therefore, nitrotyrosine may be important in RA disease progression.

Thus far, the presence of nitrotyrosine-modified proteins has not been demonstrated by immunohistochemistry in the synovial joints of patients with RA and OA. To explore further the role of RNOS in disease progression, we determined the presence of nitrated proteins in the synovial joint tissues of RA and OA patients using immunohistochemistry and Western blot analysis.

3.2.4 Hypothesis

In the inflamed arthritic joint, resident and infiltrating cells produce high amounts of RNOS such as NO and O_2 (Figure 3.3). NO and O_2^- react to form a potent oxidizing and nitrating agent peroxynitrite ($ONOO^-$), which can readily nitrate tyrosine residues. Since elevated NO levels have been detected in RA compared to OA patients, I hypothesize that nitrotyrosine should be detected in both diseases, but to a lesser extent in OA.

3.2.5 Objectives

a) To determine whether nitrotyrosine modified proteins are present in the inflamed RA and OA synovium by using immunohistochemistry with an anti-nitrotyrosine antibody

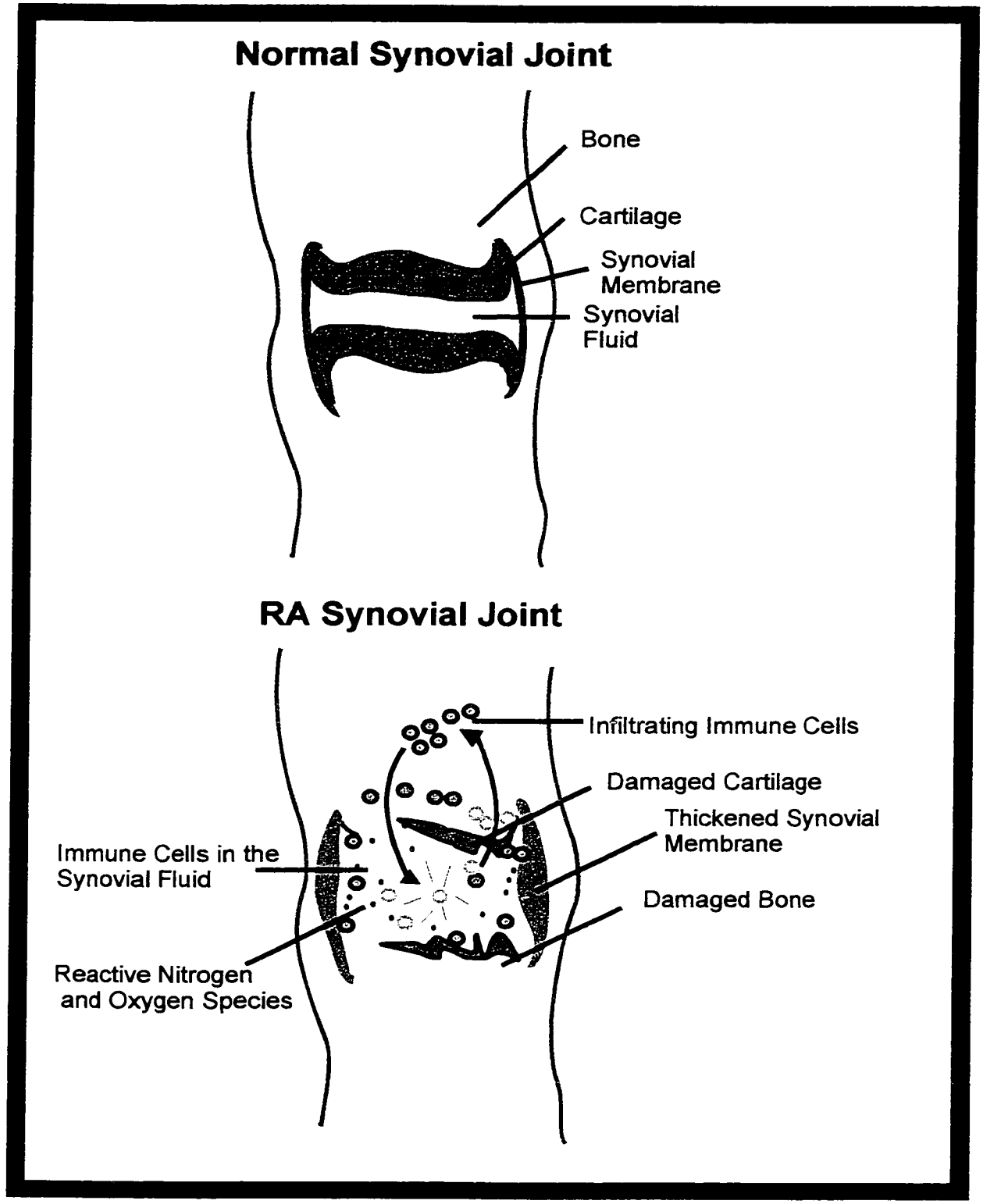
b) To determine whether nitrated proteins are present in RA and OA synovial homogenates by immunoblotting.

3.2.6 Significance

This research represents the first attempt to determine whether nitric oxide related species such as $ONOO^-$ are responsible for protein tyrosine nitration in the inflamed RA synovium. This form of chemical modification to a multiplicity of proteins is a form of damage that may contribute to cellular and tissue dysfunction. This aims to improve our current knowledge regarding the link between reactive nitrogen species, inflammation and progression of rheumatoid and other forms of arthritis. Improving our understanding

Figure 3.3: Hypothetical model of a normal and arthritic joint.

Most of the bones of the normal joints are capped with a smooth substance called cartilage. Between the bones is a joint cavity, which gives the bones enough space to move. A flexible capsule encloses the joint space between two bones. The inner lining of this capsule, the synovium, produces a thick fluid that nourishes the joint. In many forms of arthritis, the synovium becomes inflamed and thickened producing extra fluid, which contains inflammatory cells. Cells trafficking to and from the inflamed joints may produce reactive nitrogen and oxygen species that can damage the cartilage and the underlying bone.


Normal Synovial Joint
Bone
Cartilage
Synovial Membrane
Synovial Fluid
RA Synovial Joint
Infiltrating Immune Cells
Damaged Cartilage
Thickened Synovial Membrane
Immune Cells in the Synovial Fluid
Damaged Bone
Reactive Nitrogen and Oxygen Species

regarding mechanism of damage in arthritis will lead to improved design of treatments to prevent and treat inflammatory arthridites and related conditions.

3.3 Materials and Methods

3.3.1 Description of patients and controls

Synovial tissue was obtained from a patient group that consisted of 9 RA and 9 OA patients and 3 cadaver control subjects. The patients were followed at the Ottawa Hospital, General Campus. RA patients met the criteria of the American College of Rheumatology (ACR) (formerly, the American Rheumatism Association) (Arnett et al., 1988). The site of surgery, disease, age, duration, gender, and medication was noted when available. The patient's previous and current use of medication was also recorded. The control tissue was obtained from cadaver subjects with no known history of arthritis. The protocol for obtaining synovial tissue samples from patients and controls was approved by the institutional ethics review board. It should be noted that the patients studied in this chapter are different than the group studied in Chapter 2.

3.3.2 Synovial tissue processing

Synovial tissue (ST) was obtained during joint replacement surgery from group of patients with RA (n = 9), OA (n = 9) and control (n = 3). Synovial samples from RA and OA patients and cadaver were obtained from wrist, hip, knee, or shoulder joints. Briefly, each synovial specimen was cut with a sterile blade into 3 sections. Two sections were placed into cryovials, snap frozen in liquid nitrogen and placed into a –100°C freezer. The remaining synovial section was placed into 10% neutral buffered formalin and fixed

overnight. The following day the fixed tissue was placed into 70% ethanol and routinely paraffin embedded in the Department of Pathology at the University of Ottawa.

3.3.3 Nitrotyrosine immunohistochemistry

After routine paraffin embedding the tissues were cut into 4 μm sections, deparaffinized in toluene, dehydrated in ethanol and placed into H_20 for 5 min. Endogenous peroxidase activity was quenched in 3% H_2O_2 followed by washing in Tris buffered saline (50 mM Tris, 150 mM NaCl, pH 7.6). Next, non-specific IgGs were blocked with 1% normal swine serum, and 4% bovine serum albumin in TBS for 20 min. Sections were blotted and incubated with of 0.5 μg/ml of rabbit anti-nitrotyrosine polyclonal antibody (Upstate Biotechnology) in a humidified chamber at 4°C for 16 h. Sections were extensively washed in TBS and nitrotyrosine was detected using peroxidase labeled polymer conjugated to anti-mouse and anti-rabbit immunoglobulins (Dako Envision System) for 30 min at room temperature. After 3 washes in TBS, the antigen-antibody complex was visualized using a freshly prepared solution of diaminobenzidine (0.02%), H_2O_2 (0.06%) in TBS for 10 min in the dark.

The sections were counterstained in hematoxylin, dehydrated, cleared and mounted. All RA and OA samples were stained in a single procedure. Immunohistochemistry was repeated twice for some patients to ensure consistency in staining. For each tissue a positive and 3 specificity controls were performed: (1) tissues were directly treated with sodium hydrosulfite (Sigma), (2) omission of primary antibody, (3) addition of preabsorbed anti-nitrotyrosine antibody with 1 mM nitrotyrosine (Sigma) or N-BSA (10 μg/ml) for 1hr at room temperature. Positive control included

chemical nitration of tissues with H_2O_2 (1 mM) and $NaNO_2$ (1 mM) in acetate buffer (pH 5.2).

3.3.4 Semi-quantitative analysis of nitrotyrosine staining

The percentage of each tissue section that stained for nitrotyrosine was estimated from digital photomicrographs. (x200 power, 1600 x 1200 pixel) of 3 fields selected at random. Analysis for brown pixels was carried out with the aid of ImagePro Plus version 4.0 software (www.mediacy.com/ipe.htm) with colour sensitivity =4/5, colour selection = 1 x 1 pixel. A strongly staining tissue section was first selected to establish the minimum shade of brown (i.e. DAB precipitate) that would be considered positive. This template was then used to grade the amount of nitrotyrosine staining on all patients. The nitrotyrosine staining index is an estimate of percentage area staining for nitrotyrosine, with the maximum score being 100%. Percent tissue staining = total brown pixels/ total pixels * 100. Each slide was blinded to the observer

3.3.5 Statistical analysis

The nitrotyrosine staining index in tissue sections from RA synovia was compared to OA synovia using a 2-tailed Mann-Whitney U-test. Correlation. Statistical analysis was performed using GraphPad Prism version 3.02, www.graphpad.com. $P< 0.05$ was considered statistically significant.

3.3.6 Western blot analysis

Snap frozen tissue (stored at –100°C) was thawed and homogenized on ice in PBS (2.7 mM KCl, 1.5 mM KH_2PO_4, 140 mM NaCl, 8 mM Na_2HPO_4, pH 7.4) containing PMSF (1 mM). Extracts were diluted with SDS sample buffer (2% SDS, 10% glycerol, 1% 2-mercaptoethanol, 0.0005% bromophenol blue, 125 mM MOPs, pH 6.8) and heated at 100°C for 5 min. Protein was then quantified by a fluorescamine assay (Udenfriend et al., 1972). Protein samples (30 μg/lane) were resolved on a 12% SDS-PAGE at 200 V for 1 h, transferred to PVDF membrane at 15 V for 18 h in Dunn Buffer (3 mM Na_2CO_3, 10 mM $NaHCO_3$ and 10 % methanol). The membranes were Ponceau S stained to reveal the markers, washed twice in TBST (10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20, pH 8) followed by blocking in 1% BSA for 1hr at room temperature. The membrane was then incubated with 1.5 μg/ml of the anti-nitrotyrosine antibody for 1hr at room temperature. Membranes were washed 4 times in TBST and then incubated with a 1: 100 dilution of a biotinylated goat anti-rabbit secondary antibody (Vector Laboratories) for 1hr at room temperature. The membranes were washed 4 times in TBST and incubated with a 1:200 dilution of strepavidin-biotin peroxidase (Sigma) complex for 30 min. Membranes were washed 4 times in TBST and the antigen-antibody complex was visualized using in a solution of DAB (0.08%) and H_2O_2 (0.4%) for 5 min. The reaction was stopped with PBS. N-BSA (20 ng) served as a positive control.

3.3.7 BSA nitration

Nitrated bovine serum albumin was prepared by allowing BSA (6.3 mg/ml) to react with $NaNO_2$ (10 mM), $FeCl_3$ (9 μM), H_2O_2 (0.3%), and sodium acetate (16.7mM)

(pH 5.6) at room temperature for 18 h. The reaction resulted in a yellow solution containing nitrated BSA (N-BSA). The N-BSA was precipitated with 4 volumes of ethanol at –20°C for 18 h and resuspended in 16.7 mM sodium acetate and used fresh.

3.4 Results

3.4.1 Patient characteristics

Synovial tissue was obtained from a group of 9 RA and 9 OA patients that were followed at the Ottawa hospital, General Campus. Table 3.1 summarizes the average age, duration, site of surgery, gender and medication used at the time of analysis of the patient group. The average age of the RA patients (63.1 ± 10.7 years) did not differ significantly from the average age of OA (70.0 ± 9.0 years) and controls (64.3 ± 10.5 years). The RA patient group consisted mostly of females with average disease duration of 16.0 ± 8.1 years. The OA group also consisted primarily of female patients. Information concerning the disease duration of OA patients was not available. All of the RA patients were on anti-arthritic medications compared none in the OA group.

3.4.2 Synovial tissue characteristics

RA and OA synovia showed heterogeneity in terms of inflammation, angiogenesis, hyperplasia and fibrosis. Some RA synovia showed marked hyperplasia and/or inflammation evidenced by the presence of lymphocyte aggregates, monocytes and neutrophils. Other RA samples showed sparse cellular infiltrate and/or synovial hyperplasia. OA synovium displayed some proliferative changes in the synovial lining and a lower degree of inflammation as compared to RA.

Table 3-1: Characteristics of the rheumatoid arthritis (RA) patients, osteoarthritis (OA) patients and controls.

Age and duration are given as the mean in years ± standard deviation. Sex, site of surgery and medication is reported as well. The site of surgery was not available (N/A) for one RA patient. Some RA patients were on more than one type of drug.

	RA (n=9)	OA (n=9)	Control (n=3)
Age (years) ± SD	63.1 ± 10.7	70.0 ± 9.02	64.3 ± 10.5
Disease Duration (years) ±SD	16.0 ± 8.14	NA	NA
Gender	F = 6 M = 3	F = 6 M = 3	F = 2 M = 1
Site of Surgery	7 Knee 1 Hip 1 N/A	8 knee 1 toe	3 Shoulder
Current Medication (Number of Patients)			
methotrexate	6	0	0
folate	5	0	0
plaquenil	5	0	0
steroid	5	0	0

3.4.3 Nitrotyrosine is detected in the RA and OA synovium

Nitrotyrosine staining was detected in the synovial tissue of RA patients and to a lesser extent in OA patients and control cadaver samples. In all cases a positive and negative control was included to confirm the specificity of the antibody. Immunolabeling detected nitrated proteins throughout the synovium including the extracellular matrix (Figure 3.4a and b). Both cytoplasmic and nuclear staining was observed in different cell types in the RA synovium. Some lymphocytes were stained while others were not (Figure 3.4b, and c). Intense cytoplasmic staining was observed in most macrophages (Figure 3.4d) and no staining was seen in neutrophils (Figure 3.4e) except in the chemically nitrated controls. Specificity of staining was shown by the reduction of nitrotyrosine to aminotyrosine with sodium hydrosulfite (Figure 3.4f). Serial sections from RA patient 26f show that preabsorption of the polyclonal anti-nitrotyrosine antibody with N-BSA shows no staining, while immunostaining shows the presence of protein nitrotyrosine in the stroma, macrophage and some lymphocytes (Figure 3.5a and b). As expected, the most intense staining occurred in our positive control utilizing the chemical nitration of proteins in the synovium. Strong protein nitrotyrosine staining was also detected in endothelial cells (Figure 3.5c). Tissues from RA patients stained more extensively for nitrotyrosine than OA patients (median = 18.8 versus 11.8%, Figure 3.6). This difference is statistically significant (Mann-Whitney U-test; 2-tailed $p<0.008$). Too few control cadaver specimens were available to include in the statistical analysis. On general observation, nitrotyrosine staining was greater in tissues that had prominent inflammatory features. However, whether nitrotyrosine staining correlates with degree of inflammation remains to be determined.

Figure 3.4: Widespread nitrotyrosine immunostaining in an RA Patient.

Staining of synovium from RA patient 50b for nitrotyrosine. (**A**) Region demonstrating intense stromal staining; (**B**) staining of vascular endothelium (short thick arrow) and perivascular regions (thin arrows); (**C**) lymphoid aggregate (arrow) in which some lymphocytes are stained and others are not; (**D**) macrophages showing intense staining (arrows); (**E**) arrows indicate absence of staining of neutrophils, **f** indicates unstained fibrin and **s** indicates strongly stained stroma; (**F**) negative control, *in situ* chemical reduction of nitrotyrosine to aminotyrosine. A-C, F, × 100 total magnification; D-E, × 400 total magnification. Other details in Materials and Methods.

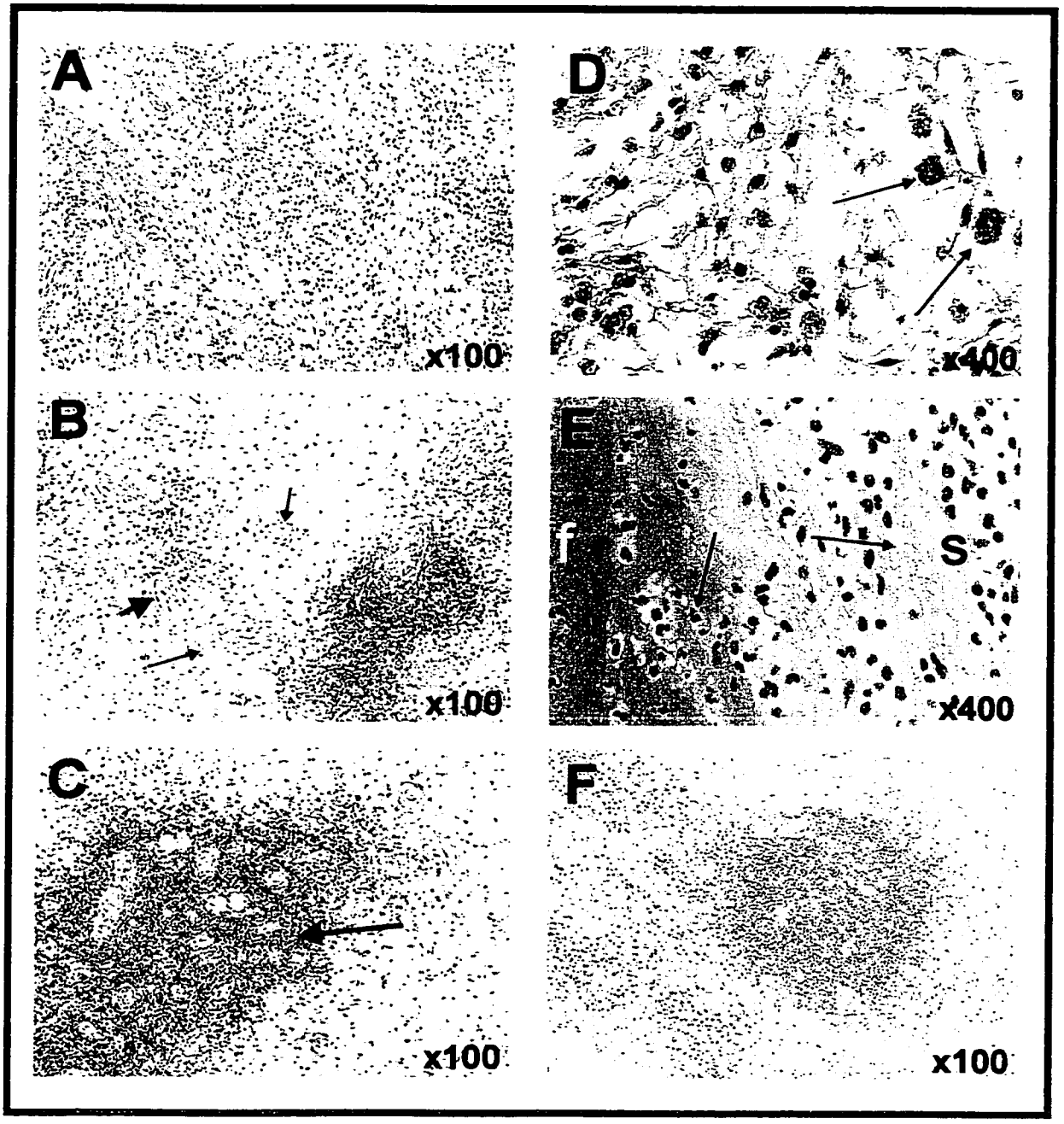

A
x100
D
x400
B
x100
E
f
S
x400
C
x100
F
x100

Figure 3.5: Nitrotyrosine immunostaining of different RA patients compared to staining in OA and Control.

Representative examples of synovia stained for nitrotyrosine. (**A**) non-arthritis subject 279, cadaver; (**B**) osteoarthritis patient 70; (**C**) RA patient 55b; (**D-E**) serial sections of synovium from RA patient 26F. (**D**) negative control, competitive inhibition of anti-nitrotyrosine antibody with nitrated BSA; (**E**) synovium stained for nitrotyrosine; (**F**) positive control, *in situ* chemically nitrated tissue section. A-C, ×100 total magnification; D-E, ×400 total magnification. Other details in Materials and Methods.

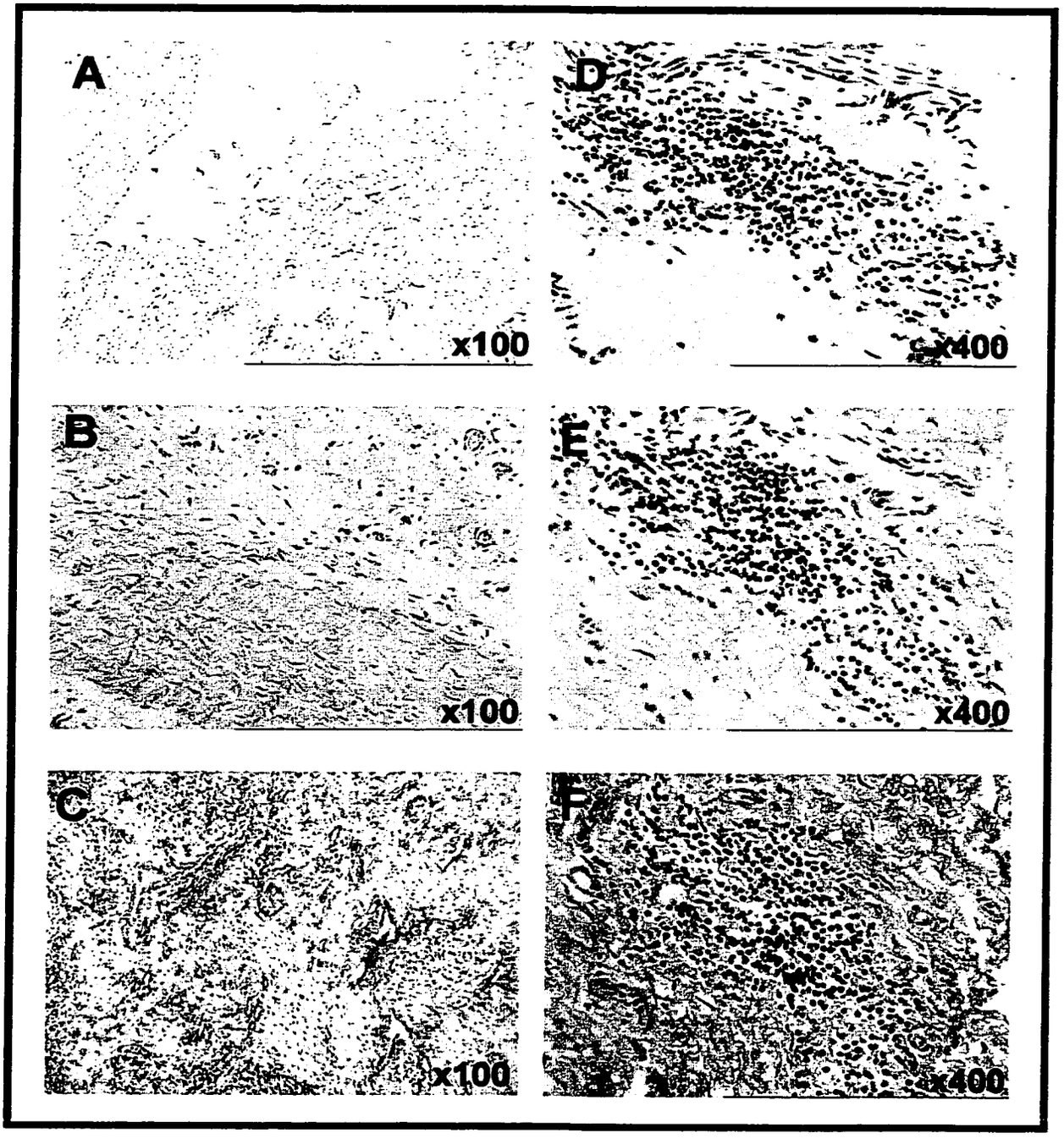
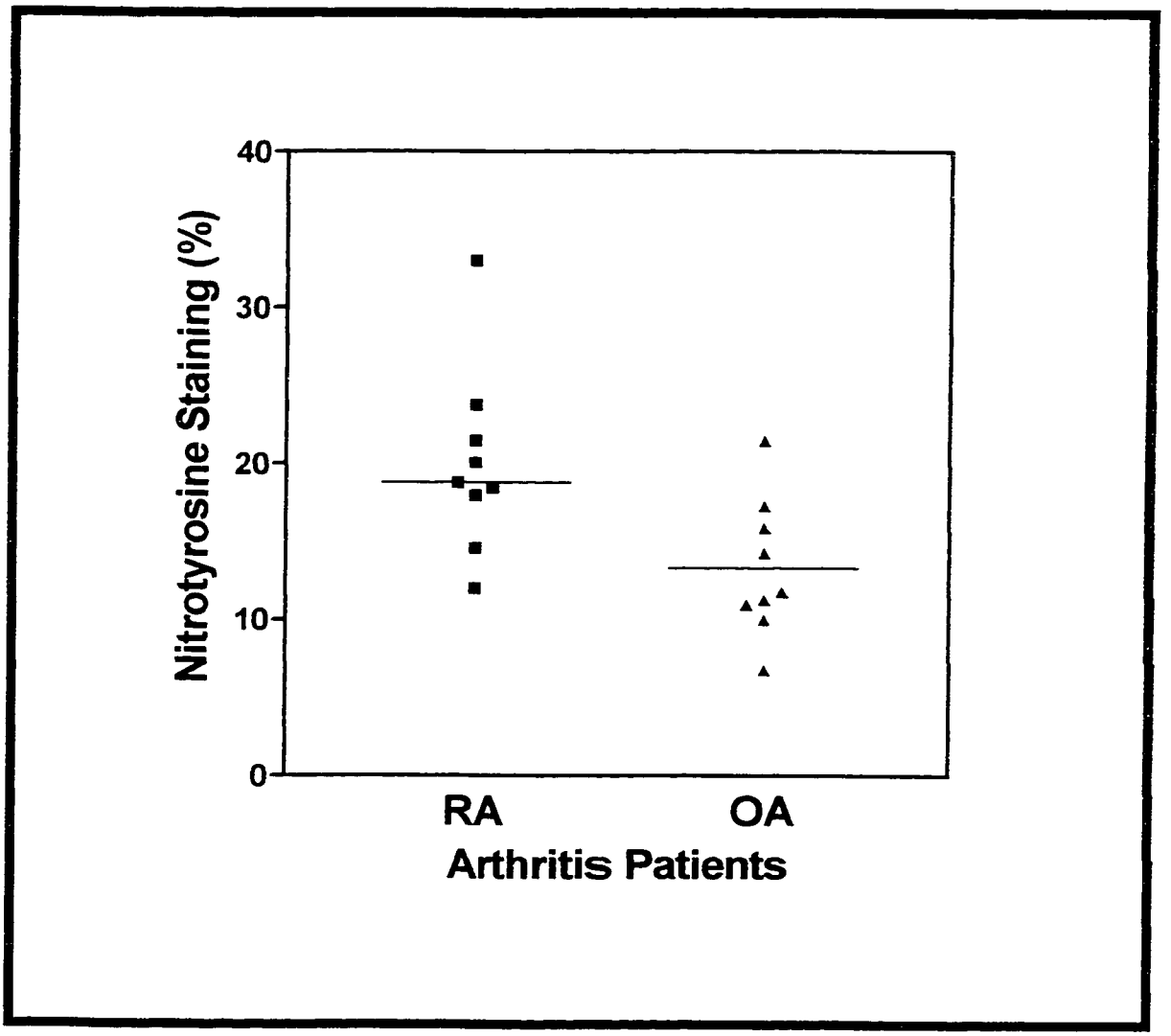

A
x100
D
x400
B
x100
E
x400
C
x100
F
x400

Figure 3.6: Percentage of area of RA and OA synovial tissue sections that stain for nitrotyrosine

Image analysis of stained sections was carried out as described in Materials and Methods. Each symbol represents tissues from a single patient; the bar represents the median for each disease group. Tissues from RA patients stained more extensively than OA patients (median = 18.8 versus 11.8%). The difference is statistically significant (Mann Whitney U test; 2 tailed $p < 0.008$). Too few control cadaver specimens were available to include in the statistical analysis.

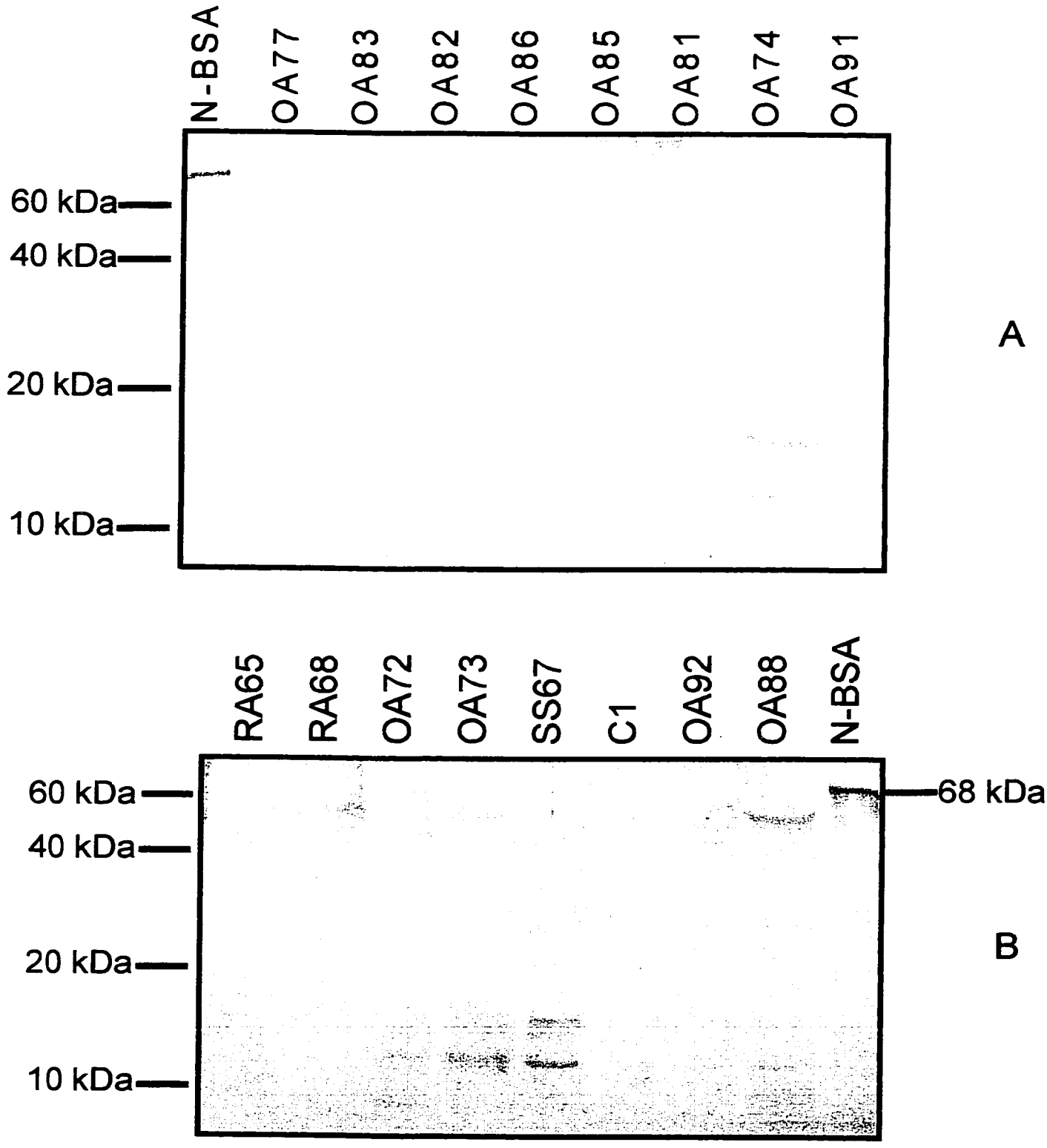

Nitrotyrosine Staining (%)
40
30
20
10
0
RA
OA
Arthritis Patients

3.4.4 Western Blot analysis detects nitrated proteins in RA and OA homogenates

To confirm our immunohistochemistry results, Western blot analysis was performed on RA and OA synovial homogenates using the polyclonal antibody directed against nitrotyrosine. Tyrosine nitration of several proteins in RA and some OA synovial homogenates was visualized by Western blot analysis (Figure 3.7a and b). The banding pattern was not the same between patients. Some high molecular mass proteins (60-70 kDa) were observed in RA 68 and OA 88. Several lower molecular mass proteins or protein fragments (10-20 kDa) are observed in OA patient 72, 73, 74 and 91. Our control patient (C1) and some OA homogenates (82, 86, 85) displayed very little immunoreactivity in synovial homogenate extracts. Nitrotyrosine immunoreactivity was readily visualized with 20 ng of nitrated bovine serum albumin (N-BSA), which indicated no technical problem with the detection of nitrotyrosine by Western blot analysis. Overall our Western blot results are keeping with the results of immunohistochemistry reported here in that our *in situ* work suggests that a variety of proteins are nitrated.

Figure 3.7: Western blot analysis for nitrotyrosine in synovial tissue homogenates from RA and OA patients.

A total of 30 μg of protein for each RA and OA homogenate was migrated on a 12% SDS-PAGE and transferred to PVDF membrane and probed with 1.5 μg/ml anti-nitrotyrosine antibody. Note the tyrosine nitration of several proteins in RA and some OA synovial homogenates. Some high molecular mass proteins (60-70 kDa) are observed in RA 68 and OA 88. Several lower molecular mass proteins or protein fragments (10-20 kDa) are observed in OA 72, 73, 74 and 91. Some patients (OA 82, 86, 85) displayed very little immunoreactivity in synovial homogenate extracts. Immunoreactivity with 20 ng of nitrated bovine serum albumin (N-BSA) indicates no technical problem with the detection of nitrotyrosine by Western blot analysis.


N-BSA
OA77
OA83
OA82
OA86
OA85
OA81
OA74
OA91
60 kDa
40 kDa
20 kDa
10 kDa
A
RA65
RA68
OA72
OA73
SS67
C1
OA92
OA88
N-BSA
60 kDa
40 kDa
20 kDa
10 kDa
68 kDa
B

3.5 Discussion

Nitric oxide is a molecule fundamental to mechanisms of normal immunity, neurotransmission and vasodilation, but at higher levels it may contribute to the pathogenesis of autoimmune diseases. Definition of the molecular basis of NO generation and its importance in contributing to joint destruction in arthritic diseases has progressed during the last decade. The generation of NO is regulated by several cellular stimuli. Cytokines are potent inducers of NO production via the iNOS pathway. IL-1β or TNF-α, which are abundantly produced at inflammatory sites such as that of the diseased RA synovium, contributes to the relatively high levels of NO in the synovial joint. Other radical species such as O_2^- (also produced at sites of inflammation) react with NO to form $ONOO^-$ which nitrates free or protein associated tyrosine residues forming nitrotyrosine. The fact that protein nitrotyrosine has been detected in various immune disorders and some animal models of arthritis prompted us to investigate whether this type of protein damage is present in the inflammatory environment of the synovial joint of arthritic patients. To this end we examined protein nitration in the RA synovium since it is an inflammatory environment where high levels of NO play a major role in the pathogenesis of the disease.

The results presented in this chapter indicate that NO mediated damage is present throughout much of the inflamed synovial tissue and a myriad of proteins may be nitrated in different cell types and the extracellular matrix of the inflamed synovial tissue. This is evidenced by Western blot detection and immunohistochemistry where intense nitrotyrosine staining was observed in mast cells, macrophages, endothelial cells, some lymphocytes and synoviocytes in RA samples. Diffuse staining of the extracellular matrix

was observed in most specimens as well. This was not due to background staining since our controls abrogated all nitrotyrosine staining in both RA and OA samples.

As indicated, we have observed protein nitrotyrosine in the extracellular matrix of sample collected from both RA patients and OA patients. Nitrotyrosine staining has been observed in the extracellular matrix of patients stricken with gastric cancers (Goto et al., 1999). In addition, Buttery and coworkers (1995) showed that in advanced atherosclerotic lesions nitrotyrosine staining was not only evident in the inflammatory cells and smooth vascular endothelial muscle but also throughout the fibrous tissue (Buttery et al., 1996). Moreover, nitrotyrosine staining was observed in the extracellular matrix of an animal model of arthritis (Szabo et al., 1998). The fact that the extracellular matrix stained may have a plausible explanation. One possibility is that tyrosine rich acidic matrix proteins (TRAMPs) are potential target of peroxynitrite-mediated damage. TRAMPs are essential 22 kDa proteins that help collagen fold into fibrils and are abundant in tissues that are relatively rich in extracellular matrix such as lung, skin and joint tissue (Forbes et al., 1994). Evidently, the reason TRAMPs would be nitrated is because they are tyrosine rich. Since nitrotyrosine modification has been shown to affect protein functions (Eiserich et al., 1999), TRAMPs function may also be affected.

Macrophage, endothelial cells and lymphocytes in lymphocyte aggregates stained for nitrotyrosine in RA tissues. Stimulated macrophage and endothelial cells are capable of producing high amounts of $ONOO^-$ that leads to nitrotyrosine formation. Our results agree with a recent study that showed that human lymph nodes taken from surgical resections of lung and colon also stained for lymphocytes, macrophage (most intense) and the interstitium (Brito et al., 1999). At present, the role of protein nitration in

lymphocytes in the RA synovium remains unclear, though it has been suggested that it can be a down modulator of immune responses by interfering with signal transduction and potentially lead to lymphocyte turnover (Brito et al., 1999).

Neutrophils are a potential source of toxic reactive oxygen and/or nitrogen species. Controversy exists whether human neutrophils express iNOS and can subsequently generate $ONOO^-$. For example, immunohistochemical studies have localized iNOS in neutrophils *in situ* in human cerebral infarcts and *in vitro* after cytokine stimulation (Forster et al., 1999; Evans et al., 1996). However, in human lung adenocarcinomas iNOS was localized to macrophage while absent in neutrophils (Birnboim and Sandhu, 2000). In correlation with the latter, protein nitrotyrosine was not detected in neutrophils of the RA synovium except in the positive controls. This contrasts with the observation that in murine neutrophils NO is produced, as demonstrated by the immunohistochemical detection of iNOS and nitrotyrosine (Sandhu et al., 2000). The possibility that human neutrophils may have a way to scavenge peroxynitrite that enters the cell might also help explain why nitrotyrosine is absent. For example, high levels of intracellular antioxidant molecules such as glutathione and superoxide dismutase may be present in activated human neutrophils in the synovium, which scavenge RNOS.

OA tissues stained for nitrotyrosine but to a lesser extent than in RA. Although OA has been primarily considered a non-inflammatory disease, secondary inflammation is hypothesized to occur as a result of tissue damage (Haraoui et al., 1991). Consequently, enhanced expression of iNOS, NO metabolites and inflammatory cytokines such as IL-1β, TNF-α, have been demonstrated in patients with OA. Moreover, evidence of

nitrotyrosine has been demonstrated in the chondrocytes and inner and outer meniscus of experimentally induced OA in rabbits and dogs (Hashimoto et al., 1999; Pelletier et al., 1999). Therefore, the presence of nitrotyrosine in the OA synovium was not an unexpected result.

The detection of nitrotyrosine in the inflammatory environment of the RA synovium and the concomitant expression of nitrated proteins in the synovial homogenates of arthritis patients further implicates NO as playing a role in the pathogenesis of RA. Buttery et al. (1994) reported that homogenates of atherosclerotic aorta revealed a number of nitrated proteins on Western blots whereas only a few bands were evident in normal aorta (Buttery et al., 1996). Multiple lower and upper molecular mass proteins were detected in mouse paw extracts of collagen-induced arthritis (Szabo et al., 1998). We report that tissue homogenates from RA and OA patients indicate that some lower and upper molecular mass proteins are nitrated *in vivo*.

The lower molecular weight bands ran in the range of 10 to 20 kDa. These bands may represent nitrated histone proteins, which typically run in this range. Recently, it has been shown that histone proteins can be nitrated *in vitro* by peroxynitrite as revealed by very strong nitrotyrosine immunoreactivity (Kuo et al., 1999). This reactivity was absent in other proteins including caspase 3 and ubiquitin treated with equal amounts of peroxynitrite indicating that histone nitration maybe dependent on the greater access to tyrosine residues. Moreover, in the Mutatect murine tumor model developed in our laboratory (Birnboim et al., 1999), nitrotyrosine immunoreactivity in histone proteins was detected by Western blot analysis (Haqqani and Birnboim, 2000). Histone modification

such as tyrosine nitration could hypothetically contribute to gene deregulation and lead to the increased levels of certain oncogenes in RA (Roivainen et al., 1996).

Though not as prominent, the upper molecular mass proteins ran in the range of 40 to 60 kDa. Many proteins resolve in this range including p53. As indicated in chapter 2, p53 is a tumor suppressor protein that serves as a critical regulator of cell proliferation and apoptosis. P53 overexpression has been well documented in the RA synovium (Firestein et al., 1996; Tak et al., 1999). Loss of p53 function through somatic mutations of the p53 gene has been suggested to contribute to increased levels of p53 seen in RA and potentially resulting in synovial hyperplasia (Firestein et al., 1997). However, an additional explanation for loss of p53 function in RA synovium could be through protein damage such as p53 nitration. Recently, it was shown that NO can nitrate tyrosine residues on the p53 protein in a human breast cancer cell line (MCF-7) (Chazotte-Aubert et al., 2000). This change has been shown to alter the conformation of p53 and significantly decrease its sequence-specific DNA binding and transcription factor activity (Calmels et al., 1997). In the RA synovium, nitrated p53 could in part lead to a hallmark of RA, synovial hyperplasia. However, whether p53 is nitrated and leads to synovial hyperplasia in RA remains to be examined in future studies. Although the identities of the nitrating agent and nitrated proteins have yet to be determined, the Western blots illustrate that a range of proteins including the possibility of histones, and p53 are susceptible to nitration in RA and OA synovium.

In summary, the results in this chapter provide the first *in situ* evidence that nitrated proteins are present in most of the synovial tissue of patients with RA and to a lesser extent OA. Moreover, these findings strengthen the body of evidence indicating

that RNOS are present in the inflamed joints of these patients and may represent major mediators in inflammation mediated protein modification and tissue injury at sites of chronic inflammation. Although we have not elucidated which proteins are nitrated, the results show generation of nitrating species *in vivo* of patients with RA and OA.

3.6 Future Direction

In future work, we will need to confirm our data in a larger series of RA and OA patients and include control synovium from joint trauma victims which we did not have access to during this study and perform correlations with current and past treatments, clinical correlations, and, correlations with degree of inflammation in the synovial specimens. Furthermore, similar studies in early RA (i.e., not only at time of joint replacement, which is late in the course and damage may have occurred in the remote past) would also be helpful for elucidating mechanisms early on in this disease.

The immunohistochemistry and Western blot data suggest the potential nitration of many proteins. To identify suspected nitrated proteins one could immunoprecipitate synovial homogenates from RA, OA and controls with a commercially available monoclonal anti-nitrotyrosine antibody. The nitrotyrosine immunoprecipitate can be subsequently resolved using 2 dimensional gel electrophoresis. Finally the nitrated proteins can be identified using mass-spectroscopy or by Western blot analysis. I have suggested that several important proteins such as p53, TRAMPs and histones could be nitrated. If confirmed, this would strengthen the important role of nitric oxide mediated pathway in the pathogenesis of RA including a role for these proteins in the disease process.

As indicated, protein nitration may be injurious via multiple mechanisms, including altered protein structure and function, increased turnover and formation of neoantigens with eventual autoimmune reactions. Since the consequence of protein nitration in the synovial joint is unknown, current work is being conducted testing the hypothesis that nitrotyrosine containing protein(s) can act as autoantigens by trying to detect the presence of anti-nitrotyrosine antibodies in the serum and synovial fluid of RA patients as compared to controls. A cycle of inflammation could be established leading to synovial joint destruction. Thus having identified nitrotyrosine as possible key player may lead to new therapies that effectively reduce inflammation while limiting toxic side effects.

CHAPTER 4: GENERAL DISCUSSION AND CONCLUSIONS

The important determining features of normal tissues homeostasis are the balance between proliferation and cell death including apoptosis. In diseased RA tissue, this balance appears to be altered either by increased proliferation and/or decrease in apoptosis. In previous studies, the rate of apoptosis was studied mainly in RA and OA synovium but was not compared to control non-arthritic synovium. If decreased apoptosis is a main contributor to synovial hyperplasia then one would expect to see a lower rate of apoptosis in RA as compared to OA and control. However, by using specific immunohistochemical markers for apoptosis, PARP p85 and activated caspase 3, we determined that the rate of apoptosis was similarly low in RA, OA and control synovium. This leads to the conclusion that increased proliferation coupled with normal low rates of apoptosis could be responsible for synovial thickening. Therefore, using immunohistochemistry techniques, we used several markers, including PCNA, Ki-67 and TS to determine the rate at which the synovial lining is proliferating. TS provided us with the advantage of resolving a long-standing disparity between Ki-67 and PCNA proliferation labeling indices in the RA synovium. We determined high PCNA labeling indices of the synovial lining in RA, OA and control synovium. The latter is not known to extensively proliferate. We determined that TS and Ki-67 proliferation labeling indices were significantly higher in RA and OA as compared to control synovium indicating a modest increase in the rate of proliferation of the synovial lining. The high PCNA labeling index did not correlate with that of Ki-67 or TS, nor did it correlate with the very low level of mitotic figures found in these synovial samples. In addition, PCNA extensively stained the control synovium. Recently, the revelation that PCNA serves

several roles in the cell cycle confounds its use as a proliferation marker. Taken together, we confirmed that TS could be used as a marker of proliferation, in that it helped determine that a modest increase in cell proliferation coupled with a normal low rate of apoptosis contributes to synovial hyperplasia. In addition, we put to question the use of PCNA in future proliferation studies.

Accumulation and proliferation of lymphocytes, synovial cells and the abundant production of RNOS, cytokines and degenerative enzymes characterize the pathogenesis of rheumatoid synovial inflammation and joint destruction. There is increasing evidence that reactive nitrogen and oxygen species are present in the inflamed RA joint and to a lesser extent in OA joints. The role that these reactive species play in disease progression is not well understood. RNOS is known to mediate genetic damage (Firestein et al., 1997) and is known to damage protein by nitrating tyrosine residues to form nitrotyrosine. The work reported in this thesis emphasizes that protein modification does occur in the RA and OA synovium as evidenced by the detection of nitrated proteins in the synovium of RA and OA patients in a variety of cell types including the extracellular matrix. Based on extracellular matrix staining we hypothesize that TRAMPs (22 kDa protein that helps fold collagen into fibrils) could be potential targets of nitration. Western blot analysis also showed multiple low and high molecular mass proteins in some RA and OA synovial homogenates. We speculate that the low mass proteins in our Western blot could represent histone proteins. This would agree with immunohistochemistry results as nuclear staining was observed in the RA synovium. Histone nitration could represent another means by which genes are regulated and conceivably lead to increased expression of certain oncogenes (Roivainen et al., 1996). However, nitrated proteins were not

detected in the 20-30 kDa range which would have suggested the nitration of TRAMPs. Future studies will address whether TRAMPs, and/or histones are preferentially nitrated and their role in RA pathology. Although the identity of nitrated proteins in this study is unknown, our results support the notion that peroxynitrite and other RNOS capable of nitrating proteins are detected *in situ* in the inflamed synovium and may potentially damage important proteins/enzymes such as histones, p53 and TRAMPs thereby leading to pathological conditions. Furthermore, my study strengthens the argument that NO is a major mediator in the pathogenesis of RA.

Overall, this study is novel in that it addresses the mechanisms involved in synovial hyperplasia and the consequence of RNOS in the synovial joint; both of which are major contributors to arthritic disease. We have observed that a modest increase proliferation coupled with normal low levels of apoptosis may be the underlying mechanism of synovial hyperplasia in rheumatoid arthritis. The presence of nitrotyrosine indicates that NO and its related species are capable of damaging proteins. Since NO is elevated in diseases other than RA, a more detailed understanding of the relationship between NO and protein damage may lead to greater understanding of its role in the pathology of arthritic diseases. Since protein damage may be important in RA pathogenesis, this study provides further evidence that targeting NO formation may have therapeutic benefits in this disease.

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