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**CHARACTERIZATION OF PROSTAGLANDIN ENDOPEROXIDE
H SYNTHASE-1 ENZYME EXPRESSION DURING THE
DIFFERENTIATION OF THE MEGAKARYOCYTIC CELL LINE,
MEG-01**

Cameron Mroske

A thesis submitted in conformity with the requirements for
the degree of Master of Science
Graduate Department of Biochemistry, Microbiology and Immunology
University of Ottawa

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ABSTRACT

Characterization of Prostaglandin Endoperoxide H Synthase-1 Enzyme Expression During the Differentiation of the Megakaryocytic Cell Line, MEG-01

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The formation of thromboxane A₂ (TXA₂), a potent aggregatory agent, is dependant upon the rate-limiting enzyme prostaglandin endoperoxide synthase-1 (PGHS-1). The specific objectives of my Master's project have been two-fold: to characterize the expression of PGHS-1 protein and mRNA within the context of megakaryopoiesis, and to identify growth factors capable of inducing such expression.

We induced MEG-01 cells to differentiate into platelet-like structures by treating them with TPA (12-0-tetradecanoylphorbol-13-acetate). MEG-01 cultures exist as nucleated cells that float, nucleated cells that adhere, and anucleated platelet-like structures. We found that PGHS-1 protein levels were highest in the platelet-like population whereas PGHS-1 mRNA levels were greatest in the adherent population. Although TPA induced PGHS-1 expression in all three populations, the relative amounts of PGHS-1 expressed between the three fractions remained unchanged. Addition of TPA (1.6×10^{-8} M) caused a time-dependant increase in the percentage of MEG-01 cells that expressed PGHS-1 protein. The percentage of MEG-01 cells expressing PGHS-1 protein was greater in the adherent populations than in the floating. Once stimulated, only the viability of the nucleated floating MEG-01 cells decreased. We assessed the differentiation of MEG-01 cells by measuring the expression of the surface antigens glycoprotein IIb/IIIa (GP IIb/IIIa) and glycoprotein Ib (GP Ib). A high percentage of control adherent MEG-01 cells labeled strongly for GP IIb/IIIa. TPA stimulation did not increase this percentage. In contrast, the expression of GP Ib was undetectable in control cells, and increased slightly in the TPA-treated adherent population.

We screened a number of recombinant hematopoietic factors for the ability to induce PGHS-1 protein expression in MEG-01 cells. We found that the combinations IL-3/IL-11/GM-CSF/SCF/TPO, IL-11/GM-CSF/SCF/TPO, IL-11/GM-CSF/TPO, IL-6/IL-11/GM-CSF/SCF, IL-6/IL-11, IL-3/IL-6, and IL-6/GM-CSF could induce PGHS-1 protein expression, but only when incubated with MEG-01 cultures that consisted solely of adherent cells. Over the course of eight days, the combination of IL-6 and IL-11 stimulated adherent MEG-01 cells to increase their expression of PGHS-1 protein. In adherent MEG-01, this combination also stimulated a 7-fold increase in PGHS-1 mRNA. In conclusion, we have revealed that PGHS-1 protein and mRNA expression correlate strongly with megakaryocyte differentiation, and that certain cytokines can act in concert to stimulate adherent MEG-01 cells to increase their expression of PGHS-1 protein. The combination of IL-6 and -11 not only stimulates MEG-01 cells to increase their expression of PGHS-1 protein, but also PGHS-1 mRNA.

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LIST OF ABBREVIATIONS

ATCC: American Type Culture Collection

BFU-Mk: burst-forming unit-megakaryocyte

BSA: Bovine serum albumin

CAMP: cyclic 3',5'-monophosphate

CCD: Charged-coupled device

CD: Cluster designation

CFU-Mk: Colony-forming unit-megakaryocyte

CML: Chronic myeloid leukemia

Cox-1: Cyclooxygenase-1

Cox-2: Cyclooxygenase-2

CSF: Colony stimulating factor

CTAP: Connective tissue activating peptide

DAPI: 4',6-diamidino-2-phenylindole

DMS: Demarcation membrane system

DMSO: Dimethylsulphoxide

ECM: Extracellular matrix

EI: enzyme-inhibitor

EPO: Erythropoietin

ER: Endoplasmic reticulum

Ets: E-twenty-six

FBS: Fetal bovine serum

FOG: Friend of GATA

G-CSF: Granulocyte colony-stimulating factor

GI: Gastrointestinal

GM-CSF: Granulocyte/macrophage colony-stimulating-factor

GP Ib: Glycoprotein Ib

GP IIb/IIIa: Glycoprotein IIb/IIIa

GP V: Glycoprotein V

GP IX: Glycoprotein IX

GP 130: Glycoprotein 130

GSH: Glutathione

15R-HETE: 15*R*-hydroxy-eicosatetranoic acid

hPGHS-1: Human prostaglandin endoperoxide synthase-1

hPGHS-2: Human prostaglandin endoperoxide synthase-2

HMWK: High-molecular weight kininogen

HUVECs: Human umbilical vein endothelial cells

IL-1 α : Interleukin-1 α

IL-1 β : Interleukin-1 β

IL-2: Interleukin-2

IL-3: Interleukin-3

IL-6: Interleukin-6

IL-6R: Interleukin-6 receptor

IL-11: Interleukin-11

IL-11R: Interleukin-11 receptor

JAKs: Janus family of protein tyrosine kinases

LIF: leukemia inhibitory factor

MALT: mucosa-associated lymphatic tissue

MAP kinase: Mitogen-activated kinase

Mk-HPP-CFC: high-proliferative-potential colony-forming cell

MPO: Myeloperoxidase

MRC: Medical Research Council

NE: Nuclear envelope

NF-E2: Nuclear factor E2

NF-IL-6: Nuclear factor interleukin-6

NF- κ B: Nuclear factor κ B

NSAID: Non-steroidal anti-inflammatory drug

PAI-1: Plasminogen activator inhibitor-1

PBS: Phosphate buffer solution

PCAM-1: Platelet and endothelial cell adhesion molecule-1

PDECGF: Platelet-derived endothelial cell growth factor

PEA: Phenylethylamine

PF 4: Platelet factor 4

PGD₂: Prostaglandin D₂

PGE₂: Prostaglandin E₂

PGF: Prostaglandin F

PGF_{2 α} : Prostaglandin F_{2 α}

PGG₂: Prostaglandin G₂

PGH₂: Prostaglandin H₂

PGH-PGE: Prostaglandin H-Prostaglandin E

PGHS-1: Prostaglandin endoperoxide synthase-1

PGHS-2: Prostaglandin endoperoxide synthase-2

PGI₂: Prostacyclin

PGI synthase: Prostacyclin synthase

PI: phosphotidyl inositol

PI 3-kinase: Phosphatidylinositol 3-kinase

PKC: Protein kinase C

PLA₂: Phospholipase A₂

PLC: Phospholipase C

PMkB: Promegakaryoblast

PRPP: Plasma-rich platelet protocol

PRI: Propidium iodide

RLP: Recirculating lymphocyte pool

RPMI 1640: Roswell Park Memorial Institute 1640

SCCS: Surface connected canalicular system

SCF: Stem cell factor

SDS: Sodium dodecylsulphate

SDS-PAGE: Sodium dodecylsulphate-polyacrylamide gel electrophoresis

SSC: Standard saline citrate

STATs: Signal transducers and activators of transcription

TBS: Tris buffer solution

TEMED: N', N',N',N'-tetramethylethylene diamine

TGF beta: Transforming growth factor beta

TLC: Thin layer chromatography

TPA: 12-0-tetradecanoylphorbol-13-acetate

TPO: Thrombopoietin

TXA₂: Thromboxane A₂

TXB₂: Thromboxane B₂

TXS: Thromboxane synthase

VnR: Vitronectin receptor

vWF: von Willebrand factor

SECTION ONE: INTRODUCTION

1.1 RATIONALE, OVERALL GOAL AND EXPERIMENTAL OBJECTIVES

The eicosanoids, which include the prostaglandins, thromboxanes, leukotrienes, and epoxides, constitute a chemical family of oxygenated fatty acids that are enzymatically derived from arachidonate. The cyclooxygenase, lipoxygenase, and P-450 pathways, collectively known as the arachidonate cascade, are the three routes by which eicosanoids are formed. Together, prostaglandins and thromboxanes make up the prostanoids, a group of hormones that possesses the ability to exert profound physiological effects at extremely low concentrations (1). Some examples of prostanoid mediated physiological events include the inflammatory response, cyto-protection of the gastric mucosa, the regulation of renal blood flow, and thrombosis and hemostasis (2).

Prostanoids are formed in most mammalian tissues by the enzyme Prostaglandin Endoperoxide H Synthase (PGHS), also known as cyclooxygenase (cox). Two PGHS isozymes, PGHS-1 (cox-1) and PGHS-2 (cox-2), are known to exist (3,4), and each is encoded by a different gene located on a separate chromosome (5,14). The major difference between the two PGHS isozymes lies in their dissimilar regulation of expression. For instance, PGHS-2 is undetectable in most resting tissues (6). However, in response to physiological conditions such as inflammation (7,8), ovulation (9-11) and mitogenesis (12), PGHS-2 expression can be induced. PGHS-2 is therefore referred to as the 'inducible' form of the enzyme. In contrast, PGHS-1 can be detected in most tissues; thus it is known as the constitutive enzyme (6). The importance of PGHS becomes

apparent when one considers that it is the pharmacological target for aspirin in the prophylaxis and therapy of cardiovascular disease. Aspirin is the most cost-effective drug for the treatment of cardiovascular disease because it derives its anti-thrombogenic activity by irreversibly inhibiting platelet PGHS-1 (13-15).

Blood platelets are the anucleated cells responsible for producing the coagulant prostanoid thromboxane A₂ (TxA₂). These cells represent one of the richest sources of PGHS-1 protein in the human body (16). Platelet protein expression, however, is the result of gene expression in a precursor cell known as the megakaryocyte. Megakaryocytes, which are found in the bone marrow, release platelets into the circulation during the final part of megakaryopoiesis, a process marked by the differentiation of a less mature blast cell into a fully mature, platelet releasing megakaryocyte (17,18). Traditionally, the study of megakaryopoiesis has been restricted by the rarity of these cells (megakaryocytes make up about 0.05% of total mononuclear cells in normal bone marrow) (19). However, the recent development of *in vitro* clonogenic culture systems, along with the isolation of immortalized megakaryocytic cell lines, has allowed researchers to gain a better understanding of the biochemistry and gene regulation associated with megakaryocyte differentiation and platelet production (19,20).

The overall objective of our research is to understand how PGHS-1 expression is regulated during megakaryopoiesis. This work is significant because an understanding of PGHS-1 regulation in the context of platelet production will reveal targets that could prove useful in the treatment of acute coronary syndromes, and in the long term prevention of atherogenesis. To this end, we have elected to use the immortalized human megakaryoblastic cell line, MEG-01, as a model system in which to study the expression

of PGHS-1 mRNA and protein in megakaryocyte cells stimulated to differentiate by the phorbol ester, TPA.

1.2 THE PHYSIOLOGICAL ROLE OF PROSTANOIDS

Ubiquitous to mammalian tissues are the prostanoids, a family of oxygenated lipids whose members function as local hormones by acting on their target cells in either an autocrine or paracrine fashion. Prostanoids are potent physiological mediators; that is, they exert their effects at extremely low concentrations. Some examples of prostanoid-mediated physiological events include inflammation in the joints, skin and eyes, fever and pain, haemostasis, the regulation of platelet aggregation, ovulation, and labor. In humans, the prostanoid precursor molecule is arachidonic acid, a C₂₀ - polyunsaturated fatty acid that possesses four unconjugated double bonds. Prostanoids are formed through the cyclooxygenase pathway, so named because the committed step is catalyzed by cyclooxygenase, or, as the enzyme is now called, PGHS (1).

1.2.1 Arachidonic acid (5,8,11,14-Eicosatetraenoic acid)

Arachidonic acid (5,8,11,14-eicosatetraenoic acid), the substrate for eicosanoids, is found esterified in the membrane phospholipids and triglycerides of all mammalian tissues. In this bound form, arachidonate serves to sustain membrane fluidity and acts as a storage pool of substrate for eicosanoid production. Because eicosanoids are extremely labile, cells cannot store them. As a consequence, the production of eicosanoids is dependent upon the release of free arachidonate acid from cellular membranes (21).

1.2.2 Arachidonate release

In most cells, the concentration of free arachidonate is well below 5 μM , the K_m for PGHS synthase (82). It is believed that stimulus-induced increases in arachidonate concentration result from the activation of phospholipases that mobilize arachidonic acid from membrane phospholipids. Current dogma encompasses the idea that the major enzyme involved in the release of arachidonic acid from the *sn*-2 position of phospholipids is phospholipase A₂ (PLA₂) (1). There are currently three known classes of mammalian phospholipases A₂ (PLA₂s): secretory PLA₂ (sPLA₂), cytosolic PLA₂ (cPLA₂) and Ca²⁺-independent PLA₂ (iPLA₂) (22). Cytosolic PLA₂ and type II sPLA₂ are the two isoforms that are currently thought to play key roles in the generation of free arachidonic acid and other lipid mediators (1). Cytosolic PLA₂ is a large 110 kD phospholipase that, when optimally activated at micromolar calcium concentrations, becomes associated with the cell membrane (23,24). It is thought that in platelets cPLA₂ is important in the generation of second messengers because this isoform is active at the intracellular Ca²⁺ concentrations reached during platelet activation (25). On the other hand, secretory PLA₂, types I and II, is a 14 kD enzyme that is either secreted from cells or remains associated with the membrane. In contrast to cPLA₂, secreted PLA₂ is active at mM Ca²⁺ concentrations (26,27). Both cPLA₂ and sPLA₂ have been described in platelets and in megakaryoblastic cell lines (28-31).

PLA₂ plays a major role in the mobilization of arachidonate in many types of cells. In thrombin-stimulated platelets, phospholipase C (PLC) is also involved in the release of arachidonic acid from the cellular pool of phosphatidyl inositol (PI). In fact, up

to 50% of the arachidonate released from the membranes of platelets treated with thrombin is due to the sequential actions of PI-specific PLC, diglyceride lipase, and monoglyceride lipase (1). Platelets contain at least two of the known families of phospholipase C: PLC β and PLC γ (32). The β forms are thought to be regulated by G proteins whereas the γ -forms are regulated by protein-protein interactions that depend on a particular state of tyrosine phosphorylation (33,34).

Once liberated from the membrane, arachidonate can be oxygenated and further metabolized along one of three distinct pathways: the PGH synthase pathway, leading to the formation of prostanoids, the lipoxygenase pathway, which leads to the production of leukotrienes, lipoxilins and hepoxilins, and the cytochrome P-450 pathway, which produces epoxides and diols (35).

1.2.3 The PGHS pathway: An overview

Prostanoid biosynthesis begins when an appropriate stimulant reaches its target cell. For instance, when platelets are stimulated by thrombin, they actively synthesize TxA₂ (301). Some other physiological agents that are able to stimulate prostanoid formation include hormones like histamine, bradykinin, and platelet activating factor (62). A stimulus molecule elicits prostanoid biosynthesis by interacting with an appropriate receptor that is coupled to a second messenger system, which is in turn linked to a set of prostanoid biosynthetic enzymes. A net increase in the intracellular concentration of arachidonic acid is achieved either through the activation of an intracellular lipase, or through an increased uptake of arachidonate from the

surroundings. As its concentration increases, arachidonate begins to saturate PGH synthase, permitting the enzyme to catalyze a *bis*-oxygenation which leads to the production of prostaglandin endoperoxide H_2 (PGH_2) (68,69,302). PGH_2 is then rapidly converted to one of the biologically active prostanoids by an enzyme unique to the particular product. For example, in platelets, PGH_2 is immediately converted to TxA_2 by the enzyme thromboxane synthetase, whereas in endothelial cells, PGH_2 is at once converted to prostacyclin by the enzyme prostaglandin I synthase (1). Depicted in Figure 1.1 is the prostanoid biosynthetic pathway as it might occur in a model cell. Although all the metabolic pathways for PGH_2 metabolism are depicted, prostanoid-forming cells generally form only one major prostanoid product (1).

1.2.4 PGH_2 metabolism and the function of prostanoids

PGH_2 is a metabolic intermediate that is instantaneously converted to one of the biologically active prostanoids by a set of tissue specific isomerases and synthases (36). The prostaglandins PGD_2 , PGE_2 and $PGF_{2\alpha}$ are formed from PGH_2 by the respective actions of PGH-PGD synthase, PGH-PGE isomerase, and PGF synthase. PGH_2 is converted to prostacyclin (PGI_2) by PGI synthase, and to thromboxane (TxA_2) by the platelet enzyme, thromboxane synthase (TXS) (36). Differentiated cells usually produce only one type of biologically active prostanoid because they tend to express a single PGH_2 metabolizing enzyme (1).

Prostaglandin D_2 is formed by the disproportionation of PGH_2 in a reaction that can be catalyzed by either a glutathione (GSH)-dependent or GSH-independent enzyme (37,38). PGD_2 is the main prostaglandin produced during the inflammatory response in

Figure 1.1 Pathways for the cellular biosynthesis of prostanoids. (Adapted from Smith, W.L., *American Journal of Physiology*, 263:F181-F191, 1992.)

skin (39), and in addition, is the major prostaglandin produced by mast cells (40). PGD_2 is also synthesized by platelets, albeit in much smaller amounts than TxA_2 . Nevertheless, PGD_2 is a potent inhibitor of platelet aggregation (41-43).

Prostaglandin E synthase, also known as PGH-PGE isomerase, catalyses the conversion of PGH_2 to PGE_2 in what appears to be a GSH-dependent mechanism; that is, GSH is required for the synthesis of PGE_2 but is not consumed during the reaction process (44). PGE_2 plays an important role in kidney function. It is produced by renal mesangial cells where it helps to regulate glomerular hemodynamics, glomerular cell proliferation and ultrafiltration (45). PGE_2 is also produced in the kidney collecting tubule where it modulates Na^+ and water resorption (46-47). When injected into skin, PGE_2 causes swelling by increasing vascular permeability. It is not known if this is due to the direct action of PGE_2 on the vasculature, or is instead caused by the PGE_2 -mediated release of vasoactive substances (48).

Two active epimers of the prostaglandin F series, $\text{PGF}_{2\alpha}$ and $9\alpha,11\beta\text{-PGF}$, have been identified in biological systems. Three pathways lead to the formation of PGF_2 analogs (49-52): PGH_2 can be reduced to $\text{PGF}_{2\alpha}$ by an endoperoxide reductase, PGD_2 can be reduced to $9\alpha,11\beta\text{-PGF}$ by an 11-keto-reductase, and PGE_2 can be reduced to $\text{PGF}_{2\alpha}$ by a 9-keto-reductase (53,54). PGF synthase has been purified from bovine lung, liver and spleen (55). It is believed that PGE_2 is not a natural substrate for the synthesis of $\text{PGF}_{2\alpha}$ *in vivo* because 9-keto-reductase has a very high K_m for PGE_2 (ca. 300 μM) (1). Like PGE_2 , $\text{PGF}_{2\alpha}$ is also produced by kidney mesangial cells (45). Furthermore, $\text{PGF}_{2\alpha}$ is one of the major prostanoids formed by vascular endothelial cells (56) and can enhance the effects of colony stimulating factor (CSF) on bone marrow colony growth (57).

Prostacyclin is synthesized from PGH_2 by the enzyme PGI synthase in a reaction that involves an acid-catalyzed heterolytic cleavage of the endoperoxide group of PGH_2 , followed by the reaction of a transiently positive oxygen at C-6 (1). PGI synthase is present in high concentrations in vascular endothelial cells and in smooth muscle (58,59). The fact that PGI synthase colocalizes with PGH synthase in the endoplasmic reticulum, nuclear membrane, and plasma membrane suggests that PGI_2 is formed in close association with PGH_2 (59). In fact, in endothelial cells and murine 3T3 cells, the expression of PGH synthase and PGI synthase protein is co-regulated (60,61). PGI_2 functions as a vasodilator and is a potent inhibitor of platelet aggregation (62).

The thromboxanes were discovered during the study of arachidonic acid metabolism in human platelets (63). Thromboxane A_2 , which is synthesized by platelets, lung, and macrophages, is a potent thrombogenic agent and vasoconstrictor (1). TxA_2 is synthesized from PGH_2 by the enzyme thromboxane synthase in a reaction marked by the transient formation of an electropositive oxygen at C-11 and the subsequent cleavage of the 9,11-peroxido group (64). Thromboxane synthase has been localized to the platelet dense tubular system (65). Because TxA_2 is extremely unstable in aqueous solution ($t_{1/2} = 30\text{s}$ at 37°C), its biological activity is short-lived (66). Thromboxane levels and the TX synthase activity associated with their production are usually measured by detecting thromboxane B_2 (TxB_2), the stable but biologically inactive hydrolysis product of TxA_2 (1). The interaction between the vascular wall and circulating platelets involves the balance between prostacyclin, a vasodilator, and TxA_2 , a procoagulant and thrombogenic agent. Thus, in thrombosis and hemostasis can be found one of the best defined roles of prostanoid function (59).

1.3 PROSTAGLANDIN ENDOPEROXIDE H SYNTHASE

The name PGHS replaced *cox* when it was learned that this molecule, which is the major rate-limiting enzyme for prostanoid biosynthesis, possessed both a cyclooxygenase and a peroxidase activity. Each activity has since been mapped to a distinct site on the protein (67). PGHS catalyses the *bis*-oxygenation of arachidonic acid to prostaglandin G₂ (PGG₂), followed by the reduction of PGG₂ to PGH₂ (68,69,302). Prior to 1991, research in the field of arachidonic acid metabolism described only one PGHS enzyme, the form now commonly referred to as prostaglandin endoperoxide H synthase-1 (PGHS-1). However, in 1989, researchers reported the existence of a mRNA species in phorbol ester and *src*-treated mouse and chicken fibroblasts possessing a deduced amino acid sequence that exhibited 60% identity to the PGHS enzyme (70). Subsequent work has revealed that this new form of PGHS, now called PGHS-2 or the inducible PGHS, is very similar to PGHS-1 in terms of structure, but differs substantially with respect to its pattern of expression and its biology (6).

1.3.1 The PGHS-1 and PGHS-2 proteins

Within a species, there is approximately 60% identity between the amino acid sequences of PGHS-1 and PGHS-2 (71-73). Fig 1.2 illustrates the primary amino acid sequence of each isoenzyme. The human PGHS-1 mRNA encodes a 599-residue polypeptide that migrates as a 70 kD band when analyzed by SDS-PAGE (36,74-76). Human PGHS-2 mRNA encodes a 604-residue polypeptide that also migrates at about 70

Figure 1.2 Sequence comparison between the human PGHS-1 and -2 polypeptides.
(Adapted from Hla, T. *et al.* 1993. *Annals New of the York Academy of Sciences*. Vol. 696. *Immunosuppressive and Antiinflammatory Drugs*. New York Academy of Sciences, New York. p. 200.)

Human-2	MLARALLCAVL-----ALSH TANPCCSHPCQNRGVCM SVGFDQYKCD
Human-1	MSR-SLLRFL LLLLLL-PPLP-VLLADPGAPTPVNPCCYYP CQHQGICVRFGLDRYQCD
	10 20 30 40 50
Human-2	CTR TGFYGENCSTPEFLTRIKFLLKPTPNTVHYILTHFKGEWNVVMNIPFLRNAIMSYVL
Human-1	CTR TGYSGPMCTIPGLWTWLRNSLRSPSPSFTHFL LTHGRWEWEFVNAT-FIREMLMLLV L
	60 70 80 90 100 110
Human-2	TSRSHLIDSPPTYNADYGYKSWEAFSMLSYYTRALPPVPDDCPTPLGVKGKKQLPDSNEI
Human-1	TVRSNLIPSPPTYN SAHDYISWESFSMVSYTRILPSVPKDCPTPMGTGKKQLPDAQLL
	120 130 140 150 160 170
Human-2	VGKLLLRKFI PDPQGSNMMAFFAQHFTHQFFKTDHKRGP AFTNGLGHGV D LNH IYGET
Human-1	ARRFLLRKFI PDPQGTNLMAFFAQHFTHQFFKTS GKMGP GFTKALGHGV D LGH IYGDN
	180 190 200 210 220 230
Human-2	LARORKIRLFKDGKMKYQI IDGEMYPPTVKDTQAEMIYPPQVPEHLRF AVGQEVFGLVPG
Human-1	LERQYQLR LFKDGK LKYQVLDGEMYP P SVEEAPVLMHYPRGIPPQSQMAVGQEVFGLLP G
	240 250 260 270 280 290
Human-2	LIMYATIWLREHNRVCDVLKQEHPEWGDEQLFQTSRLILIGETIKIVIDDYVQHLSGYHF
Human-1	LMLYATLWLREHNRVCDLLKAEHPTWGDEQLFQ TTRLILIGETIKIVIEEYVQQLSGYFL
	300 310 320 330 340 350
Human-2	KLKFDPELLFNKQFQYQNRIAAEFNTLYHWHPLLPDTFQINDQKYN YQQFIYD NSILLEH
Human-1	QLKFDPELLFGVQFQYRNRIATEFNHLYHWHPLMPDSFKVGSQEYSYEQFLNTSMLVDY
	360 370 380 390 400 410
Human-2	GITQFVESFTRQIAGRVAGGRNVPPAVQKVSQASIDQSRQMKYQSFNEYRKRFMLKPYES
Human-1	GVEALVDAFSRQIAGRIGGGRNDHHLHVAVDVIRESRMRLQPFNEYRKRFGMKPYTS
	420 430 440 450 460 470
Human-2	FEELTGEKEMSAELEALYGDIDAVELYPALLVEKPRPD AIFGETMVEVGAPFSLKGLMGN
Human-1	FQELVGEKEMAAELEELYGDIDALEFY PGLLEKCHPNSIFGESMIEIGAPFSLKGLLGN
	480 490 500 510 520 530
Human-2	VICSPAYWKPSTFGGEVGFQIINTASIQSLICNNVKGCPFTSFSVPDELIKTVTINASS
Human-1	PICSPEYWK PSTFGGEVGFNI VKTATLKKLVCLNTKTCPYVSFRVPDASQDDGPAVE---
	540 550 560 570 580 590
Human-2	SRSGLDDINPTVLLKERSTEL
Human-1	-----RPSTEL
	599

kD (11,77). PGHS-2 differs significantly from PGHS-1 at positions before amino acid residue 30. Processed PGHS-1 has 576 residues and the N-terminal sequence ADPGA due to the removal of a 25 amino acid signal peptide. Mature PGHS-2 has the N-terminal sequence ANPCC which results from the cleavage of a 17-amino acid N-terminal signal peptide. Importantly, PGHS-2 contains an 18-amino acid cassette near its C-terminus that is not present in PGHS-1. Although sequences near their N- and C-termini are relatively unique, the remaining core sequences of PGHS-1 and PGHS-2 are about 75% identical. In addition, all residues essential for the catalytic activity of PGHS-1 are conserved in PGHS-2 (12).

From its cDNA sequence, the predicted sub-unit molecular weight of PGHS-1 is 65.5 kD, excluding the signal peptide. However, when examined by SDS-PAGE, native PGHS-1 migrates as a band of about 70 kD. The increase in molecular weight is a result of the addition of three high mannose oligosaccharides ($\text{Man}_7[\text{NAcGln}]_2$ and $\text{Man}_9[\text{NAcGln}]_2$) at Asn67, Asn143, and Asn409 of the human enzyme (77,78). Native PGHS-2 contains three N-linked oligosaccharides at positions analogous to those found in PGHS-1. In addition, PGHS-2 possesses a fourth N-linked glycosylation site in its C-terminal cassette (77). It has been shown that to achieve their native conformations, PGHS-1 and -2 must be glycosylated; however, once folded correctly, the enzymes no longer need to be glycosylated to maintain a catalytically active conformation (77).

1.3.2 Mechanism of enzyme catalysis

Prostaglandin endoperoxide H synthase catalyses the committed step of prostanoid biosynthesis (6). This understanding is based on the observation that both PGHS isozymes undergo a process known as 'suicide inactivation', whereby the cyclooxygenase activity of the enzyme ceases to function after exposure to arachidonate substrate. Suicide inactivation apparently happens because there is a certain probability that an unstable PGHS protein intermediate will rearrange to an inactive species (36). On average, inactivation occurs once every 1300 turnovers (79).

PGHS-1 and -2 possess similar cyclooxygenase turnover numbers (3500 mol of arachidonate /min/mol of dimer) (80,81) and have similar K_m values for arachidonate ($\sim 5 \mu\text{M}$), and for O_2 ($\sim 5 \mu\text{M}$) (81,82). Furthermore, the key residues involved in catalysis are conserved in PGHS-1 and -2, and the crystal structures of the two isozymes are essentially superimposable (6). PGHS converts arachidonic acid to PGH_2 through a two-step process that takes place at distinct, albeit interacting catalytic sites on the protein molecule (12).

The first activity is a cyclooxygenase that catalyses the *bis*-oxygenation of arachidonate to produce PGG_2 , while the second activity is a peroxidase that catalyses a net 2-electron reduction of the 15-hydroperoxyl group of PGG_2 to form PGH_2 (68,69,302). Both the cyclooxygenase and peroxidase activities of PGHS require a heme prosthetic group (Fe^{3+} -protoporphyrin IX) to carry out catalysis. Titration of the apoenzyme with heme has indicated that there is an average of one heme per PGHS molecule. After abstracting the *pro-S* hydrogen from C-13 of arachidonate, PGHS adds

one molecule of O_2 to C-11 from the solvent side of the substrate. Serial cyclization of the incipient 11-peroxyl radical yields an endoperoxide with aliphatic chains that are trans to one another. PGHS then adds a second O_2 molecule to arachidonate at C-15 to produce PGG_2 (68,69,302).

The peroxidase activity of PGHS catalyses the two-electron reduction of the hydroperoxide PGG_2 to form PGH_2 , a process that creates a ferryl-oxo porphyrin radical as a by-product. The endogenous electron donor for the peroxidase reaction is not known; however, among the naturally occurring compounds, epinephrine and uric acid are the most likely candidates (1).

1.3.3 PGHS membrane interaction and intracellular localization

PGHS-1 and -2 are classified as integral membrane proteins because detergents, and not merely chaotropic salts, are required to extract them from membranes (36). Furthermore, proteolytic cleavage has failed to yield an active soluble fragment, suggesting that the PGHS isozymes are not simply tethered by a flexible anchor (83,84). The three-dimensional structures of both isozymes, which have been determined by x-ray crystallography (85-87), show how PGHS may interact with a potential membrane. Each isozyme possesses a compact structure with a catalytic domain similar to that of myeloperoxidase (MPO) (85-88). Due to its polar surface, the catalytic domain is most likely external to the membrane (85-87). Because PGHS-1 and 2 each possesses an N-terminal signal peptide that is cleaved in the native enzyme (84,89), it is believed that the protein is oriented towards the lumen of the ER and nuclear envelop. However, helices

A, B, C and the beginning of helix D, which are amphipathic, are situated with their hydrophobic surfaces facing outward, away from the body of the enzyme, where they form a large patch on the exterior of the molecule. It is probable that this patch anchors PGHS in the membrane in a manner analogous to the way that surface-active peptides bind, that is, with their lower surfaces interacting with the hydrophobic interior of the bilayer. The opposite sides of the helices contain a few charged residues that are well positioned to interact with phospholipid head groups. Because the binding surface of the protein is not deep enough to extend beyond one leaflet of the lipid bilayer, PGHS is said to be a monotopic membrane protein, according to Blobel's classification (85-87,90). Figure 1.3 shows the crystal structure of ovine PGHS-1.

In situ immunolabeling studies performed on murine 3T3 cells have demonstrated that PGHS-1 and PGHS-2 are integral membrane proteins associated with the endoplasmic reticulum (ER) and nuclear envelope (NE) (91,92). In megakaryocytes, PGHS-1 is associated with the endoplasmic reticulum and nuclear envelope (93,94), and in platelets, it is localized to the dense tubular system (65,94-96). At its C-terminus, each isozyme has the amino acid sequence STEL, a motif analogous to KDEL, the endoplasmic reticulum retention signal. In 1995, Smith *et al* tested the significance of the PGHS C-terminal PTEL sequence. They found that mutations in this sequence did not change the subcellular location of PGHS-1 and therefore concluded that targeting to the ER was PTEL- independent. In these original experiments, the mutant transfectants were expressed for 20 to 40 hours (91). However, in 1996, Smith's group re-tested their C-terminal PGHS-1 mutants, as well as PGHS-2 C-terminal mutants, by expressing them in

Figure 1.3. The crystal structure of ovine PGHS-1. The membrane binding domain is shown in orange; the catalytic and EGF domains in blue. Arachidonate substrate is colored violet, yellow and green. The haem group is shown in red. Although PGHS-1 exists as a dimer in crystal form, the molecular stoichiometry has not been established in intact membranes. (Adapted from Garavito, M. *et al.*, *Nature*, 367:243-249, 1994.)

cos-1 transfected cells for the shorter duration of 18 hours. In contrast to his previous findings, Smith observed that mutant PGHS-1 and PGHS-2 molecules localized to the Golgi apparatus whereas the wild-type isozymes remained in the ER. He thus concluded that “the C-terminal –S/PTEL sequences of both PGHS-1 and PGHS-2 [indeed] target the isozymes to the ER” (97).

It has been postulated that after being targeted to the ER by their C-terminal peptides, PGHS-1 and –2, like other integral ER membrane proteins that reach the nuclear envelope (98-100), diffuse laterally through the membrane, bypassing the nuclear pore complex, to arrive at the inner NE (101).

Although PGHS-1 and 2 share many similarities, results from several experiments make it difficult to argue that PGHS-2 functions solely to augment the prostanoid biosynthetic capacity of PGHS-1 (6). The different expression patterns of PGHS-1 and –2, and the apparent access of each isozyme to different pools of arachidonic acid within the cell (102-104) have led to the hypothesis that PGHS-1 and –2 represent independent prostanoid biosynthetic systems (6). The expression of PGHS-2, which is encoded by an intermediate early gene, occurs in conjunction with nuclear events such as cell replication and differentiation (2). Consequently, the inducible enzyme, especially the subset of molecules localized to the nuclear envelope, has been hypothesized to be the major source of prostanoids involved in a putative peroxisome proliferator activated receptor-mediated nuclear signaling system (105-107).

To test this model, Smith launched a series of experiments to investigate the assumption that PGHS-2 is preferentially localized to the nuclear envelope. In 1995 he published results from confocal immunolocalization experiments that he and his

colleagues performed on murine 3T3 cells, and on human and bovine endothelial cells. Smith *et al* observed that although both enzymes were present in the ER and nuclear envelope, PGHS-2 was preferentially associated with the NE. By comparison, PGHS-1 was equally distributed between the ER and the nuclear envelope. They therefore concluded that “the differential compartmentalization of the two isozymes may serve, at least in part, to separate the activities of PGHS-1 and -2 within cells” (108).

In 1998, to further test this model, Smith *et al* employed the higher resolution technique of immunoelectron microscopy to localize each isozyme *in situ*, in addition to performing Western blotting for PGHS-1 and -2 in protein isolates extracted from different subcellular fractions. In contrast to their earlier results, Smith’s group detected PGHS-1 and PGHS-2 in similar proportions on both the inner and outer nuclear membranes of NIH 3T3 cells. The Western blotting data obtained from protein isolates taken from the different subcellular fractions also revealed a similar distribution pattern for each isozyme. Finally, the analysis of products obtained by employing isozyme specific inhibitors has revealed that both enzymes generate the same products in NIH 3T3 cells (101).

Smith attributed the marked discrepancy in results from the two localization studies to differences in the affinity of each isozyme for the nuclear envelope. In his earlier investigation, Smith permeabilized the cells with 1% saponin prior to performing the analysis by confocal microscopy. Unfortunately, because the membrane binding domains of PGHS-1 and PGHS-2 differ significantly in amino acid composition (6,109), the isozymes do not have the same solubility in detergent. This fact made PGHS-2 appear more concentrated in the nuclear envelope (108). For the study published in 1998, Smith

and colleagues prepared the cells for immunoelectron microscopic analysis in the absence of saponin. Under these conditions, they found that PGHS-2 was present at the same concentration in the ER as in the nuclear envelope.

The most recent data obtained from NIH 3T3 cells, human monocytes, and HUVECs indicates that PGHS-1 and PGHS-2 are present in equal proportions on both the inner and outer membranes of the NE. Both isozymes exhibit identical distribution patterns and are present in the same subcellular compartments at comparable concentrations. Thus, unless PGHS-1 and -2 are associated with different microdomains within the ER and nuclear envelope, the results from the latest localization study rule out the possibility that the independent functioning of the two isozymes is attributed to differences in subcellular location (101). Any specific connection between PGHS-2 and the generation of products that act in the nucleus must result not from gross differences in subcellular distribution, but instead from differences in PGHS-1 and -2 activity. The most likely factors influencing such differences include varying interactions with distinct phospholipases and/or differences in PGHS isozyme kinetics.

Arm, Austen, Herschmann, and co-workers have demonstrated that there are two separate phases of PGD₂ synthesis in mast cells that are independently coupled to PGHS-1 (early phase) and to PGHS-2 (late phase) by different phospholipases A₂ (102,104,1109-112). Kinetic mechanisms may also separate the actions of PGHS-1 and PGHS-2. For example, it has been shown that PGHS-2 has a significantly lower threshold for hydroxyperoxide activation than PGHS-1, thereby enabling it to oxygenate arachidonate in the presence of lower peroxide concentrations (80,113). It has also been revealed that negative allosteric regulation of PGHS-1 by arachidonic acid at

concentrations between 0.5 nM and 1 μ M has the overall effect of causing a 2 to 4-fold greater rate of PGHS-2-mediated prostanoid formation (114). Employing a histochemical technique that detects enzyme activity in intact cells, Smith and colleagues have indeed shown that staining attributable to PGHS-1 occurs primarily in the cytoplasm, whereas staining attributable to PGHS-2 occurs both in the cytoplasm and over the surface of the nucleus (108).

1.3.4 Regulation of PGHS-1 and PGHS-2 gene expression

The PGHS-1 gene is located on chromosome 9 (9q32-q33.3), spans 22.5 kb, and contains 11 exons and 10 introns (115), while the gene encoding PGHS-2 is found on chromosome 1 (1q25.5-q25.3), spans 8.3 kb and comprises 10 exons and 9 introns (5). The first two exons of PGHS-1, which contain the translational start site and signal peptides, are condensed into a single exon in PGHS-2; the remaining intron/exon arrangements of the two genes are identical (5,115). Because the introns found in the PGHS-2 gene are much smaller than those present in PGHS-1, the gene encoding the second isozyme is only 8.3 kb in length (116). The small size of the PGHS-2 gene is consistent with its classification as an immediate early gene (117).

The single best characterized distinction between PGHS-1 and PGHS-2 is their differential regulation of expression (12). PGHS-1 is generally regarded as a housekeeping enzyme because it is expressed constitutively in low amounts in almost all tissues (6). Some examples of PGHS-1 performing such a function include the cytoprotection of the gastric mucosa (GI tract) and regulation of renal blood flow (kidney)

(12). However, this is an oversimplification because certain tissues such as platelets and endothelial cells express high amounts of PGHS-1 protein. Furthermore, there is evidence to suggest that the expression of PGHS-1 is controlled developmentally. For instance, when monocytes are stimulated to differentiate into a macrophage phenotype, the expression of PGHS-1 increases as the cells proceed along the developmental pathway (118,119). It has also been shown that PGHS-1 gene expression increases *in vivo* as ovine pulmonary artery matures during gestation and after birth (120). Like other housekeeping genes, PGHS-1 possesses a promoter with a GC-rich region that lacks a TATA box (121). The PGHS-1 promoter region also contains two widely separated Sp1 sites that, when bound to the appropriate transcription factor(s), are capable of activating basal PGHS-1 gene expression (121). Other regulatory sequences found within the PGHS-1 promoter include NF-IL-6, PEA-3, SSRE, NF- κ B, and GATA-1 (121).

With the exception of brain, the PGHS-2 protein is not usually expressed in resting tissue (122-124). Instead, PGHS-2 is expressed in response to mitogenic or inflammatory stimuli (12). For instance, PGHS-2 protein is undetectable in rat tissue that has not been stimulated (123); however, soon after rat paws are injected with carageenan, PGHS-2 protein is observed (8). PGHS-2 is not found in normal joints; it is however, present in the articular tissue of rats during staphylococcal cell wall or adjuvant-induced arthritis (125). PGHS-2 is also found in the joints of individuals inflicted with rheumatoid arthritis (125-127). Thus, in contrast to PGHS-1, PGHS-2 gene expression is highly inducible. Not surprisingly, the 5' flanking region of the human PGHS-2 gene contains a number of promoter/enhancer elements: a canonical TATA box, CArG box, NF-IL-6, PEA-1, myb, GATA-1, xenobiotic response element, cAMP response element,

NF- κ B, PEA-3, and substance 1. Additionally, there is a TPA response element buried in exon 1 (5).

1.3.5 PGHS-1 and PGHS-2 transcripts

The hPGHS-1 and hPGHS-2 open reading frames, which are both 1.8 kb in length, are 73% homologous at the nucleotide level. PGHS-1 is encoded by three different sized transcripts; the first is 2.8 kb in length (128), the second 4.5 kb (281), and the third 5.1 kb (128). All of the PGHS-1 isoforms appear to arise through alternative polyadenylation (128,281). In contrast, PGHS-2 is encoded by a 2.8 and a 4.5 kb transcript, each of which possesses an AU-rich 3' UTR (116,129). Like PGHS-1, the 2.8 kb PGHS-2 mRNA is derived from the longer 4.5 kb transcript through alternative polyadenylation (130). Studies have shown that the two PGHS-2 transcripts display differential stability in response to agents like dexamethasone and interleukin-1 β (131-133). This is because the full length 4.5 kb PGHS-2 mRNA transcript contains 22 copies of the Shaw-Kamen instability motif AUUUA whereas the 2.8 kb transcript contains only 7 copies (3). When the expression pattern of the PGHS-2 gene is taken into account, there seems to be a functional role for both transcriptional and post-transcriptional regulation.

Because the PGHS-1 gene contains only one AUUUA instability motif within the known 3'UTRs of each of its transcripts, PGHS-1 mRNA is much more stable than PGHS-2 in cultured cells (128). It is not known whether the regulation of mRNA stability is important for PGHS-1 expression. It has been suggested, however, that because only low levels of PGHS-1 transcriptional activation have been reported, post-

transcriptional regulation centered at the 3'UTR of PGHS-1 mRNA may play an important role in the expression of this isozyme (14,134). In summary, it appears that specific sequences contained within the 3'UTRs of both the PGHS-1 and PGHS-2 transcripts make important contributions towards the expression pattern of each PGHS gene.

1.3.6 PGHS-1 and PGHS-2 inhibitors

Glucocorticoids and non-steroidal anti-inflammatory drugs (NSAIDs) comprise the two major chemical families that derive their therapeutic effect by targeting PGHS and thereby blocking the formation of prostanoids. The discovery of PGHS-2 in 1991 (3) has had a profound impact on the development and prescription NSAIDs. The pharmacological significance of the two PGHS isozymes lies chiefly in their dissimilar mode of regulation. It is now widely accepted that PGHS-1 is the physiological form of the enzyme because it is expressed constitutively in most tissues (12). In contrast, PGHS-2 is usually expressed only in response to cytokines and inflammatory stimuli (12). Current dogma encompasses the notion that the anti-inflammatory actions of NSAIDs are produced by the inhibition of PGHS-2, whereas the unwanted side effects, such as irritation of the stomach lining, are caused by inhibition of PGHS-1 (135). However, there is one important benefit procured from NSAID inhibition of PGHS-1. When administered at a low dose, aspirin is absorbed in a fashion that limits its activity to the inhibition of PGHS-1 in blood platelets. PGHS-1 inhibition ultimately blocks the

formation of the coagulant molecule TxA_2 , thereby producing the anti-thrombogenic effect necessary for the prevention and treatment of cardiovascular disease (136-138).

Glucocorticoids act as anti-inflammatory agents by completely inhibiting the expression of PGHS-2 without blocking PGHS-1 (6). This action is accomplished by at least two mechanisms. Glucocorticoids like dexamethasone inhibit PGHS-2 by suppressing PGHS-2 gene transcription (139), and by reducing the stability of PGHS-2 mRNA (140). There is also evidence to support the notion that glucocorticoids block the translation of PGHS-2 mRNA (132,141). In contrast, NSAIDs achieve their therapeutic effect by blocking the activity of both PGHS isozymes (12), although it has recently become apparent that certain NSAIDs are selective towards a specific PGHS isozyme (137).

The class of “aspirin-like drugs” now termed NSAIDs first came into being when salicylic acid and salicylates were obtained from natural sources and used as medicaments. Salicylic acid was chemically synthesized in 1860 and originally employed as an antiseptic, antipyretic and antirheumatic drug. Almost forty years later aspirin was developed in order to provide a more palatable form of salicylate (135). Since the development of aspirin, many new drugs possessing similar actions have been developed and classified. NSAIDs inhibit the activity of PGHS by specifically targeting the cyclooxygenase active site that is located in the long hydrophobic channel present in both isozymes (12,85,86). There are presently three ways of classifying NSAIDs.

The first system of nomenclature categorizes NSAIDs according to their chemical structure. NSAIDs can be grouped into the following categories: p-aminophenols, indols, heteroaryl acids, aryl propionic acids, and enolic acids (137). A second and perhaps

more useful way to categorize NSAIDs is according to their mode of PGHS inhibition. Class I NSAIDs are simple competitive PGHS inhibitors that compete reversibly with arachidonate for binding to the cyclooxygenase active site. Included in this group of compounds are piroxicam, flufenamate, sulindac sulfide, and ibuprofen (12). Class II NSAIDs are time-dependent competitive inhibitors of PGHS cyclooxygenase activity (82,142-145). Such an agent will bind rapidly and reversibly in an initial phase to form an enzyme-inhibitor (EI) complex. However, if retained in the cyclooxygenase active site for a sufficient length of time, a class II NSAID will cause a conformational change in the protein that is associated with tighter (but noncovalent) binding. Once bound in this tighter form, a time-dependent NSAID can only slowly dissociate from the cyclooxygenase active site (82,142-145). Examples of Class II NSAIDs are indomethacin, flurbiprofen, meclofenamate, diclofenac, NS-398, and DuP 697 (12). Class III NSAIDs inhibit cyclooxygenase by forming an irreversible EI complex through the direct covalent modification of PGHS. The best known Class III NSAID is aspirin. When bound to the cyclooxygenase active site, aspirin transfers its acetyl group from salicylate to serine 530 of hPGHS-1 and to serine 516 of hPGHS-2. Acetylation of PGHS-1 by aspirin completely inhibits cyclooxygenase activity without affecting peroxidase activity (146,147). Studies suggest that the aspirin catalyzed acetylation of PGHS-1 places a bulky group at Ser530, preventing arachidonate from binding to the cyclooxygenase active site (148,149). In contrast, acetylation of PGHS-2 by aspirin does not inhibit the PGHS-2 oxygenase activity but instead modifies the isoenzyme so that PGHS-2 produces 15R-hydroxyeicosatetraenoic acid (15R-HETE) instead of PGG₂ (150-152,303). As shown in Figure 1.4, the acetylation of PGHS-2 by aspirin does not prevent

Figure 1.4 Comparison of the effects of aspirin acetylation on human PGH synthase-1 and synthase-2. (Adapted from Smith, W.L. *et al.*, *Seminars in Nephrology*. 15:179-194, 1995.)

arachidonate from binding to the cyclooxygenase active site; instead, it prevents the addition of O₂ to C-11 of arachidonate, leading to the formation of 15R-HETE (150).

The third and perhaps most useful way of classifying NSAIDs is according to the affinity with which a given drug will inhibit PGHS-2 relative to PGHS-1 (137). This classification scheme is important because NSAIDs, which are the most frequently prescribed drugs in the world, can irritate the gastrointestinal tract and cause kidney damage. Because PGHS-2 is responsible for the elevated production of prostaglandins during inflammation, it is thought that most of the negative side-effects of NSAIDs are due to the inhibition of the physiological PGHS-1, whereas the therapeutic anti-inflammatory effects result from the inhibition of PGHS-2. In theory, NSAIDs that are highly selective towards PGHS-2 should be GI and renal sparing. On this basis, NSAIDs can be classified as selective PGHS-1 inhibitors, non-selective PGHS inhibitors, selective PGHS-2 inhibitors, and highly selective PGHS-2 inhibitors (135). The category into which a particular inhibitor will belong is determined by its PGHS-2: PGHS-1 IC₅₀ ratio. Inhibitors that have a low ratio are highly PGHS-2 selective whereas drugs that have a high ratio are PGHS-1 selective. Some examples of NSAIDs that belong to these different categories are given in Table 1.1 (137,287,288,304).

It is a subtle difference between the cyclooxygenase active sites of PGHS-1 and – 2 that accounts for the different NSAID selectivity profiles. Because of the substitution of valine for isoleucine, the NSAID binding site is much larger in PGHS-2 than it is in PGHS-1. The deletion of a methylene group at position 523 in PGHS-2 allows the inhibitor access to a pocket that leads directly to the solvent. The selective PGHS-2 inhibitor exploits this additional pocket for enhanced binding through a

TABLE 1.1
Some examples of PGHS isozyme inhibitors

SELECTIVE PGHS-1 INHIBITORS	NONSELECTIVE PGHS INHIBITORS	SELECTIVE PGHS-2 INHIBITORS	HIGHLY SELECTIVE PGHS-2 INHIBITORS
<p>Low dose aspirin</p> <p>Valeryl-SA</p>	<p>High dose aspirin</p> <p>Indomethacin</p> <p>Piroxicam</p> <p>Diclofenac</p> <p>Ibuprofen</p>	<p>Meloxicam</p> <p>Salicylate</p> <p>nimesulfide</p>	<p>Celecoxib</p> <p>Rofecoxib</p> <p>Etodolac</p>

phenylsulphonamide moiety. Selective inhibition of PGHS-2 is a slow, time-dependent process whose kinetics seem to be correlated with the molecular complexities that accompany the penetration of a portion of the inhibitor into this new pocket of the inducible enzyme. Because this pocket is restricted in PGHS-1, PGHS-2 selective NSAIDs inhibit the constitutive enzyme in a simple competitive manner (86).

1.3.7 Phenotypes of PGHS-1 and PGHS-2 deficient mice

In 1995, Langenbach and Morham published complementary studies in *Cell* detailing the construction and phenotypic characterization of transgenic mice deficient in PGHS-1 and PGHS-2 (153,154). Prior to these studies, it was generally assumed that a PGHS-1 deficient mouse would suffer greater pathology than a mouse lacking PGHS-2 because PGHS-1 is expressed at some level in almost every tissue. However, to everyone's surprise, it was PGHS-2 gene ablation, not PGHS-1 disruption, that caused the most severe physiologic defects in mice (155).

Morham *et al* showed that homozygous mice deficient in PGHS-2 ($ptgs2^{-/-}$) rarely survived past 16 weeks due to aborted kidney development. Kidney maturation ceased after birth, with only a small percentage of nephrons developing. The vast majority of glomeruli and tubules remained small and immature. All other tissues examined in these PGHS-2-deficient mice were normal (153). In contrast, in the PGHS-1 knockout mice engineered and studied by Langenbach *et al*, renal structure and function was sound (154). Another unexpected observation made by Langerbach's group was the

absence of stomach ulceration in the PGHS-1 deficient mice. Furthermore, the stomachs of these mice displayed a reduced sensitivity to indomethacin induced ulceration (154).

Langerbach's PGHS-1 deficient mice also demonstrated a reduced responsiveness to arachidonic acid in the ear swelling assay, a measure of acute inflammation (154). Though the current model of inflammation attributes the formation of inflammatory prostaglandins to PGHS-2, the process does involve an early response phase that happens prior to two hours, during which time inflammatory cells are recruited and an edema forms. Apparently, prostaglandins produced by PGHS-1 play an essential role during these early events. The arachidonic acid ear inflammation assays performed by Langerbach *et al* were scored at 2 hours. At later times, however, as monocytes are recruited and cytokine production is increased, PGHS-2 is induced, and prostaglandins made by the second isozyme likely predominate the inflammatory reaction. The results obtained from Langerbach's ear-inflammation assays make sense in that PGHS-1 is the only enzyme present when the inflammatory response begins. A significant induction of PGHS-2 does not occur at sites of inflammation until after two hours (155). Thus, it could be predicted that mice deficient in PGHS-2 would have a normal response to the same 2 hr. arachidonate ear-swelling assay. This is in fact what Morham *et al* found (153).

As anticipated, Langerbach *et al* found that platelets extracted from PGHS-1 deficient mice were unresponsive to arachidonic acid induced aggregation, confirming the idea that only PGHS-1 is expressed in blood platelets (154). Because all platelets are anucleated, the cells that Langerbach's group isolated from the PGHS-1 deficient mice had no means of expressing PGHS-2 to compensate for the deleted PGHS-1.

Perhaps the most serious deficiency suffered by the PGHS-1 knockout mice was their inability to produce viable offspring when homozygotes ($Ptgs1^{-/-}$) were crossbred. Heterozygotes ($Ptgs1^{+/-}$) of either sex, when mated with homozygotes ($Ptgs1^{-/-}$), were able to produce healthy offspring (154). Therefore, PGHS-1 is an essential requirement for parturition, but not for ovulation or spermatogenesis. Recent studies have suggested that pup death is caused by dystocia, a result of the lengthening of female gestation by one to two days (155). PGHS-2 deficient mice are infertile due to ovulatory failure (153).

The development of mice engineered to be deficient in either PGHS-1 or PGHS-2 has produced some unexpected results regarding the roles played by each isozyme in the developing and adult organism. Moreover, these PGHS gene knockout experiments have reaffirmed the hypothesis that PGHS-1 and -2 are not redundant.

1.3.8 The pathophysiology of impaired TxA_2 formation

In platelets, any biochemical defect that occurs along the axis of thromboxane formation has the potential to disrupt TxA_2 synthesis and throw hemostasis out of balance. It has been demonstrated that certain mild bleeding disorders, which are associated with the impaired formation of TxA_2 , result from a deficiency in the human PGHS-1 protein (305,335). Matijevic-Aleksic *et al* have demonstrated two kinds of PGHS-1 dysfunction. There is a type I defect, manifested by an absence of PGHS-1 protein, and a type II defect, caused not by a lack of enzyme, but instead by a subnormal PGHS-1 catalytic activity (305). In both cases, platelet aggregation in response to adenosine diphosphate (ADP), collagen and epinephrine is subnormal. Furthermore,

platelets marked by such a defect undergo an abnormal second-wave aggregation, and also have a propensity for disaggregation (305).

Recently, Drouin and colleagues characterized a new type of PGHS-1 dysfunction (335). In this case, PGHS-1 enzyme is present in platelets, albeit in greatly reduced amounts. Nevertheless, the enzyme retains its catalytic activity (335). Patients with this type of PGHS-1 deficiency suffer from bleeding diathesis (335).

The inability of platelets to actively synthesize TxA_2 can also result from defects in TXS itself. A number of researchers have documented patients who have an increased tendency to bleed because they possess a partial deficiency in platelet thromboxane synthase (306-308).

Finally, an impaired platelet aggregatory response can occur when a defect exists in the intracellular signaling process that normally becomes activated after TxA_2 binds to its receptor on the platelet surface. Hirata *et al* have characterized a TxA_2 receptor defect in which the single amino acid substitution of Arg60 to Leu in the first cytoplasmic loop of the molecule results in an impaired platelet response to TxA_2 (309). This defect has no impact on the affinity with which TxA_2 binds to its receptor; instead, the mutation results in decreased GTPase and second messenger activity on the cytoplasmic side of the membrane (309-312). This amino acid substitution has the overall effect of lowering the level of PLC activation following the binding of TxA_2 to its receptor (309-312).

1.4 THE BIOLOGY OF MEGAKARYOCYTES

1.4.1 Megakaryocyte differentiation

Megakaryocytes are the cells found in bone marrow that release platelets into the general circulation (17). The process of platelet release is only the final part of a continuum of developmental events marked by the differentiation of a less mature blast cell into a fully mature megakaryocyte (17,18). Termed megakaryopoiesis, this differentiation process may also be defined as the sequential or coordinate expression of platelet proteins from a committed progenitor to platelets (156). The cellular hierarchy of megakaryocytes can be artificially divided into three developmental stages: progenitor cells, immature megakaryocytes (promegakaryoblasts; PMkB), and mature megakaryocytes (157). Figure 1.5 is a schematic illustrating the various stages of megakaryopoiesis.

1.4.1.1 Megakaryocyte progenitors

Megakaryocyte progenitor cells are a heterogeneous population that consists of at least three distinct cell types, each of which has a different capacity for proliferation. *In vitro* studies have shown that as differentiation proceeds, megakaryocyte progenitor cells progressively lose their proliferative potential (157). The most primitive progenitor cell in the megakaryocyte lineage is the megakaryocyte high-proliferative-potential colony-forming cell (Mk-HPP-CFC). This cell type propagates *in vitro* to the extent that its

Figure 1.5 Cellular hierarchy of megakaryocyte maturation. As they differentiate, megakaryocytes can acquire a DNA content of up to 128N, where 2N is the DNA content of a normal somatic cell. (Adapted from Abraham, H., *Stem Cells*, 11:499-510, 1993.)

colonies are macroscopically visible (157). The existence of the MK-HPP-CFC has recently been documented by the groups of Long (158,159), Quesensberry (160), and Bruno (161). Such cells are primitive and respond to a variety of hematopoietic regulators. Mk-HPP-CFCs produce colonies of a few thousand megakaryocytes (157).

The burst-forming unit-megakaryocyte (BFU-Mk) is more mature than the Mk-HPP-CFC; however, it retains a high proliferative capacity, being able to develop multiple 'bursts' of megakaryocyte clusters consisting of approximately 100 to 500 cells (162). BFU megakaryocytes, which are believed to be the progeny of the Mk-HPP-CFC, are the immediate ancestors of the colony-forming cell (158). *In vitro* BFU-Mk colonies morphologically resemble their erythroid counterparts in that they consist of multiple foci of development, each of which is presumably due to the presence of a single colony forming unit megakaryocyte (CFU-Mk) (157).

The most differentiated of the megakaryocyte progenitor cells, the CFU-Mk, was the first to be assayed *in vitro* (163-166). CFU-Mks have restricted proliferative potential and generate only 4 to 32 megakaryocytes (167). Increases in CFU-Mk numbers in response to thrombocytopenia occur long after platelet numbers have decreased, suggesting that these cells are somehow regulated by megakaryocyte and not platelet mass (168,169).

1.4.1.2 Immature megakaryocytes

Promegakaryoblasts are transitional cells that occupy an intermediary position between proliferating progenitor cells and post-mitotic, mature megakaryocytes

(170,171). Although immature megakaryocytes are not easily observed *in vitro* or in bone marrow specimens, they can be identified by their expression of megakaryocyte/platelet-specific markers such as platelet peroxidase, platelet glycoprotein IIb/IIIa, and von Willebrand's factor (172). Because they are at the developmental stage where megakaryocytes increase their DNA content, PMkBs have a restricted or entirely absent proliferative potential. Thus, immature megakaryocytes are said to be endomitotic (a mechanism of acquiring polyploid nuclei, *vide infra*) (157). A number of animal studies have documented the responsiveness of PMkBs *in vivo*. PMkBs react quickly to thrombopoietic demand; they are the first cells to increase in number during thrombocytopenia, and are the first to decrease in number during thrombocytosis (170,173). Subsequent increases or decreases in the number of mature megakaryocytes have also been documented, confirming the developmental relationship between PMkBs and their more differentiated progeny (157).

1.4.1.3 Mature megakaryocytes

Morphologically, three to four stages of mature megakaryocytes can be identified. The earliest morphologically recognizable cell within this group is the stage I megakaryocyte, also known as the megakaryoblast. Megakaryoblasts have a high nucleus to cytoplasm ratio, and a scanty, basophilic cytoplasm resulting from a high level of protein synthesis. The stage II megakaryocyte, or promegakaryocyte, exhibits an increased nuclear and cytoplasmic volume, as well as an increased number of platelet-specific granules. Granular or 'platelet-shedding' megakaryocytes make up stages III and

IV. They are the most mature cells, possessing an abundant, fully developed cytoplasm, and a large, eccentrically located nucleus that is distinctly lobulated (157).

1.4.2 Anatomical location

In adult humans, the principal hematopoietic tissues that produce blood cells are the bone marrow, thymus, lymph nodes, mucosa-associated lymphatic tissue (MALT), spleen, the immunologically competent pool of recirculating lymphocytes (RLP), and blood itself (174-179). The marrow contains the vast majority of hematopoietic stem cells and provides the diverse microenvironments that induce their differentiation into each type of blood cell (178,180-182). Bone marrow can be viewed as having two components: a stroma, or supporting meshwork of cells that includes reticulocytes, adipocytes, macrophages, and endothelial cells, and a hematopoietic compartment made up of stem and progenitor cells, along with their progeny. At the marrow center is an area known as the vascular compartment, which is composed of large, thin-walled veins termed vascular sinuses (183). Mature megakaryocytes reside against these sinuses but originate in deeper areas of the marrow as small lymphoid-like cells (184). As the megakaryocytes develop, they move to the outside surface of the vascular sinus where, in that hematopoietic environment, they continue to differentiate into mature cells. It has been suggested that during the early stages of megakaryocyte differentiation, reticular or barrier cells reach out from their pericytic location in the wall of a venous sinus to a megakaryocyte some distance away, bringing the progenitor to its characteristic mural position (184). Set against an aperture wall, a mature megakaryocyte may deliver

platelets efficiently into the vascular sinus, while at the same time, 'stoppering' the fissure so that the vessel remains competent (184). On the other hand, completion of megakaryopoiesis may require emigration of the megakaryocyte from the marrow into other tissues, such as the lung (185).

1.4.3 Nuclear maturation

Megakaryocytes, along with certain plant and insect cells, undergo an altered cell cycle marked by polyploidization (186,187), a process which can be defined as the acquisition of elevated DNA content by a cell regardless of the mechanism by which such changes occur. Megakaryocytes are unique among mammalian marrow cells in that they leave the diploid state ($2N$) to differentiate, synthesizing 4 to 64 times the normal DNA content within a single nucleus. The process of polyploidization occurs during the differentiation of these cells from an immature blast stage into a fully mature megakaryocyte that is capable of releasing platelets. Thus, megakaryocyte cell cycle control is atypical in the sense that it does not have a normal $2N \rightarrow 4N \rightarrow \text{mitosis}$ progression. Nevertheless, polyploidization is tightly regulated. During each replication event, the entire DNA content is duplicated. As a result, megakaryocytes contain multiples of the normal diploid DNA content; they are either $4N$, $8N$, $16N$, $32N$, or $64N$, where $2N$ is the normal DNA content of a cell in the G_0/G_1 phase. Thus, even though mitosis is dissociated from the completion of S-phase, global control of DNA replication is retained (188).

1.4.4 Markers of megakaryocyte differentiation

Megakaryocytes and their precursors express a number of developmentally regulated cell surface antigens. One such marker, CD34, is present on all hematopoietic progenitor cells. CD34 is expressed on megakaryocyte progenitors from the BFU-Mk to the megakaryoblast (157). As CD34 expression decreases and eventually disappears, surface antigens specific to megakaryocytes and platelets begin to be expressed at detectable levels. One such megakaryocytic marker, glycoprotein IIb/IIIa (GP IIb/IIIa or CD41), is expressed on cells from approximately the CFU-Mk stage through to the mature megakaryocyte, and finally on to platelets (172,189). Another surface antigen whose expression increases as megakaryocyte differentiation proceeds is glycoprotein Ib (GP Ib or CD42). Like GP IIb/IIIa, GP Ib is thought to be present at high levels on the surface of mature megakaryocytes and platelets. However, GP Ib expression follows that of GP IIb/IIIa; that is, GP Ib first appears on megakaryocytes that have proceeded slightly further down the maturational pathway than those initially expressing GP IIb/IIIa (190-193).

1.4.4.1 Glycoprotein IIb/IIIa

GP IIb/IIIa is a heterodimeric protein that serves as the platelet receptor for fibrinogen (194-197). GP IIb/IIIa is a member of the integrin family of surface adhesion molecules, and as such, possesses an α (α_{IIb}) and a β (β_3) subunit, each of which is encoded by a separate gene located on chromosome 17 (198). The β_3 (β_{IIIa}) integrin

subunit is not specific to megakaryocytes. It is also found in osteoclasts, endothelial cells, placenta cells and macrophages. However, GP IIb mRNA has only been detected in megakaryocytes (199). Within a megakaryocyte, neither subunit accumulates in isolation. That is, the processing of the α and β subunits is tightly controlled so that GP IIb/IIIa is exposed to the cell surface as a fully formed heterodimer (199). Although the 5' flanking region of the α IIb gene does not have an obvious TATA box, it does possess *cis* elements that have recently been shown to recognize the transcription factors Sp1 and fli-1 (199-201). Deficiency or dysfunction of platelet membrane GP IIb/IIIa results in a rare bleeding disorder known as Glanzmann's thrombasthenia (202).

1.4.4.2 Glycoprotein Ib

GP Ib is a major component of the platelet membrane receptor for von Willebrand factor. Designated the GP Ib-IX-V complex, this receptor is actually made up of four membrane spanning polypeptides: the disulfide-linked GP Ib α and GP Ib β , and the noncovalently associated GP IX and GP V (203). These subunits possess the alternative cluster designation nomenclature: GP IX = CD42a, GP Ib α = CD42b, GP Ib β = CD42c, GP V = CD42d. The GP Ib-IX-V complex is thought to play a critical role in the aggregation and adhesion of platelets at sites of blood vessel injury where fluid shear forces are high (203). The GP Ib component of the von Willebrand receptor consists of two subunits, GP Ib α and GP Ib β , each of which is synthesized by a separate gene. The gene encoding GP Ib α has been localized to chromosome 17 (204). It has recently been

shown that Ets* and GATA elements within the 5' flanking regions of each subunit gene play a key role in the transcriptional regulation of GP Ib, GP IX and GP V (205-207). Deficiency or dysfunction of the platelet GP Ib-IX-V membrane complex results in Bernard-Soulier syndrome, a disease manifested by prolonged bleeding time, very large platelets, and thrombocytopenia (208-210).

1.4.5 Mechanism of platelet release

1.4.5.1 The demarcation membrane system (DMS)

Megakaryocytes possess a smooth membrane system that is distinct from the endoplasmic reticulum. Termed the demarcation membrane system (DMS), this megakaryocyte-specific organelle is in continuity with the extracellular space and divides the cytoplasm of mature cells into 'platelet fields' (211-214). Combined cytophotometric, autoradiographic, and ultrastructural studies have indicated that the DMS begins to form at the onset of endomitosis and DNA replication (215), features that correspond to the megakaryoblast stage of development. However, in promegakaryoblasts, some smooth tubules at the region adjacent to the Golgi apparatus could represent the first development of the DMS (18). At the present time, the true origin of the DMS remains unknown. For many years, the majority of investigators assumed that the DMS delineated platelet territories and that its membranes constituted the plasma membranes of future platelets. According to this model, putative platelets

* An Ets (~~E~~-twenty-six) element is a DNA sequence that possesses a consensus core of 5'-GGAA/T-3' that is recognized by the Ets family of transcription factors, namely Tel, Erg, Ets-1, Ets-2 and Fli-1.

would fragment from the DMS and enter the circulation from the bone marrow. However, because the effort to construct a 3-D model showing how a flat membrane can arise from a tubular membrane has so far been unsuccessful, the true mechanism of platelet release remains unknown (18). An alternative system of platelet formation has been postulated; it is termed the mechanism of proplatelet release.

1.4.5.2 Proplatelet release

It has been shown that megakaryocytes send out long cytoplasmic extensions into marrow sinuses, giving mature cells an octopus-like appearance (214,216,217). Wright was the first to demonstrate that these filaments can liberate platelets by fragmenting at constrictions that occur along their lengths at irregular intervals (218,219). According to this concept, the DMS is not involved in platelet release.

1.4.6 Platelets

Because the number of platelet precursor cells is very low compared to the number of platelets in circulation, researchers have estimated that one megakaryocyte can produce up to 3000 platelets (217,338). Overall, the body's total daily platelet production is on the order of 2×10^{11} (220). Platelets were first described and illustrated in the late 19th century by William Oster and Georges Hayem (313). In 1882, in addition to coining the term platelet (Blutplättchen), Julius Bizzozzero noted the shape change that these cells undergo when they become activated, and described their involvement in hemostasis

(313). In a normal person, quiescent discoid platelets spend on average 7-10 days in the circulation before they are removed by the reticuloendothelial system (282). Although not spherical, platelets can be considered to have a diameter of about 2-3 μm (213,314). Figure 1.6 is a schematic representing the ultrastructural features of a typical resting platelet. Each cell contains a single peripheral microtubule coil (315), which, along with a subplasmalemmal actin membrane skeleton (316), likely serves to control the discoid shape of the platelet. Peripheral to the microtubule coil, the platelet cytoplasm is poor in organelles, save for the plasma membrane itself and its surface invaginations, otherwise known as the surface-connected canalicular system (SCCS) (282). Both membranes are richly invested by a glycocalyx that is comprised of several integral membrane glycoproteins: GP Ib/IX, GP V, GP IIb/IIIa, and GP IIIb, all of which participate in platelet adhesion and aggregation (282). Within the confines of their microtubule coil, platelets contain secretory α and dense granules, mitochondria, peroxisomes, lysosomes, stores of glycogen, and the two membrane systems, the SCCS and the dense tubular system (282).

Platelets can influence hemostasis in a number of ways. The main prostanoid that they synthesize, TxA_2 , is a potent vasoconstrictor that activates other platelets, as well as vascular smooth muscle cells (317). In contrast, vascular endothelial cells synthesize prostacyclin, which is a potent platelet antagonist and a vasodilator (46,318). The equilibrium between the amount of TxA_2 and prostacyclin present in the circulation therefore influences how readily platelets can become activated, and also influences vascular muscle tone (59).

Figure 1.6 A schematic of platelet organelles. (Adapted from Bentfeld-Barker *et al*, *J. Ultrastruct. Res.* 61:201, 1977.)

Perhaps the best characterized and most important role of platelets in hemostasis is their participation in the phenomenon of blood coagulation. Platelets ordinarily circulate in blood vessels as individual entities that interact with neither each other, nor with other cell types (319). However, if exposed to the appropriate stimulus, platelets undergo a rapid transition from a nonadhesive to an adhesive state (319). Any disruption in the endothelial wall that occurs following a vascular injury will cause platelets to become activated as they come into contact with the negatively charged constituents of the ECM that underlie the damaged layer. Once adherent to the subendothelium, the platelets spread out on its surface, and additional platelets, brought to the scene by flowing blood and chemoattractants secreted from the activated cells, adhere first to the basal layer, and eventually to one another, forming a mass of aggregated platelets (319). This 'platelet plug' helps to stopper the leak and provides a large surface area of negatively charged membrane upon which the reactions of the clotting cascade, a process which ultimately leads to the formation of a fibrin plug, can take place. Many of the enzymes necessary for coagulation are contained within platelet granules and are secreted by the cells upon their activation (31,282,319).

As the endothelial cells bordering the lesion begin to divide, the platelet mass actually contracts, helping to bring the endothelial cells at either end of the fissure into contact (282). Activated platelets ultimately release factors that help to terminate the coagulation phase, and release enzymes that dissolve the platelet-fibrin plug. Platelet dense granules contain agonists such as ADP, ATP, serotonin and calcium. Platelet α -granules contain adhesive proteins like fibrinogen, fibronectin, vWF, thrombospondin, and vitronectin; growth modulators such as PDGF, PF 4, CTAP, thrombospondin, and

TGF Beta, and the following coagulation factors: Factor V, HMWK, C1 inhibitor, fibrinogen, Factor XI, protein S and PAI-1. Within their cytoplasm platelets contain Factor XIII and PDECGF; they also possess lysosomes (319).

In addition to playing a key role in thrombosis and hemostasis, blood platelets also take part in the coordinate processes of inflammation and subsequent tissue repair. For this purpose, platelets collaborate closely with all types of leukocytes (320). Activated platelets secrete chemotactic substances, facilitate the binding of leukocytes to the endothelium, along with their subsequent extravasation, and may also influence the inflammatory responses of leukocytes in both stimulating and inhibiting ways (320). Because platelets themselves contain an array of potent proinflammatory substances, they can be regarded as mediator and effector cells in the inflammatory process. Platelets also secrete IgE, a phenomenon that provides a functional basis for their involvement in allergic responses, particularly in the skin and airways (320).

1.4.7 Regulation of megakaryopoiesis

Megakaryocyte development is a complex process in which a variety of regulatory signals work in concert to direct a highly specific response to thrombopoietic demand. The complex nature of the megakaryocyte developmental hierarchy is underscored by the diversity of hematopoietic factors that are able to stimulate these cells. Thrombopoiesis is further modulated by the interaction of megakaryocytes with extracellular molecules and neighboring stromal cells. Each of these key elements defines a highly organized and localized regulatory system known as the megakaryocytic

microenvironment, a system that coordinately regulates megakaryocyte development and the daily production of approximately 2×10^{11} platelets (220).

1.4.7.1 Hematopoietic factors

Stromal cells, resident bone marrow macrophages, and megakaryocytes themselves produce a variety of cytokines and hematopoietic factors that regulate megakaryopoiesis (221). The most important factor involved in this regulatory process is thrombopoietin (TPO), a polypeptide that exerts its biological effects through the receptor c-mpl (222). C-mpl is a proto-oncogene that belongs to the same hematopoietic cytokine receptor family as glycoprotein 130 (GP 130) (223,224). It is expressed in hematopoietic tissues, particularly in CD34+ hematopoietic progenitor cells, megakaryocytes, and platelets (224). TPO, the c-mpl ligand, is detected primarily in the liver, kidney and smooth muscle, with lesser amounts present in the spleen and bone marrow (225). It has been shown that daily infusions of TPO into mice or nonhuman primates induces a marked increase in platelet counts and in the number of megakaryocytes and megakaryocyte progenitor cells (226,227). Furthermore, c-mpl and TPO-deficient mice generated by gene targeting exhibit a striking decrease in their number of platelets and megakaryocytic progenitor cells (228,229). These findings indicate that the TPO-c-mpl system is a physiological regulator of platelet and megakaryocyte production. Upon binding to TPO, c-mpl transmits a series of biochemical events including the tyrosine phosphorylation and activation of the Janus family of protein tyrosine kinases (JAKs), the activation of signal transducers and activators of transcription (STATs), along with the

activation of phosphatidylinositol 3-kinase (PI 3-kinase), and Shc (230-234). It has recently been demonstrated in megakaryocytes that ligand binding to c-mpl activates STAT5, which in turn increases the expression of p21, thereby inducing differentiation (235).

Like TPO, the glycoprotein IL-3 is a potent hematopoietic factor capable of inducing megakaryocyte proliferation (236-238). However, unlike TPO, it possesses a limited capacity to induce megakaryocyte differentiation. Although exogenous IL-3 stimulates colony expansion of the three classes of megakaryocyte progenitor cells *in vivo*, this cytokine has little effect on platelet production (239). The pleiotropic cytokine GM-CSF also functions as a megakaryocyte colony stimulating factor, although with an activity approximately 100 times less potent than that of IL-3 (238). The actions of GM-CSF and IL-3 are additive (240).

Glycoprotein 130 is the common signal transducing component for the interleukin 6 (IL-6) family of functional receptor complexes. The cytokines that recognize this class of receptor are IL-6, IL-11, leukemia inhibitory factor (LIF), oncostatin M, ciliary neurotrophic factor, and cardiotrophin-1 (241). Specifically, SCF, IL-1 α , IL-6, and IL-11 function as co-regulators that serve to augment megakaryocyte size, DNA content and antigen expression (237,243-245). Out of this group, IL-6 and IL-11 seem to have the greatest effect on megakaryocyte maturation *in vivo*. Neben *et al* have shown that in mice, the administration of recombinant IL-11 results in increased numbers of megakaryocyte progenitors, increased megakaryocyte ploidy, and increased peripheral platelet counts (246). Studies employing IL-6 have yielded similar results (245,247-254).

The current model describing the regulation of megakaryopoiesis depicts different combinations of cytokines acting in synergy over the course of megakaryocyte development to induce a high level of cellular differentiation. It is not known if such factors have a biochemical role in the terminal phase of megakaryocyte differentiation, that is, in platelet release. However, there is an increasing body of evidence to support the notion that cell-cell and cell-matrix interactions play key roles in the regulation of megakaryocyte differentiation and platelet release.

1.4.7.2 Cell adhesion and cell-cell interactions

In the bone marrow environment, developing megakaryocytes have the potential to interact with extracellular matrix components like collagen, fibrinogen, and fibronectin, in addition to neighboring stromal cells such as macrophages and endothelial cells. Various groups have demonstrated the importance of megakaryocyte adhesion to the molecular constituents of the ECM through different cell surface receptors. In 1995, Leven showed that guinea pig megakaryocytes interact with type I collagen through the vitronectin receptor (VnR) to release proplatelets in a cAMP-dependent manner *in vitro* (255). He also demonstrated that megakaryocytes stimulated with either thrombin or TPA attach and spread to plastic through the GP IIb/IIIa receptor in a process marked by increased PKC activity (255). Berthier *et al* have shown that *in vitro*, human megakaryocytes stimulated with TPO adhere specifically to fibronectin through $\beta 1$ integrins and subsequently undergo events that appear to be indicative of terminal differentiation (256,257). Zauli *et al* have shown that TPO enhances the GP IIb/IIIa-

dependent adhesion of megakaryocytic cells to fibrinogen and fibronectin through PI-3-kinase (258).

Megakaryocytes also make important contacts with bone marrow stromal cells. Hagiwara *et al* have shown that PECAM-1 (CD31), an adhesion molecule which is a member of the immunoglobulin superfamily, accumulates selectively at the tip of megakaryocyte cytoplasmic extensions. They have suggested that the homodimerization of CD31 at this location with CD31 present on the surface of endothelial cells may be important for the extension of these processes and for the migration of megakaryocytes (259). A different team of researchers, lead by Hava Avraham, has also studied the adhesive interactions between megakaryocytes and endothelial cells. They demonstrated that human umbilical vein endothelial cells (HUVECs) treated with either INF- γ or the phorbol ester TPA adhere to megakaryocytes in a time and dose-dependent manner. Similarly, when Avraham *et al* stimulated CMK cells with the cytokines IL-1 β , GM-CSF, IL-6, and IL-3, and with TPA, the megakaryocytes adhered to the endothelium. In both cases, adhesion promoted megakaryocyte maturation as measured by increases in the expression of GP Ib and GP IIb/IIIa, and by an increase in cellular DNA content (260). Finally, research by Yuka Nagata and colleagues has revealed that megakaryocytes co-cultured with stromal cells and TPO differentiate and adhere to the stroma *in vitro*. Interestingly, in this system, although attachment induced the formation of beaded, cytoplasmic processes, adhesion actually inhibited proplatelet release compared to free megakaryocytes treated with TPO (261). From these examples, it is clear that both cytokine signals and the state of megakaryocyte adhesion play critical

roles in the regulation of thrombopoiesis. However, the exact biochemical mechanism by which such factors induce platelet formation remains unknown.

1.4.8 Molecular control of megakaryocyte development

The exact nature of the master control genes regulating megakaryocyte lineage-specific development remains obscure. Nevertheless, a number of *cis*-regulatory DNA elements within the control regions of megakaryocyte-specific genes have been identified. Through extensive work, Orkin *et al* have shown that the DNA sequence GATA is an important control element for erythroid and megakaryocyte development (262). Two transcription factors that recognize this sequence, GATA-1 and GATA-2, are found in megakaryocytes (262,263). Transfection studies have shown that although GATA-1 is involved in certain aspects of megakaryocyte development, over expression of this factor is not sufficient to drive full differentiation (157,264). GATA-1 null mice develop marked thrombocytopenia, deregulated megakaryocytic proliferation, and deficient cytoplasmic maturation (265).

Another transcription factor, NF-E2, is important in the later phases of megakaryocyte development. NF-E2 is an obligate heterodimer that consists of a 45 kD subunit that is restricted to hematopoietic cells, and a ubiquitously expressed 18 kD subunit (266). The p45 subunit is co-expressed with GATA-1 in hematopoietic cells, megakaryocytes and mast cells. Mice engineered to be deficient in p45 exhibit high mortality due to hemorrhage that occurs secondary to thrombocytopenia. Moreover, the megakaryocytes from p45-null mice undergo cytoplasmic arrest, lack platelet granules,

and possess a DMS that is reduced in quantity and has an altered structure (266). It has recently been shown that the thromboxane synthase (TXS) gene is directly regulated by p45 NF-E2 (267).

Orkin *et al* have recently demonstrated a functional relationship between GATA-1 and NF-E2 in megakaryocyte development. GATA-1 specifically interacts with a novel zinc-finger protein known as Friend of GATA (FOG) (268). The high specificity of this interaction suggests that the function of DNA-bound GATA-1 is to recruit FOG (and other factors) for the assembly of cell-specific transcriptional complexes. FOG is co-expressed with GATA-1 exclusively in megakaryocytic and erythroid cells. Moreover, GATA-1 and FOG act in synergy to activate the p45 NF-E2 gene (268).

1.5 SUMMARY, SPECIFIC AIMS AND EXPERIMENTAL MODEL

Cardiovascular disease, such as myocardial infarction and stroke, is the leading cause of death in North America. The therapeutic basis of low-dose aspirin administration for the treatment and prevention of cardiovascular disease is inhibition of PGHS-1 in platelets. At present, there are few safe alternatives to such therapy. However, despite its effectiveness, the long-term administration of aspirin is not without side effects. Gastric ulceration and renal toxicity are just two of the possible side effects that can arise from the inhibition of prostanoid formation. Furthermore, there are many bleeding disorders that are marked by the inability of platelets to aggregate properly, as well as conditions that result from a decrease in the total number of platelets in the general circulation. In light of such facts, there is an urgent need to understand how

PGHS-1 expression is regulated during platelet production, and more importantly, to understand how the process of thrombogenesis is coordinated so that new treatments capable of targeting abnormal thrombosis and hemostasis may be developed.

Because platelets lack a nucleus, their protein content is a consequence of gene expression in their precursor cell, the megakaryocyte. One cannot understand the expression of PGHS-1, or any other platelet enzyme for that matter, without understanding megakaryocyte differentiation. It is to this end that our overall goal is the characterization of PGHS-1 expression during the differentiation of megakaryocytes into platelets. Specifically, this Master's project has had two objectives:

1. The characterization of PGHS-1 protein and mRNA expression relative to platelet specific antigens that are known to be markers of megakaryocyte differentiation.
2. The identification of endogenous factor(s) that are capable of increasing PGHS-1 expression in megakaryocytes.

The study of megakaryopoiesis is difficult because megakaryocytes are extremely rare. Since they only make up about 0.05% of the total bone marrow population, megakaryocytes cannot be obtained in sufficient quantity in a cost-effective manner (19). Furthermore, primary bone marrow megakaryocytes have a limited life span in cell culture.

In order to achieve our experimental goals, we have elected to use the chronic myeloid leukemic (CML) cell line MEG-01 (269) as a model system in which to study PGHS-1 expression because it has been previously shown that *in vitro* MEG-01 cells

undergo a form of differentiation that closely mimics the *in vivo* process of megakaryopoiesis (270-272). Furthermore, it has been demonstrated that the phorbol ester TPA is able to induce the cells of a related CML line, CMK, to differentiate into mature forms that express PGHS-1 (93). MEG-01 cells express the platelet specific antigens GP IIb/IIIa and GP Ib (269,273), both of which are known to be markers of maturation within the megakaryocytic lineage (18,20,274). GP IIb/IIIa is thought to appear on the surface of cells at the earlier stages of megakaryopoiesis (275,276) whereas GP Ib is thought to appear on the outer membrane of very mature megakaryocytes and platelets (190-193). Another important feature of the MEG-01 line is the observation that its cells exist as three populations: nucleated cells that float in the liquid culture media, nucleated cells that adhere to the bottom of the tissue culture flask, and anucleated platelet-like structures. It has been suggested that floating MEG-01 represent cells that are at the early stage of megakaryopoiesis, whereas adherent MEG-01 are cells that have differentiated into a slightly later stage (269).

To achieve our first experimental objective, we stimulated MEG-01 cells to differentiate into mature forms with nanomolar concentrations of TPA and then, through Northern and Western blotting, measured the amount of PGHS-1 protein and mRNA in cell lysates from each of the three MEG-01 populations. In addition, utilizing antibodies specific for PGHS-1, GP IIb/IIIa, and GP Ib, we performed indirect immunofluorescence to measure the expression of PGHS-1 *in situ* on a cell-by-cell basis. We then made a quantitative comparison between the expression of PGHS-1 protein and the expression of the GP IIb/IIIa and GP Ib antigens within cells of the floating and adherent MEG-01 populations by employing digital microscopy and computer-analysis software.

To meet our second experimental goal, we treated MEG-01 cultures with a number of different hematopoietic factors, either alone or in combination, and then measured the expression of PGHS-1 protein and mRNA in cell lysates by Western and Northern blotting.

SECTION II: MATERIALS AND METHODS

2.1 Chemicals and solutions

The following chemicals were obtained from Sigma-Aldrich (Oakville, ON): paraformaldehyde, saturated picric acid solution, saponin, poly-L-lysine and TPA (12-0-tetradecanoylphorbol-13-acetate). The prostanoid standards PGE₂, PGF_{2α}, and TxB₂ were bought from the Cayman Chemical Company (Ann Arbor, MI). Tween 20, bis-acrylamide, TEMED, and acrylamide were obtained from BioRad (Mississauga, ON). TRIzol reagent was purchased from GibcoBRL (Burlington, ON), while [³²P]-dCTP and 1-[¹⁴C]-arachidonic acid were bought from Amersham (Oakville, ON). Prime-It II Random Primer Labeling Kit was purchased from Stratagene (La Jolla, CA). Oligotex poly (A)+ mRNA extraction kit was bought from Quiagen (Chatsworth, CA). Permafluor mounting media was obtained from Lipshaw Immunon (Pittsburgh, PA). Propidium iodide was purchased from Molecular Probes (Eugene, Oregon). DAPI (4',6-diamidino-2-phenylindole) stock (2 mg/mL) was a gift from the lab of Dr. D. Brown (University of Ottawa, Dept. of Biology).

2.2 Hematopoietic factors

All hematopoietic factors were recombinant human peptides. The following agents were purchased from Boehringer Mannheim (Laval, PQ): IL-3, IL-6, IL-11, and GM-CSF. SCF was purchased from Sigma. IL-2, G-CSF, and EPO were gifts from Dr.

H. Atkins (Ottawa Regional Cancer Center). TPO was obtained from Genentech (San Francisco, CA). Hematopoietic factors were reconstituted in 0.2 μ m-filter-sterilized PBS/0.1% BSA (bovine serum albumin). Stock concentrations* were as follows: [IL-2] 5 μ g/mL, [IL-3] 5 μ g/mL, [IL-6] 5 μ g/mL, [IL-11] 5 μ g/mL, [G-CSF] 5 μ g/mL, [GM-CSF] 5 μ g/mL, [SCF] 10 μ g/mL, [EPO] 5 μ g/mL, [TPO] 5 μ g/mL.

2.3 Antibodies

Polyclonal rabbit-anti hPGHS-1 IgG (gift from Dr.'s W.L. Smith and D.L. DeWitt, University of Michigan) was raised against the ovine PGHS-1 amino acid sequence L272-Q283 (LMHYPRGIPPQ-C). This peptide is 100% identical to the corresponding human sequence. Antibody stock solution was at a concentration of 0.5 mg/mL. Polyclonal rabbit-anti hPGHS-2 IgG, also a gift from Dr. DeWitt, was raised against the human PGHS-2 amino acid sequence S584-K598. For Western blotting experiments, rabbit antiserum was immunoaffinity purified.

Monoclonal mouse anti-human platelet GP IIb/IIIa, CD41 (clone 5B12) and monoclonal mouse anti-human platelet GP 1b, CD 42b (clone AN51) were obtained from DAKO (Carpintera, CA). Both antibodies were supplied in liquid form as tissue culture supernatant (RPMI 1640 medium containing fetal calf serum) that had been dialyzed against 0.05 M Tris/HCl, pH 7.2, 15 mM NaN₃. The Ig concentration in the monoclonal

*Hematopoietic factor concentrations are reported as weight/volume ratios. Naturally occurring factors are glycosylated proteins; the agents we used were non-glycosylated recombinant peptides. Though it has been shown that glycosylation does not impact hematopoietic factor activity, it does make the molecular weights of naturally occurring compounds greater than those of recombinant peptides. Reporting the concentrations in molarity would therefore be inaccurate.

mouse anti-human platelet GP IIb/IIIa solution was 120 mg/L, whereas in the mouse anti-human platelet GP Ib stock it was 125 mg/L.

Cy3-conjugated secondary donkey-anti-mouse IgG (H+L) and rhodamine conjugated donkey-anti-rabbit (H+L) were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Each had a stock IgG concentration of 0.6 mg/mL. The antibodies, supplied in powder form, were reconstituted with 0.8 mL water/glycerol (1:1). This solution contained 0.01M sodium phosphate; 0.15M NaCl, pH 7.6; 15 mg/mL Bovine Serum Albumin; 0.01% Thimerosal, and 0.05% Sodium Azide. Goat anti-rabbit and anti-mouse IgG (H+L) horseradish peroxidase conjugates were purchased from Promega (Nepean, ON).

2.4 Cell culture

MEG-01 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA).

2.4.1 Culture preparation for the study of PGHS-1 expression during MEG-01 differentiation

Cultures slated for immunolabelling studies on their adherent cellular compartment were seeded at a concentration of 2×10^5 cells/mL into Falcon 6-well tissue culture dishes containing 18 mm^2 glass coverslips. The cells were grown in 5 mL of RPMI 1640 supplemented with 10% FBS. Cultures slated for immunofluorescent studies on their floating cell population were seeded at a density of 2×10^5 cells/mL into 75 cm^2

tissue culture flasks that contained 40 mL of RPMI 1640 supplemented with 10% FBS. For the initial PGHS-1 and -2 protein time course experiments, as well as for the PGHS-1 enzyme activity assay, MEG-01 cells were seeded at a density of 2×10^5 cells/mL into 75 cm² tissue culture flasks that contain 40 mL of RPMI 1640 supplemented with 10% FBS. For all other Western and Northern blotting experiments, MEG-01 cells were seeded at a density of 2×10^5 cells/mL into 175 cm² tissue culture flasks containing 100 mL of media. In each case, the media was partially replaced with fresh RPMI 1640 supplemented with 10% FBS every 4 days. Prior to analysis, the cells were cultured in a humidified atmosphere of 5% CO₂/95% air at 37°C for two weeks. Where indicated, cells were treated with TPA dissolved in dimethylsulphoxide (DMSO) at a final concentration of 1.6×10^{-8} M. Control cultures were treated with DMSO alone.

2.4.2 Culture preparation for the screening of hematopoietic factors capable of inducing PGHS-1 expression

Different types of MEG-01 cultures were prepared for experiments designed to identify PGHS-1 inducing thrombopoietic factors. All cultures were seeded at a density of 2×10^5 cells/mL into 75 cm² tissue culture flasks containing 10 mL of RPMI, with or without FBS. FBS concentrations ranged from 1% to 10%. Prior to growth factor treatment, the cells were cultured in a humidified atmosphere of 5% CO₂/95% air at 37°C for 7 days. The media was partially replaced every 3 days with fresh RPMI 1640 containing the appropriate concentration of FBS. Prior to growth factor treatment, the floating MEG-01 were removed and the adherent cells gently rinsed with RPMI 1640 at 37 °C. Ten mL of fresh RPMI 1640 were added to the flask, along with different

hematopoietic factors. These cultures were then incubated in a humidified atmosphere of 5% CO₂/95% air at 37°C for a specified length of time. The second set of culture conditions was identical to the first, except that at the time of growth factor addition, the floating MEG-01 were not removed; instead, their media was partially replaced with RPMI 1640 and FBS. In the third set of culture conditions, floating MEG-01 cells were transferred into new 75 cm² flasks before being incubated with hematopoietic factors.

Hematopoietic factors were diluted from their stock concentrations in sterile PBS/0.1% BSA to the following final concentrations: [IL-2] 50 ng/mL, [IL-3] 50 ng/mL, [IL-6] 50 ng/mL, [IL-11] 50 ng/mL, [G-CSF] 50 ng/mL, [GM-CSF] 50 ng/mL, [SCF] 50 ng/mL, [EPO] 10 ng/mL, [TPO] 50 ng/mL.

2.5 Cell counts and viability

To achieve the correct seeding density, cells were counted on an inverted microscope coupled to a hemocytometer. Cell viability was assessed by staining samples with 0.4% trypan blue exclusion dye.

2.6 Cell isolation

2.6.1 Cells isolated for PGHS-1 and PGHS-2 protein time course experiments

Floating and adherent MEG-01 were collected together. Adherent cells were scraped from the bottom of the tissue culture flasks with a plastic policeman. The

harvested cultures were subsequently spun at 900g for 5 min. The media was then aspirated and the pellets washed three times by resuspension in 10 mL of 37°C PBS followed by centrifugation for 5 min. at 900g. After washing, pellets were resuspended in different volumes of 0.1M Tris, pH 7.4, and lysed by sonication for 5 sec. with a Microson Ultrason cell disrupter set at 80%. Protein concentrations in MEG-01 cell pellets suspensions were determined using the Bio-Rad Protein Kit, an assay based on the Bradford method (278).

2.6.2 Cells isolated for the study of PGHS-1 expression during MEG-01 differentiation

MEG-01 cells were isolated according to the plasma-rich platelet (PRP) protocol (277). Briefly, the floating fractions of TPA-treated and control MEG-01 cultures were collected and spun at 100g for 5 min. The supernatants obtained from this slow spin were then re-spun at 900g for 15 min. (fast spin). Upon completion of both slow and fast spins, individual pellets were washed three times by resuspension in 10 mL of 37°C PBS followed by centrifugation for 5 min. at 900g. After washing, pellets were resuspended in different volumes of 0.1 M Tris, pH 7.4 and lysed by sonication for 5 sec. with a Microson Ultrason cell disrupter set at 80%. The adherent fractions of TPA-treated and control MEG-01 cultures were scraped with a plastic policeman and resuspended in 40 mL of fresh media. The adherent cells were then harvested in exactly the same fashion as the floating MEG-01 cultures. Protein concentrations in MEG-01 cell pellets suspensions were determined using the Bio-Rad Protein Kit.

2.6.3 Cells isolated for the screening of thrombopoietic factors capable of inducing PGHS-1 expression

Following incubation with various hematopoietic factors, MEG-01 cultures were collected and spun at 900g for 5 min. The floating and adherent populations present in the different test groups were not separated. Following centrifugation, pellets were washed 3 times with 37°C PBS, resuspended in 0.1 M Tris, pH 7.4, and finally lysed by sonication for 5 min. The protein concentrations of the cell pellet suspensions were determined with the BioRad Protein Assay.

2.7 Protein determination

A dilution series was prepared from a BSA protein standard (Bio-Rad) that had been reconstituted in H₂O. Various amounts of BSA were mixed with water to a final volume of 20 µL. The final BSA concentrations calculated were 0.0 [calibration blank], 0.2, 0.3, 0.5, 0.7, and 0.9 mg/mL. One milliliter of 1X Coomassie Brilliant Blue Dye was then added to each tube and the resulting mixture incubated for 5-60 min. at room temperature. After the incubation period, the BSA standards were transferred to plastic cuvettes and the absorbency of each solution was read by a Beckman DU-65 spectrophotometer set at a wavelength of 595 nm. The resulting absorbencies were automatically transferred to a Quant II linear memory chip that calculated the standard concentration curve against which subsequent sample protein concentrations were determined.

The various test samples were prepared by diluting cell homogenates suspended in 0.1 M Tris, pH 7.4, to a final volume of 20 μ L in H₂O. For each test point, a duplicate sample at a different concentration was prepared. One mL of 1X Coomassie Brilliant Blue Dye was added to each tube and the resulting mixture incubated at room temperature for 5-60 min. The diluted samples were then transferred to plastic cuvettes and their concentrations calculated by the spectrophotometer equipped with the Quant II linear module. Finally, sample concentrations were manually corrected by the appropriate dilution factor, and the final concentration for each test point calculated as the average of two different dilutions.

2.8 Assay for PGHS-1 enzyme activity

Samples consisted of MEG-01 cultures treated with TPA (1.6×10^{-8} M) over eight days, and control MEG-01 cells treated with a DMSO vehicle. Both floating and adherent MEG-01 cells were harvested together. Adherent cells were scraped from the bottom of the tissue culture flasks with a plastic policeman. Cells were separated from the media by centrifugation at 900g for 5 min. The conditioned media was then aspirated and the pellets washed in PBS at 37°C. The resulting suspensions were centrifuged at 900g for 5 min., the supernatants aspirated, and the cell pellets resuspended in 500 μ L of fresh RPMI 1640 at 37°C. Next, 1-[¹⁴C] arachidonate was added at a final concentration of 10 μ M (42 μ Ci/mmol) to the samples which were subsequently incubated for 30 min. at 37°C. Following the incubation period, the cells were collected using a tabletop microcentrifuge and the supernatants transferred to glass test tubes. The cell pellets were

washed in PBS at 37°C and collected by micro-centrifugation. Supernatants were aspirated and the pellets resuspended in 0.1 M Tris pH 7.4. MEG-01 cells in the resulting suspensions were lysed by sonication for 5 sec. Sample protein concentrations were determined using the BioRad Protein Assay.

To the supernatants contained in the test tubes were added 1.5 mL of the lipid extraction solvent, diethyl ether/methanol/0.2 M citric acid (30:4:1,v:v:v). The tubes were capped and the samples mixed by hand before being centrifuged at 900g for 5 min. The resulting upper organic phase of each sample was transferred to a new tube; the lower aqueous phase was discarded. The organic solvent phase of each sample was then evaporated under a stream of N₂ in a water bath at 37°C. The evaporated lipid samples were then resolubilized in 20 µL of mobile solvent, ethyl acetate/2.2.4-trimethyl pentane/acetic acid (11:5:2,v:v:v).

Lipid samples were spotted onto separate lanes of a Silica Gel 60 thin layer chromatography (TLC) plate that had been saturated with the mobile solvent system. The following cold standards were also spotted onto the plate: PGE₂, PGF_{2α}, and TxB₂ (Cayman Chemical Co.). The plate was then placed in an air tight glass chamber that contained a large piece of Whatman filter paper and mobile solvent filled to a level just below the loading origin. Radioactive lipid samples and cold standards were then separated by TLC; the plate was removed from the chamber once the solvent had reached the top. The silica gel was air-dried and the cold standards were visualized under iodine vapor. After the standard locations were marked on the gel, the silica plate was exposed to Kodak XAR-5 autoradiographic film in a cassette for 48 hrs. After the exposure period, the plate was removed from the cassette and sample bands corresponding to TxB₂

were scraped from the plate and placed into vials containing liquid scintillation fluid. The amount of radioactivity in each sample was quantified by liquid scintillation counting. Signal density was expressed as a ratio of CPM (counts per minute) to μg protein.

2.9 SDS-PAGE and Western blot analysis

For all samples, 50 μg of protein lysate were diluted to a final volume of 50 μL in loading buffer (125 mM Tris-HCl, pH 6.8, 12.5% (v/v) 2-mercaptoethanol, 22.5% (v/v) glycerol, 5.8% (w/v) SDS, 0.25% bromophenol blue). Twenty-two micrograms of purified PGHS-1 or PGHS-2 protein standard were diluted to a final volume of 30 μL in the same concentration of loading buffer. Prior to loading, protein samples and PGHS protein standards were boiled for 3 min.

All samples were loaded onto a vertical, Bio-Rad Mini-Protean II polyacrylamide gel system prepared according to the method of Laemmli (279). The upper stacking gel consisted of 5% acrylamide, 0.14% bis-acrylamide, 0.1% SDS, and 125 mM Tris-Cl, pH 6.9. The lower resolving gel was made up of 10% acrylamide, 0.14% bis-acrylamide, 0.1% SDS, and 375 mM Tris-Cl, pH 8.7. The upper electrode buffer was 0.05 M Tris-Cl, 0.38 M glycine, 0.1% SDS, while the lower electrode buffer consisted of 0.05 M Tris-Cl, pH 8.9, and 0.1% SDS. In addition to the protein samples and PGHS standards, 10 μL of molecular weight size marker purchased from BioRad were also loaded onto each gel. Prior to loading, the marker was heated at 37 °C for 1 min. When a current of 150 V was

applied to the system for 60 min., sample proteins separated according to molecular weight.

Following band separation, the protein in the gel was transferred at 150 V for 30 min. to a nitrocellulose membrane (Bio-Rad) using a Bio-Rad Mini Trans-Blot Electrophoretic Transfer Cell. The transfer buffer consisted of 45 mM Tris-Cl, 39 mM glycine, 0.037% SDS and 20% methanol.

Once the transfer was complete, the membranes were blocked overnight at 4°C in a solution of 0.1% Tween 20/TBS (tris buffer solution)/3% milk. Membranes were incubated with hPGHS-1 primary antibody at room temperature for 1.5 hours in a solution of 0.1% Tween 20/TBS/1% milk/0.02% NaN₃ and with secondary antibody for 1 hour in a solution of 0.1% Tween 20/TBS/1% milk. The primary antibody, polyclonal rabbit-anti-human PGHS-1 (hPGHS-1), was diluted 1:1900 (0.26 µg/mL). The secondary antibody, goat-anti-rabbit linked to horseradish peroxidase (Promega), was diluted 1:2000 (0.25 µg/mL). Following antibody incubation, membranes were washed in 0.1% Tween 20/TBS/1% milk and finally rinsed in TBS. To confirm band identity, protein samples were run against a hPGHS-1 standard. Protein bands were visualized using the Boehringer Mannheim Chemiluminescence Blotting Substrate detection system (POD) and photographed on Polaroid black and white Polapan 667 film. Human PGHS-1 protein bands were digitally scanned and their net intensity determined using the Kodak 1D image analysis software system.

2.10 RNA isolation and quantification

2.10.1 RNA isolated for the study of PGHS-1 expression during MEG-01 differentiation

The floating and adherent fractions of TPA-treated and control MEG-01 cultures were isolated according to the PRP protocol. Total RNA was extracted from the cell pellets with TRIzol reagent according to the protocol supplied by GibcoBRL (see Appendix A1.1). Total RNA pellets were dissolved in DEPC water and RNA concentrations determined by measuring absorbency at 260 nm on a Beckman DU-65 spectrophotometer. Poly (A)+ RNA was then isolated from 150 µg of the total RNA pool using the Oligotex poly (A)+ mRNA extraction kit purchased from Quiagen (Mississauga, ON). The poly (A)+ mRNA isolation was performed according to the supplier's instructions (Appendix A1.2).

2.10.2 RNA isolated for the identification of thrombopoietic factors capable of inducing PGHS-1 expression

MEG-01 cells were collected from their respective tissue culture flasks and spun at 900g for 5 min. Total and messenger RNA were extracted from sample pellets as described in section 2.8.1. Total RNA concentration was measured as previously described.

2.11 Northern blot hybridization

Poly (A)+ mRNA samples isolated from the different fractions of TPA-treated and control MEG-01 cultures, along with samples isolated from MEG-01 cells that had been treated with hematopoietic factors, were subjected to electrophoresis on a denaturing formaldehyde (2.2 M)-agarose (1%) gel. The RNA was then transferred to a Biodyne nylon membrane (Gibco) through capillary action and baked at 80°C for 2 hours. Following RNA transfer, the membrane was prehybridized at 42°C for 1 hour in (5X SSC/5X Denhardt's solution/50% formamide/1% SDS). The complementary DNA hPGHS-1 probe consisted of the entire hPGHS-1 coding region. It was radiolabeled with [³²P]-dCTP by random-primer extension (Prime-it II kit, Stratagene, Appendix A1.3) and hybridized to the membranes by incubation overnight at 42°C in (5X SSC/5X Denhardt's solution/50% formamide/ 1% SDS). Membranes were then washed three times at room temperature in 2X SSC/0.1% SDS and finally once for 30 min. at 65°C in 0.1X SSC/0.1% SDS. Blots were then exposed to radiographic film in a cassette at -80°C for 48 hrs. hPGHS-1 mRNA bands were digitally scanned and net band intensity determined using the Kodak 1D image analysis software system. Following hPGHS mRNA detection, membranes were stripped of probe by washing for three min. in DEPC water at 95°C, and reprobed with human α -tubulin cDNA. α -tubulin mRNA was detected and quantified in the same manner as PGHS-1 mRNA in order to control for the amount of mRNA loaded in each lane.

2.12 Immunocytochemistry

Detection of intracellular hPGHS-1 and cell surface antigens GP IIb/IIIa and GP Ib was carried out by indirect immunofluorescence. The nuclei of MEG-01 cells were stained directly with DAPI. Adherent MEG-01 cells were rinsed briefly in PBS at room temperature and then fixed in either 2% paraformaldehyde/PBS (for hPGHS-1 detection) or Zamboni's fixative (for GP IIb/IIIa and GP Ib detection) (280).

2.12.1 hPGHS-1 detection

Fixed cells were washed in PBS/10% FBS three times for 5 min. and incubated in a humid chamber with polyclonal rabbit anti-hPGHS-1 diluted 1:40 (0.01 mg/mL) in PBS, 2% saponin, and 10% FBS. The cells were subsequently washed 3 times in PBS/10% FBS before being incubated in a humid chamber for 45 min. with rhodamine-labeled secondary donkey anti-rabbit IgG diluted 1:30 in PBS/2% saponin/10% FBS. Following three 5 min. washes in PBS/10% FBS, the cells were incubated for 2 min. with 5 µg/mL DAPI in PBS/2% saponin/10% FBS. After 3 final washes the coverslips were mounted onto microscope slides in 15 µL of Permafluor mounting media. In order to assess nonspecific binding of the secondary antibody to cellular antigens, the same procedure was carried out in the absence of primary antibody.

2.12.2 GP IIb/IIIa and GP Ib detection

Fixed cells were washed and incubated in a humid chamber with either monoclonal mouse anti-human GP IIb/IIIa (CD41), or monoclonal mouse anti-human GP Ib (CD42b). GP IIb/IIIa IgG was diluted in PBS 1:100, while GP Ib IgG was diluted 1:25. After three 5 min. washes in PBS the cells were incubated in a humid chamber for 45 min. with Cy3-labelled secondary monoclonal donkey anti-mouse IgG (0.6 mg/mL) diluted 1:150 in PBS. The cells were finally incubated with DAPI (5 µg/ml in PBS), rinsed three times in PBS for 5 min., and mounted onto microscope slides in 15 µL of Permafluor mounting media. Nonspecific binding of the secondary antibody was assessed by performing the procedure in the absence of primary antibody.

To assess the expression of hPGHS-1, GP IIb/IIIa and GP Ib on floating MEG-01 cells, a slightly different procedure was followed. The 40 mL volumes of RPMI 1640 supplemented with 10% FBS that contained the floating MEG-01 cells were collected and centrifuged at 330g for 5 min. The cell pellets were gently resuspended in 10 mL of PBS at 37°C and re-centrifuged at 330g for 5 min. The pellets obtained from this second spin were resuspended in 5 mL of PBS. Next, 0.5 mL aliquots of the cell suspensions were applied to 18 mm² glass coverslips coated with 1 mg/mL poly-L-lysine. Incubating the suspensions for 1 min. at room temperature caused the floating MEG-01 to become attached to the coverslips. The cells were then fixed and stained according to the two previously described methods.

2.13 Immunofluorescence Viability Assay

MEG-01 cells grown on glass coverslips were incubated with propidium iodide (PRI) at a concentration of 50 $\mu\text{g/mL}$ for 5 min. in a six well dish. Samples were then rinsed in PBS three times for 5 min. Following PRI staining, the samples were fixed in 2% paraformaldehyde in PBS for 45 min., washed three times in PBS for 5 min., and mounted in 15 μL of permafluor mounting media onto microscope slides.

To assess the viability of floating MEG-01 cells via fluorescence microscopy, floating samples were collected as previously described, rinsed in PBS, and then incubated with 2 mL of 50 $\mu\text{g/mL}$ propidium iodide in PBS for 5 min. Following three 5 min. rinses in PBS, the cells were attached to poly-L-lysine coated coverslips, fixed in 2% paraformaldehyde in PBS for 45 min., and mounted onto microscope slides in 15 μL of Permafluor mounting media.

2.14 Image Analysis

Cells mounted on coverslips were viewed with an Olympus BX50 microscope under direct or indirect fluorescence. Images were captured with a Sony CCD monochrome camera and analyzed using Image-Pro Plus (Media Cybernetics, Silver Spring, Maryland). All images were viewed and captured at 200X magnification. Triplicate slides were prepared at each time point, and for each slide, three 425 x 425 micron fields were randomly selected. To determine total cells in a given field, DAPI fluorescence of nuclei was visualized by excitation at 330-385 nm with a 420 nm barrier

filter. Rhodamine fluorescence (excitation 510-550 nm, barrier 590 nm) of the same field was then used to identify cells expressing PGHS-1, GP IIb/IIIa and GP Ib. To eliminate background fluorescence, control slides were labeled with rhodamine conjugated secondary antibody in the absence of primary antibody in order to establish the appropriate cut-off thresholds. The procedure was automated so that many fields could be counted at each time point. Data were exported to Microsoft Excel and the results expressed graphically with Sigma Plot.

SECTION THREE: RESULTS

3.1 CHARACTERIZATION OF PGHS-1 mRNA and PROTEIN EXPRESSION IN MEG-01 CELLS STIMULATED TO DIFFERENTIATE WITH TPA

3.1.1 PGHS-1 protein expression

It is accepted that platelets possess significant amounts of PGHS-1 protein but contain substantially less PGHS-2. Through Western blotting* we have demonstrated that MEG-01 cells express PGHS-1 protein when stimulated to differentiate into mature forms by the addition of 1×10^{-8} M TPA. Figure 3.1, panel (A) shows the PGHS-1 protein expression profile for both TPA-stimulated and control MEG-01 cells. Compared to the level in controls, there is a significant increase in the amount of PGHS-1 protein expressed in phorbol ester-treated MEG-01. PGHS-1 protein levels reach a maximum when MEG-01 cells have been incubated with TPA for 4 to 8 days. The PGHS-1 standard depicted in panel (A) appears as a doublet, the consequence of being produced in a cos-1 expression system whereby the enzyme is actualized in two different glycosylation states (321). Panel (B) reveals that neither control nor TPA-treated MEG-01 express PGHS-2. The polyclonal antibodies we employed recognize the PGHS-1 and -2 standards without cross-reacting, a fact which demonstrates their specificity.

Figure 3.2 shows the time profile of PGHS-1 enzyme activity in MEG-01 cells incubated with 1.6×10^{-8} M TPA over a period of eight days, and in control MEG-01.

*We have observed that β actin expression is not constant during MEG-01 differentiation. Thus, we did not employ this marker to probe for the amount of total protein. We controlled for protein by first measuring total protein concentration in each sample, and then loaded an equal amount of protein onto each gel lane.

Figure 3.1 Time course of hPGHS-1 and -2 protein expression in MEG-01 cells treated with 1.6×10^{-8} M TPA. Control samples were treated with a DMSO vehicle. Bands were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and probed with either a hPGHS-1 or hPGHS-2 specific polyclonal antibody. Samples were run against both human PGHS-1 and-2 standards. (A) PGHS-1 protein expression over the course of 8 days. (B) PGHS-2 protein expression profile. Note that the antibodies only recognize their corresponding PGHS standard; there is no cross-reaction.

A **B**

Figure 3.2 Time profile of PGHS-1 enzyme activity in MEG-01 cells incubated with 1.6×10^{-8} M TPA over an eight day period. Control MEG-01 cells were incubated with a DMSO vehicle. Floating and adherent cells were harvested together. Activity was assessed by measuring 1- ^{14}C -Tx B_2 formation and is expressed as a ratio of counts per min.(CPM) to 0.73 mg of protein. Values are the mean $\pm$ SEM of two separate trials.

Over the 8 day time course, we observe a dramatic increase in PGHS-1 enzymatic activity within the TPA-treated MEG-01 cells. This increase in activity mirrors the increase that we observe in PGHS-1 protein expression in the TPA-treated MEG-01. These data suggest that the PGHS-1 protein expressed by TPA-treated MEG-01 cells is enzymatically active.

Figure 3.3 illustrates the expression profile of PGHS-1 protein in the different MEG-01 populations that were isolated according to the PRP protocol. Shown are both control MEG-01 cultures and MEG-01 cells incubated in the presence of 1.6×10^{-8} M TPA for eight days. Eight days incubation was chosen because the total amount of PGHS-1 protein became maximal at this time (Fig 3.1). In both control and TPA-treated samples, the amount of PGHS-1 protein is lowest in the fraction comprising the MEG-01 cells that grew in suspension, is intermediate in the fraction consisting of megakaryocytes that adhered to the tissue culture flask, and is greatest in the fraction containing the platelet-like structures. Specifically, there is a 2-fold increase in the amount of PGHS-1 protein expressed in the untreated adherent population compared to the untreated floating population, and a very small increase in the TPA-treated adherent fraction compared to the TPA-treated floating fraction. The increase in the amount of PGHS-1 protein found in the control and TPA-treated platelet fractions compared to their respective floating fractions is even higher: 5-fold for the control samples and 1.6-fold for the TPA-treated samples. MEG-01 cells that adhere to the bottom of the tissue culture flask are thought to be more differentiated than those that float in suspension (269). Thus, there is a positive correlation between the amount of PGHS-1 protein found in a given population of MEG-01 cells and the overall level of cellular differentiation of that population.

Figure 3.3 Protein expression of hPGHS-1 in the floating, adherent, and platelet fractions of control and TPA-treated MEG-01 cultures. hPGHS-1 levels were determined through Western blotting and subsequent densitometric analysis. (A) Representative Western blot of control and TPA-treated samples. (B) Histogram showing the hPGHS-1 protein expression profile for control and MEG-01 cultures incubated with TPA over an eight day period. Values are the mean $\pm$ SEM of three separate trials.

A **B**

3.1.2 PGHS-1 mRNA Expression

We next sought to determine whether PGHS-1 mRNA expression was related to the level of differentiation in each of the three MEG-01 cell populations. We had previously shown that PGHS-1 mRNA reaches maximal levels 24 hours after treatment with 1.6×10^{-8} M TPA (281). Poly-A⁺ mRNA was isolated from each of the three MEG-01 populations and analyzed by Northern blotting with a [³²P]-labeled probe corresponding to the entire coding region of hPGHS-1. Figure 3.4 shows that in control and TPA-treated MEG-01 cultures there are approximately 3- and 2-fold increases in the amounts of PGHS-1 mRNA present in the populations of adherent cells compared to the respective floating populations. Thus, in the adherent MEG-01 populations the increase in PGHS-1 mRNA mirrors the increase in PGHS-1 protein. Not surprisingly, PGHS-1 mRNA levels actually drop slightly by a factor of 1.3x in the control platelet-like population, and by a factor of 2.3x in the TPA-treated platelet-like population. The decrease is due to the fact that platelets, which are anucleate cells, synthesize protein inefficiently since they contain negligible amounts of RNA (282).

3.1.3 Megakaryocyte differentiation and PGHS-1 protein expression

In order to further refine the expression profile of PGHS-1 protein in the MEG-01 cell line, we measured PGHS-1 protein expression on a cell-by-cell basis by indirect immunofluorescence and compared its pattern of expression to the profiles of two cell surface antigens known to be markers of megakaryocyte differentiation, GP IIb/IIIa and

Figure 3.4 Expression profile of the 2.8 kb hPGHS-1 mRNA transcript in the floating, adherent and platelet fractions of control and TPA-treated MEG-01 cultures. (A) Representative Northern blot of control and TPA-treated samples. (B) Histogram showing the hPGHS-1 2.8 kb mRNA transcript expression profile for control cultures and cultures incubated with TPA over a 24 hour period. Data are expressed as a ratio of hPGHS-1 band density over α -tubulin band density in order to control for the total amount of mRNA loaded in each lane. Values are the mean $\pm$ SEM of three separate trials.

A **B**

GP Ib. Figure 3.5, panels (A) and (B) show the expression profile of PGHS-1 protein on a cell-by-cell basis in both the adherent and floating MEG-01 populations. As can be seen, 30% of adherent MEG-01 cells express PGHS-1 before treatment with TPA. Furthermore, the expression of PGHS-1 in the untreated adherent MEG-01 population remains relatively constant with about 20% of control adherent cells expressing PGHS-1 over the eight day time course. Upon treatment with TPA, the percentage of cells expressing PGHS-1 increases to 45% after one day, and reaches a plateau of about 70% after 4 days. The percentage of floating MEG-01 cells that express PGHS-1 rises dramatically to 55% between 2 and 4 days incubation with TPA.

Compared to PGHS-1, GP IIb/IIIa is expressed in both the adherent and floating MEG-01 cell populations at a high level from the onset of the time course. Prior to TPA stimulation, 64% of adherent MEG-01 cells express GP IIb/IIIa (Fig. 3.5, panel C). TPA stimulation has little effect on this percentage over the course of eight days. In fact, GP IIb/IIIa expression is greater in the absence of TPA. Specifically, after eight days, 80% of untreated MEG-01 cells express GP IIb/IIIa. The floating MEG-01 cells have a much lower expression of GP IIb/IIIa than the adherent cells. Nevertheless, TPA does cause an increase in GP IIb/IIIa expression over 8 days compared to the untreated floating MEG-01 population, where GP IIb/IIIa expression remains relatively constant at a value of about 15% (Fig. 3.5, panel D). Glycoprotein Ib has the lowest expression profile of the three antigens we examined. Prior to treatment with TPA, there was no detectable GP Ib protein expression in the adherent MEG-01 population. The percentage of untreated adherent MEG-01 that expressed GP Ib rose to 15 % by the end of the eight day time course (Fig. 3.5, panel E). Following treatment with TPA, the percentage of adherent

Figure 3.5 Immunofluorescent detection of the antigens PGHS-1, GP IIb/IIIa, and GP Ib in MEG-01 cultures incubated with the phorbol ester TPA over an eight day period. Panels (A), (C) and (E) represent adherent MEG-01 cultures while panels (B), (D) and (F) depict floating MEG-01 cells. (A) and (B) show PGHS-1 protein expression profile, (C) and (D) show GP IIb/ IIIa protein expression profile, (E) and (F) show GP Ib protein expression profile. Solid line, solid circle = TPA-treated samples, dashed line, open triangle = control. Data represent three separate trials plus or minus the standard error of the mean.

MEG-01 cells expressing GP Ib increased to 27% by 8 days. We were unable to detect any significant amount of GP Ib in either the TPA treated or untreated floating MEG-01 population (Fig. 3.5, panel F).

3.1.4 MEG-01 viability

To determine if the MEG-01 cells remained viable over the eight day time course, we labeled cells in both the adherent and floating MEG-01 populations that had been treated with either TPA or DMSO vehicle with the fluorescent viability stain PRI. Because PRI is taken up by cells that have lost membrane integrity, only nonviable cells will fluoresce red (322,323). As can be seen in Figure 3.6, solely the MEG-01 cells in the floating population treated with TPA show a significant loss of viability. The percentage of dead cells in the TPA treated floating population increases from about 10% after one day to a maximum of 79% after 6 days incubation with TPA.

3.1.5 MEG-01 growth profile

Figure 3.7 illustrates the growth pattern of adherent and floating MEG-01 cells. The number of adherent MEG-01 cells dramatically increases from 28 cells / 425 μm^2 to 235 cells / 425 μm^2 two days after treatment with TPA. The number of adherent MEG-01 cells then decreases to 94 cells / 425 μm^2 after 8 days of incubation with TPA. In contrast, the number of untreated adherent MEG-01 cells increases to 75 cells / 425 μm^2 after 2 days and then remains constant for the remainder of the time course. After 1 day

Figure 3.6 Viability of control and TPA-treated MEG-01 cells. Both floating and adherent MEG-01 cultures were stained before fixation with 50 ng/mL of the fluorescent dye, PRI. Cells staining positive for PRI are nonviable. Note that only the TPA-treated floating population shows any significant sign of compromised viability. Solid line, solid circle = TPA-treated, floating population; dashed line, solid triangle = control, floating population; solid line, open circle = TPA-treated, adherent fraction; solid line, open triangle = control, adherent fraction. Values are the mean $\pm$ SEM of three separate trials.

Figure 3.7 Cellular growth profile of the adherent and floating MEG-01 culture populations that were either left untreated, or incubated with TPA (1.6×10^{-8} M) over a period of eight days. Cell counts were obtained by computer analysis of samples stained with the fluorescent dye, DAPI. (A). Number of cells per $425 \mu\text{m}^2$ in the adherent population. The number of cells reaches a maximum of 245 cells/ $425 \mu\text{m}^2$ 2 days after TPA treatment and then steadily declines whereas the number of control cells remains constant. (B) Number of cells per $425 \mu\text{m}^2$ in the floating population. After one day of TPA treatment, the number of floating cells per $425 \mu\text{m}^2$ drops from 80 to 20. The number cells in the untreated floating MEG-01 population increases slightly over the eight day period. Solid bar = TPA-treated samples, open bar = control. Values are the mean $\pm$ SEM of nine separate trials.

of TPA treatment, the number of cells in the floating MEG-01 population decreases from 79 cells/ 425 μm^2 to 21 cells / 425 μm^2 and then remains relatively constant for the next 7 days (Fig 3.7, panel B). Over the eight day time course, the number of cells in the untreated floating MEG-01 population rises slightly from 79 cells / 425 μm^2 to 108 cells / 425 μm^2 .

3.1.6 Protein Localization

We detected PGHS-1 protein primarily in the cytoplasm of floating and adherent MEG-01 cells, both TPA-treated and control (Fig. 3.8). A distinct ring of PGHS-1 could often be seen circumscribing the MEG-01 nuclei. The arrow in Figure 3.8, panel D points to such an example of PGHS-1 perinuclear staining. Unlike PGHS-1, we usually detected GP IIb/IIIa around the peripheries of MEG-01 cells (Fig. 3.9). Cytoplasmic protrusions and budding structures frequently labeled for GP IIb/IIIa (Fig. 3.9, panel B), as did numerous long, thin tendrils emanating from the bodies of the adherent megakaryocytes (Fig. 3.10). We also detected GP IIb/IIIa in areas of cytoplasm that seemed to be ‘exploding’ or breaking apart. This pattern of GP IIb/IIIa localization was greater in the adherent MEG-01 population, and seemed to increase in frequency as incubation time with TPA progressed. Like GP IIb/IIIa, we usually detected GP Ib around the peripheries of adherent MEG-01 cells stimulated with TPA (Fig. 3.11, panel B). The regions of cytoplasm that seemed to be breaking apart frequently stained positive for GP Ib (Fig. 3.11 panels D,F).

Figure 3.8 Immunodetection of PGHS-1 in MEG-01 cells incubated with TPA (1.6×10^{-8} M) for 4 days. Panels A, C, and E are phase contrast images while panels B, D and F are the corresponding fluorescent micrographs. The nucleus is labeled blue, PGHS-1 red. Panels (A) and (B) show an adherent MEG-01 cell with an eccentrically positioned, tri-lobed nucleus. (C) and (D) illustrate a different adherent MEG-01 cell that stains positive for PGHS-1 and has 2 large nuclei. Note that PGHS-1 is located in the cytoplasm and around the nucleus of the adherent cells (arrow). Panels (E) and (F) show a floating MEG-01 cell. Like the adherent MEG-01, the floating MEG-01 cells also exhibit cytoplasmic and perinuclear PGHS-1 staining. Note that the floating MEG-01 are much more spherical than the adherent cells. Bar = 10 μ m.

Figure 3.9 Immunodetection of GPIIb/IIIa. The MEG-01 cells illustrated in panels (A), (B), (E), and (F) were incubated with TPA (1.6×10^{-8} M) for 4 days whereas the cells depicted in panels (C) and (D) were treated with DMSO. The nucleus is shown in blue, GP IIb/IIIa in red. The adherent cell depicted in panels (A) and (B) labels for GP IIb/IIIa specifically around its surface. Budding structures emanating from this cell label for GP IIb/IIIa (arrows). The two unstimulated adherent cells shown in (C) and (D) also label for GP IIb/IIIa around their outer perimeters, although the cell on the right fluoresces much more intensely. Panels (E) and (F) show a floating MEG-01 that has labeled for GP IIb/IIIa at its exterior. Although spherical, this floating cell has developed blebs that protrude out from its surface (arrow). Bar = 10 μ m.

Figure 3.10 GP IIb/IIIa detection on cytoplasmic processes. Panels (A) and (B) show a large adherent MEG-01 cell that was incubated with 1.6×10^{-8} TPA for 4 days. Notice that this megakaryocyte has a distinct network of branched filopodia (cytoplasmic processes) that label for GP IIb/IIIa (arrow). The fluorescence in panel (B) was deliberately overexposed in order to reveal the fine detail. Bar = 10 μm .

Figure 3.11 Immunodetection of GP Ib in adherent MEG-01 cells incubated with TPA (1.6×10^{-8} M) for 4 days. The nucleus is depicted in blue, GP Ib in red. Panels (A) and (B) illustrate a cell with a GP Ib positive periphery. The cells depicted in panels (C), (D) and panels (E), (F) each exhibit perimeter GP Ib staining (arrow, panel D), but also label with strong intensity at cytoplasmic areas that appear to be 'exploding' (arrow, panel F). Bar = 10 μ m.

3.2 IDENTIFICATION OF HEMATOPOETIC FACTORS CAPABLE OF INDUCING PGHS-1 EXPRESSION IN MEG-01 CELLS

3.2.1 Hematopoietic factor screening

We sought to determine which cytokines, if any, were able to induce the expression of PGHS-1 protein in MEG-01 cells. We tested the following hematopoietic factors: TPO, EPO, IL-2, IL-3, IL-6, IL-11, G-CSF, GM-CSF and SCF. We screened for any possible increase in PGHS-1 expression by subjecting cell isolates from the different test samples to gel electrophoresis followed by Western blotting with a PGHS-1-specific antibody. A complete list of the hematopoietic factor combinations that we tested, along with their ability to induce PGHS-1 protein expression, is shown in TABLES 3.1 to 3.3. No single factor was able to induce PGHS-1 protein expression; however, when their adherent population was stimulated with certain combinations of hematopoietic factors, MEG-01 cells expressed significantly higher amounts of PGHS-1 protein compared to cultures treated with a PBS/BSA vehicle. As shown in Table 3.3 and Figure 3.12, the combinations IL-3/IL-11/GM-CSF/SCF/TPO, IL-11/GM-CSF/SCF/TPO, IL-6/IL-11/GM-CSF/SCF, and IL-6/IL-11 caused MEG-01 cells to markedly increase their expression of PGHS-1 protein. The combinations IL-3/IL-6 and IL-6/GM-CSF induced MEG-01 to slightly increase their expression of PGHS-1.

TABLE 3.1
THE EXPRESSION OF PGHS-1 PROTEIN IN FLOATING MEG-01 CELLS
INCUBATED WITH DIFFERENT COMBINATIONS OF HEMATOPOIETIC
FACTORS*

HEMATOPOIETIC FACTOR(S)	PGHS-1 PROTEIN EXPRESSION
IL-3/IL-11/SCF/GM-CSF/TPO	-
IL-11/SCF/GM-CSF/TPO	-

*Hematopoietic factors were incubated with floating MEG-01 cultures for 7 days

++ Large increase in PGHS-1 expression, + intermediate increase, +/- threshold increase, - no increase in expression relative to control

TABLE 3.2
THE EXPRESSION OF PGHS-1 PROTEIN IN ADHERENT AND FLOATING MEG-01
CELLS INCUBATED TOGETHER WITH DIFFERENT COMBINATIONS OF
HEMATOPOIETIC FACTORS*

HEMATOPOIETIC FACTOR(S)	PGHS-1 PROTEIN EXPRESSION	HEMATOPOIETIC FACTOR(S)	PGHS-1 PROTEIN EXPRESSION
TPO	-	SCF/IL-11	-
EPO	-	GM-CSF/IL-11	-
IL-2	-	GM-CSF/IL-3 [†]	-
IL-3	-	SCF/IL-3 [†]	-
IL-6	-	IL-11/IL-3 [†]	-
IL-11	-	IL-3/IL-3 [†]	-
G-CSF	-	TPO/IL-3 [†]	-
GM-CSF	-	GM-CSF/IL-11 [†]	-
SCF	-	SCF/IL-11 [†]	-
TPO/IL-3	-	IL-11/IL-11 [†]	-
TPO/SCF	-	IL-3/IL-11 [†]	-
TPO/GM-CSF	-	TPO/IL-11 [†]	-
TPO/IL-11	-	IL-3/IL-11/SCF/ GM-CSF/TPO	-
IL-3/IL-11	-	TPO/GM-CSF/SCF/ IL-11	-

*MEG-01 cells (both floating and adherent) were incubated with hematopoietic factors for 7 days, unless indicated otherwise

[†] The first cytokine in this combination was incubated with the cells for 4 days, the second for 7 days

++ Large increase in PGHS-1 expression, + intermediate increase, +/- threshold increase, - no increase in expression relative to control

TABLE 3.3 THE EXPRESSION OF PGHS-1 PROTEIN IN ADHERENT MEG-01 CELLS INCUBATED WITH DIFFERENT COMBINATIONS OF HEMATOPOIETIC FACTORS*

HEMATOPOIETIC FACTOR(S)	PGHS-1 PROTEIN EXPRESSION	HEMATOPOIETIC FACTOR(S)	PGHS-1 PROTEIN EXPRESSION
TPO	-	GM-CSF/ IL-6	+/-
EPO	-	SCF/IL-11/IL-6	-
IL-2	-	SCF/GM-CSF/IL-11/ IL-6	+
IL-3	-	GM-CSF/TPO/SCF/ IL-3/IL-11	++
IL-6	-	GM-CSF/TPO/SCF/ IL-11	++
IL-11	-	GM-CSF TPO/IL-11 TPO [†]	++
G-CSF	-	IL-6/IL-11/ G-CSF/GM-CSF/TPO/ EPO	-
GM-CSF	-	IL-2/ IL-11/ G-CSF/GM-CSF/TPO/ EPO	-
SCF	-	IL-2/ IL-6/ G-CSF/GM-CSF/TPO/ EPO	-
TPO/IL-6	-	IL-2/ IL-6/IL-11/ GM-CSF/TPO/ EPO	-
IL-3/IL-6	+/-	IL-2/ IL-6/IL-11/ G-CSF/ TPO/ EPO	-
IL-11/IL-6	+	IL-2/ IL-6/IL-11/ G-CSF/GM-CSF/ EPO	-
SCF/IL-6	-	IL-2/ IL-6/IL-11/ G-CSF/GM-CSF/TPO/	-
		IL-2/ IL-6/IL-11/ G-CSF/GM-CSF/TPO/ EPO	-

* Adherent MEG-01 were incubated with hematopoietic factors for 5 days, unless indicated otherwise

† Adherent MEG-01 were incubated with GM-CSF for 5 days, IL-11 and TPO for 4 days, and with a second administration of TPO for 3 days

++ Large increase in PGHS-1 expression, + intermediate increase, +/- threshold increase, - no increase in expression relative to control

Figure 3.12 PGHS-1 protein expression in adherent MEG-01 cultures incubated with various combinations of hematopoietic factors. hPGHS-1 protein was resolved by SDS-PAGE and detected by Western blotting. Hematopoietic factors were incubated with the adherent MEG-01 for 5 days unless indicated. The numbers represent 1) DMSO control, 2) IL-3/IL-11/GM-CSF/SCF/TPO, 3) IL-11/GM-CSF/SCF/TPO, 4) GM-CSF (5 day incubation)/TPO/IL-11 (4 day incubation)/second administration of TPO (3 day incubation), 5) TPA, 6) DMSO control, 7) TPO, 8) IL-6/GM-CSF 9) IL-3/IL-6, 10) IL-6/IL-11/GM-CSF/SCF, 11) IL-6/IL-11.

3.2.2 Partial characterization of PGHS-1 expression in MEG-01 cells stimulated with IL-6 + IL-11

3.2.2.1 PGHS-1 protein expression profile

We proceeded to characterize in further detail the effect that IL-6 and -11 had on the expression of PGHS-1 protein in MEG-01 cells. We chose this particular cocktail because it represented the combination with the fewest number of hematopoietic factors that was capable of stimulating the cells to increase their expression of PGHS-1 protein relative to PBS/0.1% controls (Fig. 3.12, lower panel). Figure 3.13 shows the expression of PGHS-1 protein in MEG-01 cultures whose adherent populations were incubated with IL-6 and IL-11 (50 ng/mL each) for eight days. The cells begin to express PGHS-1 after 6 days incubation. They continue to increase their expression of PGHS-1 protein over the next two days (upper panel). We do not detect PGHS-1 protein in control cultures (lower panel). It is clear that the combination of IL-6 and IL-11 is not able to stimulate MEG-01 cells to increase their expression of PGHS-1 protein to the same extent that TPA is able to (compare Fig. 3.1, panel A to Fig. 3.13). Nevertheless, in relation to control cultures incubated with a PBS/0.1%BSA vehicle, together IL-6 and IL-11 do stimulate MEG-01 cells to increase their expression of PGHS-1 protein to levels detectable by Western blotting.

Figure 3.13 PGHS-1 protein expression in adherent MEG-01 cells incubated with 50 ng/mL IL-6 and 50 ng/mL IL-11 over an 8 day period. hPGHS-1 protein in IL-6 + IL-11-treated samples (upper panel) and in PBS/0.1% BSA controls (lower panel) was resolved by SDS-PAGE and revealed by Western blotting.

3.2.2.2 PGHS-1 mRNA expression profile

After showing that IL-6 and IL-11, through their combined action, cause MEG-01 cells to increase their expression of PGHS-1 protein, we sought to determine the effect that this mixture had on the expression of PGHS-1 mRNA. As can be seen in Figure 3.14, adherent MEG-01 incubated with IL-6/IL-11 increase their level of PGHS-1 mRNA expression 7-fold over the course of 2 days. By comparison, in control cells treated with a PBS/0.1%BSA vehicle, PGHS-1 mRNA levels do not increase to any notable degree.

Figure 3.14 Expression profile of the 2.8 kb hPGHS-1 mRNA transcript in adherent MEG-01 cells incubated with 50 ng/mL IL-6 and 50 ng/mL IL-11 over 72 hours. (A) Northern blot of control and IL-6 + IL-11-treated samples. (B) Histogram showing the hPGHS-1 2.8 kb mRNA transcript expression profile for control cultures and MEG-01 incubated with IL-6 and IL-11 for 72 hours. Data are expressed as a ratio of hPGHS-1 band density over α -tubulin band density in order to control for the total amount of mRNA loaded in each lane. One trial was performed.

A **B**

SECTION FOUR: DISCUSSION

4.1 Characterization of PGHS-1 expression within the context of TPA-stimulated MEG-01 differentiation

Since low amounts of PGHS-1 protein can be detected in most tissues, it is commonly referred to as the constitutive form of the enzyme (6). However, this is an over simplification because certain tissues such as platelets express high amounts of PGHS-1 protein (1). We studied the expression of PGHS-1 mRNA and protein within the context of megakaryocyte differentiation to gain a better understanding of how the PGHS-1 enzyme is regulated during the process of platelet production. We chose the MEG-01 cell line as a model system in which to study PGHS-1 expression because it has been previously shown that *in vitro* MEG-01 cell cultures undergo differentiation events that mimic the *in vivo* process of megakaryopoiesis (270-272). Furthermore, it has been demonstrated that the phorbol ester TPA is able to induce the cells of a related CML line, CMK, to differentiate into mature forms that express PGHS-1 (93). MEG-01 cells exist as three populations: nucleated cells that float in the liquid culture media, nucleated cells that adhere to the bottom of the tissue culture flask, and anucleated platelet-like structures. It has been suggested that floating MEG-01 are cells at the early stage of megakaryopoiesis and that adherent MEG-01 are cells that have differentiated into a slightly later stage (269).

In our initial experiments, we separated the floating, adherent and platelet-like MEG-01 populations according to the PRP protocol and measured the amounts of PGHS-1 protein and mRNA in each compartment. The amounts of PGHS-1 protein and mRNA

correlate strongly with the overall level of differentiation in each population. That is, the floating MEG-01 population which contains the less differentiated MEG-01 cells also contains the lowest amounts of PGHS-1 protein and mRNA, whereas the adherent population, which consists of more differentiated megakaryocytes, contains a greater amount of PGHS-1 protein and mRNA. The platelet-like population, which represents MEG-01 cells at the latest possible stage of differentiation, contains the highest amount of PGHS-1 protein. Interestingly, PGHS-1 mRNA levels are negligible in the platelet-like compartment. This finding is expected since it is a well known fact that platelets do not contain significant amounts of either DNA or RNA (282). The overall effect of TPA-stimulation is to increase the amount of PGHS-1 protein and mRNA in all three populations; however, TPA treatment does not change the relative relationship of PGHS-1 protein or mRNA expression between the floating, adherent, and platelet-like populations.

We then investigated whether the differences in the amount of PGHS-1 protein found between the control and TPA-treated floating and adherent populations were due to an increase in the number of cells expressing PGHS-1, or were simply due to an increase in the size and ploidy of MEG-01 cells that already expressed high levels of the PGHS-1 enzyme. By employing indirect immunofluorescence to measure PGHS-1 protein expression on a cell-by-cell basis, we observed that the percentage of MEG-01 cells expressing PGHS-1 was greater in the adherent population than in the floating. Overall, the PGHS-1 fluorescence signal was much weaker in the floating MEG-01 cells than in the adherent. Following TPA stimulation, the percentage of adherent cells expressing the PGHS-1 enzyme reached a maximum of 70%. In contrast, the percentage of floating

MEG-01 that expressed PGHS-1 only increased to 55% after 4 days of TPA treatment. These results strongly suggest that the differences in PGHS-1 protein expression found between the three MEG-01 populations are in fact due to an increase in the number of cells expressing PGHS-1 and not simply due to fewer, larger cells possessing higher amounts of the enzyme. PGHS-1 protein was present in the MEG-01 cytoplasm and around the nuclear periphery. This staining pattern is in agreement with the accepted localization of PGHS-1, an enzyme which is anchored in the inner leaflet of the lipid bilayer and which faces the lumen of the endoplasmic reticulum and nuclear envelop (85,101,108).

Although less differentiated than the adherent MEG-01, the floating MEG-01 cells still express high amounts of PGHS-1 protein. Specifically, 55% of floating MEG-01 cells express PGHS-1 four days after TPA treatment. We cannot exclude the possibility that this peak in PGHS-1 expression represents MEG-01 cells that have, upon reaching the terminal stage of differentiation, detached from the substratum. Soon after their release, these cells shed their cytoplasm and become nonviable in the process. There is recent evidence to support this notion. Levine *et al* have shown that *in vivo* human megakaryocytes detach from the ECM and migrate into the pulmonary circulation where they purportedly release platelets (185). In addition, the final stage of erythropoiesis is marked by the loss of adhesion between the mature red blood cell and the ECM (283). Even after treatment with TPA, MEG-01 cultures remain heterogeneous in the sense that cells representing all stages of development within the continuum of megakaryocyte differentiation can be found. Moreover, the global effect that TPA has on

MEG-01 cultures is to shift the balance of megakaryopoiesis towards the later stages of differentiation.

Two days after TPA stimulation we detected a dramatic decrease in the percentage of adherent MEG-01 cells that expressed PGHS-1. The decrease in PGHS-1 protein expression corresponds to the time at which MEG-01 adherence is maximal. These data are in agreement with our observation that TPA stimulation causes the migration and adherence of floating MEG-01 cells (the less differentiated cells) to the bottom of the tissue culture flask. Thus, on the level of an individual MEG-01 cell, adherence precedes the increase in PGHS-1 protein expression. That is why after two days incubation with TPA there is a drop in the number of cells expressing PGHS-1 protein. Although these MEG-01 cells have recently become adherent, they have not had sufficient time to upregulate their expression of PGHS-1 protein. However, after two more days incubation with TPA, these newly adherent cells do increase their level of PGHS-1 and thus the number of adherent cells expressing PGHS-1 protein becomes maximal.

After 4 days incubation with TPA, the percentage of cells that express PGHS-1 in the floating compartment drops rapidly. Interestingly, this is the same time at which the floating MEG-01 cells begin to die. These data suggest that dying MEG-01 cells maintain high levels of GP IIb/IIIa expression but not high levels of PGHS-1. PRI staining reveals that the viability of floating MEG-01 cells begins to decrease after 2 days treatment with TPA and continues to do so until after 6 days, 80% of floating MEG-01 cells are non-viable. In contrast, the untreated floating and adherent MEG-01 populations, along with the TPA treated adherent population, retain their viability over

eight days. Our data suggest that adhesion is required to maintain the viability of MEG-01 cells stimulated to differentiate with TPA. Two different groups have recently published studies examining megakaryocyte viability during thrombopoiesis. Zauli *et al* found that during megakaryopoiesis, isolated human megakaryocytes progressively underwent apoptotic cell death (284). Berthier's group found the opposite was true: isolated human megakaryocytes underwent thrombopoiesis without the onset of apoptosis (257). Our data may reconcile these seemingly contradictory findings. That is, Berthier's model consisted solely of megakaryocytes that adhered to fibronectin whereas Zauli's model consisted of floating megakaryocytes. Clearly, adhesion alters some aspect of megakaryocyte biology that confers protection from cell death.

Differentiation of MEG-01 cells was evaluated by the expression of the platelet specific cell surface antigens GP IIb/IIIa and GP Ib, both of which are known to be markers of maturation within the megakaryocytic lineage (18,20,274). GP IIb/IIIa is thought to appear on the surface of cells at the earlier stages of megakaryopoiesis, whereas GP Ib, which is often only detectable at much lower levels than GP IIb/IIIa, is thought to appear on the surface of very mature megakaryocytes (190-193,275,276). We found that even before stimulation with TPA, the GP IIb/IIIa antigen is highly expressed on the surface of MEG-01 cells. Furthermore, within the adherent population, this expression level changes little over the eight day time course. In fact, in the adherent control population, a greater percentage of MEG-01 cells express GP IIb/IIIa than do cells in the TPA-treated adherent population. This observation is explained by the fact that a significant amount of GP IIb/IIIa is already expressed at the earliest stages of megakaryopoiesis (CFU-Mk) (172,189). In fact, GP IIb/IIIa is used to isolate progenitor

cells that are committed to the megakaryocyte lineage (285,286). Because there are so few adherent MEG-01 cells in the control group compared to the TPA treated group, especially over the first two days of TPA treatment, and because by definition adherent cells are at a later stage of differentiation than floating cells (269), the majority of untreated adherent MEG-01 express GP IIb/IIIa. This fact is represented by the high 'percent-labeled' control curve shown in Figure 3.5, panel C. Most cells labeled strongly for GP IIb/IIIa around their periphery. Some adherent MEG-01 cells stained for GP IIb/IIIa in areas of cytoplasm that seemed to have a 'fractionated' or 'exploding' appearance. Berthier's group has reported the localization of GP IIb/IIIa at areas of fragmented cytoplasm in cultured megakaryocytes (257). These regions of exploding cytoplasm could represent areas of the demarcation membrane system (DMS) that are accessible to antibodies which label surface antigens. The long, branched networks of filopodia emanating from the cell bodies of adherent MEG-01 cells stained positive for GP IIb/IIIa.

The fluorescent signal representing the GP Ib antigen was much weaker than the GP IIb/IIIa signal. The percentage of adherent MEG-01 cells expressing GP Ib increases slightly over the course of an eight day incubation with TPA, indicating that the adherent MEG-01 cells are differentiating over time, and that they are generally more differentiated than the floating MEG-01 cells. Like GP IIb/IIIa, we usually detected GP Ib around the outer periphery of MEG-01 cells. However, some adherent MEG-01 cells also stained for GP Ib in areas of 'exploding' cytoplasm.

In conclusion, this part of the study has demonstrated that PGHS-1 protein and mRNA expression are strongly correlated with megakaryocyte differentiation. Furthermore, PGHS-1 protein can now be used as an additional and perhaps better index

by which to survey megakaryocyte differentiation. GP IIb/IIIa is already quite highly expressed early on in megakaryopoiesis, whereas GP Ib is difficult to detect and is only upregulated towards the very end of megakaryocyte differentiation. PGHS-1 shows a consistent increase over the entire course of differentiation, that is, from blast cell to platelet-shedding megakaryocyte.

4.2 Certain combinations of hematopoietic factors stimulate increases in PGHS-1 protein and mRNA expression in MEG-01 cells

MEG-01 cells stimulated with TPA is a useful system in which to study megakaryocyte development. Utilizing this model, we have shown (1) that adherent MEG-01 represent highly differentiated cells and that (2) the expression of PGHS-1 protein and mRNA correlates strongly with megakaryocyte maturation. However, TPA is not an endogenous hormone. It has been shown that several hematopoietic factors* regulate megakaryocyte development, the most notable being TPO (222,336,337). We investigated whether IL-2, IL-3, IL-6, IL-11, G-CSF, GM-CSF, SCF, EPO, and TPO, either alone or in combination, could increase the expression of PGHS-1 protein in MEG-01 cells. We tested these hematopoietic factors at concentrations ranging from 10 to 50

*Many peptide hormones act on cells to promote either growth and division, or cellular differentiation. Often a single hormone may exhibit either activity, depending on the particular context in which it is presented. The receptors that a cell expresses at a particular time, the presence of other growth and differentiating factors, along with many other variables, can influence the type of activity that a given peptide hormone will exert upon its target. TPO, for instance, can promote both mitogenesis and megakaryocyte differentiation. Often, peptide hormones acting on developing blood cells in the bone marrow are referred to simply as 'growth factors'. However, this term does not acknowledge the fact that a particular factor may possess both growth and maturation-promoting activities. To be clear, I will refer to the peptide hormones that regulate megakaryopoiesis as either hematopoietic factors or cytokines.

ng/mL, values that have been shown to approximate the concentration of hematopoietic hormones present in the bone marrow microenvironment.

As can be seen in Tables 3.1 through 3.3, individual factors were unable to stimulate PGHS-1 protein expression in either floating MEG-01 cells, adherent MEG-01, or floating and adherent MEG-01 cells cultured together. This lack of singular activity could perhaps be expected for most hematopoietic factors; however, it is interesting that TPO was unable to stimulate PGHS-1 protein expression in our MEG-01 cultures. TPO is known to be the most potent thrombopoietic hormone: it has both megakaryocyte colony-stimulating and maturation-promoting activities (289-292). One must recognize, however, that MEG-01 cells originate from a patient who was inflicted with CML, and therefore may not respond to hematopoietic factors in the same way that normal megakaryocytes do *in vivo*. Nevertheless, our accumulated knowledge of megakaryocyte biology can be accredited, in large part, to the study of megakaryocytic cell lines, and to the investigation of clonogenic cultures derived from normal bone marrow megakaryocytes. It should be noted that neither immortalized cell lines, nor clonogenic megakaryocytes, produce platelets as efficiently *in vitro* as do normal megakaryocytes *in vivo* (324). A single, mature megakaryocyte can produce up to 3000 platelets in a healthy individual (217,338). Despite this shortcoming, chronic myeloid leukemia cell lines and clonogenic cultures continue to provide a convenient window through which we can study the morphological and biochemical changes associated with megakaryocyte differentiation (19).

Recent research has shown that certain pleiotropic hematopoietic factors, namely IL-3, IL-6, IL-11, GM-CSF, and TPO, can regulate megakaryopoiesis. Most of these

factors have both colony-stimulating and megakaryocyte-maturation promoting activities, though not to the same extent. For example, although IL-3 has a potent effect on megakaryocyte proliferation, it exerts a relatively weak effect on megakaryocyte maturation (294-298). In contrast, TPO, which is thought to be a key regulator of thrombopoiesis, has both strong colony-stimulating and megakaryocyte maturation-promoting activities (289-292). In addition, the activities of any given cytokine can be modulated by the concerted action of different hematopoietic factors (299).

It has been reported that adherent MEG-01 cells are more differentiated than the floating MEG-01 (269). Our results confirm this notion. More cells in the adherent population (control and TPA-treated) express the differentiation markers GP IIb/IIIa and GP Ib, as well as PGHS-1, than in the floating MEG-01 population (control and TPA-treated) (Figure 3.5). Furthermore, we found that certain combinations of hematopoietic factors could induce PGHS-1 protein expression in MEG-01 cells only when the floating population was removed from culture. When incubated with adherent MEG-01, the combinations IL-3/IL-11/GM-CSF/SCF/TPO, IL-11/GM-CSF/SCF/TPO, IL-11/GM-CSF/TPO, IL-6/IL-11/GM-CSF/SCF, IL-6/IL-11, IL-3/IL-6, IL-6/GM-CSF (Table 3.3) stimulated the cells to increase their level of PGHS-1 protein expression relative to controls. The increase in PGHS-1 protein induced by these cytokine combinations, although notable, is not as great as the increase induced by TPA.

As can be seen in Table 3.3, TPO did stimulate an increase in PGHS-1 protein expression when it was administered to adherent MEG-01 in conjunction with IL-11, SCF, GM-CSF and IL-3. Our data suggest that these factors can act in synergy to promote megakaryocyte differentiation and PGHS-1 protein expression.

When we incubated interleukins-6 and 11 together with adherent MEG-01 cultures in our initial screening process, we observed that this combination was able to induce the cells to increase their expression of PGHS-1 protein. IL-6 and 11 are both thought to be important regulators of megakaryopoiesis (245-254). In fact, these cytokines are currently being used in clinical trials to treat patients who suffer from either chemotherapy or bone marrow transplantation induced-thrombocytopenia (325,326). In this regard, IL-11 seems to hold great promise in elevating the platelet counts of cancer patients undergoing radiation therapy (327). Because it is more cost-effective to study two cytokines than to study four, and given that IL-6 and IL-11 have both been implicated in the regulation of megakaryocyte differentiation, we elected to perform a full time-course with these growth factors.

Figure 3.13 shows the IL-6/IL-11 induced expression profile of PGHS-1 protein over the course of eight days, as determined by Western blotting. Although the increase in expression is not as great as that induced by TPA, it is nevertheless detectable. It must be pointed out that in our initial screening process, we detected PGHS-1 protein in MEG-01 cells that had been incubated with IL-6 and 11 for 5 days (Figure 3.12, lower panel). However, in subsequent experiments, we could not detect PGHS-1 this early. In the time course shown in Figure 3.13, MEG-01 cells do not express detectable levels of PGHS-1 protein prior to 6 days incubation with IL-6 and 11 (upper panel). Even after eight days, the cells do not express as much PGHS-1 protein as the MEG-01 did when they were incubated with IL-6/-11 for 5 days in our initial screening trial. Despite having repeated the experiment on several occasions, we were unable to detect PGHS-1 protein by

Western blotting as early as 5 days into the time course, or by a band as intense as that shown in the lower panel of Figure 3.12.

One factor that could account for our inability to detect PGHS-1 protein as early as 5 days in later IL-6/-11 experiments is oxygen tension. LaLuppa and colleagues have recently demonstrated that the effects of cytokines on megakaryoblastic cell cultures often depend on the tension of O₂ in the surrounding atmosphere (293). They demonstrated that thrombopoietin-containing cultures under a gas phase of 20% oxygen tension produce 1.4 to 2.2-fold more megakaryocytes than those grown under 5% O₂. Furthermore, cells cultured under 20% O₂ are much larger than megakaryocytes grown in 5% oxygen. Thus, it seems that oxygen tension can greatly influence both hematopoietic cell proliferation and differentiation (293). In our culture system, the O₂ level was set at 5%. However, even with constant monitoring, it is difficult to maintain a steady level of oxygen in the tissue culture incubators because they are supplied with O₂ from air pumps. Perhaps the reason we could not detect the increase in PGHS-1 protein in IL-6/-11-stimulated MEG-01 as early or as intensely in our later trials was because the O₂ tension in the incubator had changed slightly.

Another explanation as to why we could not detect PGHS-1 protein in our later trials as early as 5 days after IL-6/-11-treatment lies in the stability of the recombinant cytokines that we administered. Initially, our concern was that proteases present in the FBS would either bind to or degrade the hematopoietic factors. We therefore repeated the various time course experiments with serum concentrations that ranged from 0 to 10%. However varying the FBS concentration in the tissue culture media had no effect on the pattern of PGHS-1 protein expression. Even so, it is possible that during certain

trials, the megakaryocytes themselves secreted protein factors that bound to the administered cytokines, effectively reducing their concentration in the tissue culture media.

More striking is the increase in PGHS-1 mRNA expression (Fig. 3.14). By the second day of incubation, adherent MEG-01 cells treated with IL-6 (50 ng/mL) and IL-11 (50 ng/mL) increased their PGHS-1 mRNA expression by a factor of 7 relative to control cultures. Two days after treatment with IL-6/IL-11, PGHS-1 mRNA levels rise 7-fold, whereas protein levels do not begin to increase until about 5 or 6 days after stimulation. A similar scenario occurs in TPA-treated MEG-01 cells. Twenty-four hours after TPA stimulation, PGHS-1 mRNA levels become maximal; however, it can take up to 6 to 8 days before PGHS-1 protein levels reach their peak (333).

Interestingly, NF-IL-6, a nuclear factor required for the expression of IL-6, is one of the molecules that is phosphorylated and activated by MAP kinase in accordance with GP 130 stimulation (339). As pointed out earlier, the gene encoding PGHS-1 possesses a NF-IL-6 response element in its 5' flanking region (121). It is therefore possible that the rise in PGHS-1 mRNA and protein that we observe in adherent MEG-01 stimulated with IL-6 and 11 is due, at least in part, to the transcriptional activation of PGHS-1 by NF-IL-6. In addition, we have postulated that the PGHS-1 mRNA transcripts are subject to post-transcriptional regulation (334). Specifically, there may be one or more mRNA binding proteins that possess both a mRNA stabilizing activity, and an activity capable of modulating mRNA translatability (334).

Interleukins 6 and 11 elicit their effects by binding to distinct cell surface receptors. Although the IL-6 and 11 receptors (IL-6R and IL-11R) are individual peptide

chains, they possess a similar domain structure and fall into a large group known as the hematopoietic cytokine receptor family (328). The domain of similarity comprises about 200 amino acid residues and a WSXWS (Trp-Ser-any amino acid-Trp-Ser) motif (241). IL-6 binds to the IL-6R, and the resultant complex of IL-6 and IL-6R then associates with GP 130 at the extracellular region. The IL-6 signal is transduced inside the cell solely through the cytoplasmic region of GP 130. Thus, the signaling-competent form of the IL-6R complex is composed of two functionally different chains, cytokine-binding (IL-6R) and signal-transducing (GP 130) (241). The important event that instantly occurs after association of the IL-6/IL-6R complex with GP 130 is homodimerization of GP 130 (329). This action is prerequisite for IL-6 signal transduction (241). It has been postulated that IL-6 induces a hexameric complex composed of IL-6, IL-6R and GP 130 molecules (330). Like the IL-6R complex, the IL-11R-associated GP 130 homodimer is also thought to be induced by binding to its cognate ligand, IL-11 (331).

Upon homodimerization, GP 130 activates a set of cytoplasmic proteins known as JAKs. The recruitment of the JAKs to the cytoplasmic side of the GP 130 complex leads directly to their phosphorylation (340,341). To date, four members of the JAK family have been identified in mammalian cells: JAK1, JAK2, JAK3, and TYK2 (241). JAK1, JAK2 and TYK2 have all been shown to associate with the GP 130 homodimer receptor complex that forms after IL-6 and IL-11 have bound to their respective receptor chains (340,341). Once activated, the JAKs phosphorylate one or more tyrosine residues in the cytoplasmic region of the receptor complex (340,341). Such phosphorylation attracts a set of latent cytoplasmic transcription factors known as STATs (241). The receptor docked STATs are then tyrosine phosphorylated by their neighboring JAKs, which

allows them to form either homodimers made up of the same STAT species, or heterodimers made up of different STAT species. The dimerization of STATs occurs through the intermolecular interaction of phosphotyrosine-containing SH2 domains (241). Now dimerized, the activated STATs translocate from the cytoplasm to the nucleus, where they bind to target gene sequences, an event that results in transcriptional activation (332,342,343). The STAT species known to interact with the GP 130 receptor complex are STAT1, STAT3, and STAT5 (241).

In addition to the JAKs and STATs, several other signaling molecules are either activated or regulated downstream of GP 130. Among them are a number of SH2 domain-containing signal molecules that are tyrosine-phosphorylated, most likely by JAKs, following GP 130 activation (241). Specifically, stimulation of GP 130 leads to the tyrosine-phosphorylation and/or recruitment of SHP-2 (SH2 domain-containing tyrosine phosphatase 2), Shc, Vav, and the p85 subunit of PI3-kinase (340,344,345). The former three molecules are thought to function as adapters that link GP 130 to the Ras/MAP kinase pathway (241). Among the three molecules, SHP-2 appears to be the most critical for MAP kinase activation in GP 130-mediated signal transduction (346). After SHP-2 is recruited to a particular phosphotyrosine residue in the stimulated GP 130 complex, it itself is tyrosine-phosphorylated by the JAKs. Tyrosine-phosphorylated SHP-2 then attracts a complex of Grb-2 (another SH2 domain-containing adapter molecule) and Sos (guanosine 5'-triphosphate, guanosine 5' diphosphate exchange factor for Ras activation). It is generally known that the translocation of the Grb2/Sos complex from the cytoplasm to the membrane leads to the activation of the small G-protein Ras, which in turn activates Raf kinase (241). Activated Raf then phosphorylates a

downstream molecule known as MAP kinase kinase, which in turn activates its substrate, MAP kinase, through the dual phosphorylation of tyrosine and threonine residues (347).

Although c-mpl and GP 130 are part of the same receptor family (223,224), the c-mpl ligand TPO promotes both megakaryocyte proliferation and maturation, whereas GP 130-stimulating factors evoke solely maturational effects (289-291,348,349). Such functional differences suggest that c-mpl and GP 130 do not physically interact (241). Unlike the IL-6 and IL-11 receptors, c-mpl does not need other dimerpartners to transduce its signal. Upon binding to TPO, c-mpl forms a homodimer that acts as both a cytokine binding chain and a signal transducing molecule (332).

The fact that megakaryocytes and platelets are retained in TPO and c-mpl-deficient mice could be explained by the actions of IL-6, IL-11 or other thrombopoietic factors that signal through a non-c-mpl receptor system. Results from gene disruption experiments have revealed that TPO and c-mpl deficient mice exhibit a striking decrease in their number of platelets and megakaryocytic progenitor cells (228,229). Even so, platelet production still occurs, indicating that TPO alone is not responsible for terminal differentiation. Furthermore, *in vitro* studies have demonstrated that when megakaryocytes adhere to stromal cells, they cannot be stimulated by TPO to undergo terminal differentiation (261). Because mice engineered to have a continuously active GP 130 show a significant increase in plasma platelet number (241,300), we cannot discount the possibility that IL-6 and IL-11, which signal through this receptor complex, may impact directly on the very late stages of megakaryopoiesis.

PGHS-1-deficient mice do not exhibit impaired platelet production; instead, they show a decrease in their platelet aggregatory response (154). Thus, it appears that the

expression of PGHS-1 is not required for terminal megakaryocyte differentiation. To date, the only gene that has been shown essential for platelet production is the one that encodes the transcription factor NF-E2 (266). NF-E2-deficient mice rarely survive birth. Essentially, they hemorrhage to death because their megakaryocytes cannot produce platelets (266). It has recently become apparent that the TXS gene is a target for the p45 subunit of NF-E2 (267). Whether this interaction has any specific impact on platelet production is unknown.

A possible explanation as to why only adherent MEG-01 increase their level of PGHS-1 protein expression in response to cytokine stimulation lies in the fact that these cells are at a later stage of development, whereas floating MEG-01 are at a relatively early stage of differentiation. The floating MEG-01 cells probably do bind the cytokines that we administered; however, because these cells are at a relatively early stage of differentiation, they do not possess the correct set of intracellular signaling proteins necessary for differentiation to proceed. Only after the MEG-01 cells have adhered to the surface do genes encoding such proteins become expressed. The end result is that the floating MEG-01 bind to the hematopoietic factors, effectively reducing their availability to adherent MEG-01. When the floating MEG-01 cells are removed, however, the factors are able to function 'undiluted' and thus stimulate the adherent MEG-01 cells to differentiate and increase their expression of PGHS-1 protein.

Another explanation as to why MEG-01 cultures with large floating and adherent populations do not increase their level of PGHS-1 protein in response to hematopoietic factor stimulation concerns the presence of negative regulatory factors. It is possible that the less differentiated floating cells release soluble agents that have an inhibitory effect

on the more differentiated adherent cells, thereby blocking the actions of the administered agents. In fact, *in vivo*, both of these models may act in concert.

In the bone marrow, non-adherent megakaryocytes adhere to the ECM and/or stromal cells and differentiate in response to a finely tuned and as yet uncharacterized mechanism. I hypothesize that when enough of the less-differentiated megakaryocytes attach, their former compartment becomes depleted of cells, and as a consequence, maturation-inducing factors that were formerly bound-up by the less differentiated and unresponsive cells now become available to the responsive adherent megakaryocytes. At the same time, the decrease in number of the less differentiated cells in the non-adherent compartment would cause a drop in the secretion of inhibitory agents into the microenvironment, thereby lifting a potential block on differentiation and allowing the adherent megakaryocytes to increase their expression of PGHS-1 protein.

4.3 Concluding Remarks

By stimulating MEG-01 cells to differentiate into mature forms with TPA, we have shown that PGHS-1 expression is closely linked to megakaryocyte differentiation. Adherent MEG-01 cells, which are more mature than their non-adherent counterparts, express more PGHS-1 protein and mRNA than floating MEG-01. Furthermore, we have demonstrated that IL-6 and IL-11 can induce adherent MEG-01 to increase their expression of PGHS-1 protein and mRNA. Therefore, it is not unreasonable to suggest that through their combined action, IL-6 and IL-11 can affect the very late stages of megakaryopoiesis. To confirm this hypothesis, it will be necessary to perform a more

detailed analysis of PGHS-1 expression, and a more comprehensive characterization of the level of differentiation in MEG-01 cells stimulated by IL-6 and IL-11.

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APPENDIX

A1 Experimental protocols

A1.1 Isolation of total RNA

The GibcoBRL protocol for isolation of total RNA, which is based on the procedure developed by Chomczynski and Sacchi (350), utilizes the patented reagent TRIzol, a monophasic solution of phenol and guanidine isothiocyanate. The protocol was performed as follows:

Cell pellets were suspended in 1 mL of TRIzol reagent per 1×10^8 cells and incubated at room temperature for 5 min. Next, 200 μ L chloroform were added, the tubes shaken vigorously, and the resulting mixtures incubated at room temperature for 2 min. The samples were then centrifuged at 7500g for 15 min. at 4°C. The clear, upper aqueous phase from each sample was extracted and combined with 500 μ L isopropyl alcohol. The resulting solutions were mixed by hand and then incubated at room temperature for 10 minutes. Total RNA was collected by centrifuging the samples at 7500g for 10 min. at 4°C. Following centrifugation, the isopropyl alcohol was aspirated and the pellets washed with 1 mL of ice-cold 70% ethanol in order to remove excess salt. Samples were then centrifuged at 3000g, 4°C for an additional 5 min. The resulting supernatants were aspirated and the pellets air-dried at room temperature for 5-10 min. Depending upon the size of the pellet, the sample RNA was resuspended in 100 to 200

μ L of sterile diethyl pyrocarbonated (DEPC) treated ddH₂O. Total RNA was stored at –80°C.

A1.2 Purification of poly (A)+ mRNA from total RNA

Poly (A)+ RNA was purified from total RNA utilizing the Quiagen poly (A)+ extraction kit. The procedure was performed as follows:

Up to 250 μ g total RNA in 250 μ L DEPC ddH₂O were combined with 250 μ L 2X binding buffer (20 mM Tris-HCl, pH 7.5, 1000 mM NaCl, 2 mM EDTA, 0.2% SDS and 0.1% NaN₃) and 15 μ L of Oligotex suspension (10% (w/v) Oligotex particles, 10 mM Tris-HCl, pH 7.5, 500 mM NaCl, 1 mM EDTA, 0.1% SDS, 0.1% NaN₃). After an initial 3 min. incubation at 65°C to disrupt RNA secondary structure, the samples were incubated at room temperature for 10 min. in order to allow mRNA poly (A)+ tails to hybridize with the Oligotex particles. Following incubation, the Oligotex particles were collected by centrifugation at 10,000g for 2 min. at room temperature. The supernatants were then removed and the pellets resuspended in 400 μ L of OW2 wash buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA). The resulting suspensions were transferred onto Oligotex spin columns and centrifuged at 10,000g for 30 sec. The spin columns were then transferred to new RNase-free tubes and the beads washed with 400 μ L of OW2 washing buffer. The samples were again centrifuged at 10,000g for 30 sec. Next, the spin columns were transferred to new RNase-free tubes and the mRNA eluted from the beads by centrifugation at 10,000g for 30 sec. in the presence of 20 μ L of

preheated (70°C) elution buffer (5 mM Tris-HCl, pH 7.5). Purified poly (A)+ mRNA samples were stored at -80°C.

A1.3 Radioactive labeling of DNA probes

Utilizing the Stratagene Prime-It II Random Primer Labeling Kit, DNA probes were labeled with [α -³²P] dCTP by random primer extension. Specifically, 25 ng of unlabeled, gel-purified DNA probe in 25 μ L ddH₂O were mixed with 10 μ L of random 9-mer primers. DNA in the resulting mixture was denatured by heat (100°C for 5 min.). The mixture was then briefly cooled at room temperature before being mixed with 10 μ L of 5X primer buffer, 5 μ L of [α -³²P]-dCTP (50 μ Ci) and 1 μ L of Exo (-) klenow enzyme (5 U/ μ L). Following a 10 min. incubation at 37 °C, the ensuing reaction was stopped by adding 2 μ L of 0.5 M EDTA, pH 8.0. In addition, 170 μ L of Tris-HCl-EDTA (TE), pH 8.0 (10 mM Tris-HCl pH 8.0, 1 mM EDTA) were added to the tube. One μ L of this solution was removed in order to confirm radioactive labeling.

The remaining mixture was combined with 5 μ L of salmon sperm DNA (10 mg/mL) and 5 μ L of 0.1 M spermine before being incubated on ice for 10 min. The DNA precipitate was recovered by centrifugation at 8500g at 4°C. The pellet was resuspended in 135 μ L TE, 15 μ L 4 N NaOH, and 15 μ L of 5 M NaCl. Prior to membrane incubation, the probes were denatured by heat (95°C for 5 min.).

The DNA present in the 1 μ L of solution that had been removed was precipitated by incubation with 2 mL 5% TCA and 100 mL 0.2% BSA on ice for 10 min. The resulting solution was filtered under suction through a Whatman GF/C microfiber glass

filter. To determine the amount of radiolabeled probe on the filter, the paper was transferred to a scintillation tube, liquid scintillation solution added, and the radioactivity counted on a Beckman LS5000 scintillation counter.

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WORK EXPERIENCE

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Employed fluorescent microscopy and digital imaging techniques to study protein and mRNA expression on a cell by cell basis in differentiating megakaryocytes.

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Tasks included the application of fundamental molecular biology techniques to study hPGHS-1 gene expression in a megakaryoblastic cell system. Proficient at culturing different cell lines without antibiotics and under sterile conditions.

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Responsibilities included the sampling and analysis of the research reactor helium systems, analysis of the reactor heavy and light water systems using FTIR spectroscopy, and chlorine analysis of environmental water samples. Also, prepared solutions used in the extraction process of Molybdenum 99 isotope sold commercially to Nordion International.

EDUCATION

UNIVERSITY OF OTTAWA, OTTAWA, ONTARIO, CANADA

- May 1997 – present **Candidate for Master of Science in Biochemistry**
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 - Title of thesis: *Characterization of Prostaglandin Endoperoxide synthase-1 Enzyme Expression during the Differentiation of the Megakaryocytic Cell Line, MEG-01*
- Sep 1996 – Enrolled in courses of general interest
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- Sep 1992 – **Obtained Baccalaureate of Science, Honors in Biochemistry**
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- Sep 1991 – Student in B.Sc., Honors Physics
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- Sep 1989 – Student in Baccalaureate of Music program
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RESEARCH TECHNIQUES

MICROSCOPY

Fully competent with the following light-microscopy techniques: fluorescent, phase-contrast, bright-field, dark-field, and Nomarski interference. Able to prepare cell and tissue samples for analysis according to a variety of different fixation and permeabilization techniques. Fluent in digital imaging and computer analysis software. Also, able to employ digital filters and computer algorithms to analyze images and produce high quality micrographs.

ELECTROPHORESIS

Proficient at one-dimensional gel electrophoresis, Western and Northern blotting.

TISSUE CULTURE

Ability to perform tissue culture under sterile conditions without using antibiotics.

GENERAL

I am also proficient at the following techniques: cell isolation; protein, DNA and total RNA extraction, mRNA purification, small scale preparation of plasmid DNA, restriction digests, lipid extraction, and thin layer chromatography.

SKILLS

EXCELLENT WRITING AND VERBAL COMMUNICATION SKILLS

LEADERSHIP ABILITY

In Air Cadets, I attained a rank of Warrant Officer 2nd Class and received my glider pilots license. I am trained to either lead, or be part of a team that is dedicated to accomplishing the task at hand.

BILINGUAL: French/English

FLUENT IN THE FOLLOWING PC-BASED SOFTWARE:

- Word processing and spread-sheets: Microsoft Word, Power-point and Excel, Corel Word-Perfect and Presentations
- Statistics and graphing: Sigma-plot
- Digital-imaging: Adobe PhotoShop, Image-Pro Plus and Paint-shop Pro

PROGRAMMING LANGUAGES: Fortran and Pascal

CHEMISTRY SKILLS

I am proficient with the following instruments: Fourier Transform Infrared Spectrometer (FTIR), gas chromatographs, digital pH and conductivity meters, digital titrators, and beta radiation/automated liquid scintillation counters. In addition, I can perform up to six distillations simultaneously.

PUBLICATIONS

1. Chan, A.C., Wagner, M., Kennedy, C., Mroske, C., Proulx, P., Laneuville, O., Tran, K., and Choy, P.C. Vitamin E Up-Regulates Phospholipase A₂, Arachidonic Acid Release and Cyclooxygenase in Endothelial Cells. *Akt. Ernähr.-Med.* 23:152-159, 1998.
2. Mroske, C., Plant, M., Franks, D., and Laneuville, O. Characterization of Prostaglandin Endoperoxide H Synthase-1 Enzyme Expression during Differentiation of the Megakaryocytic Cell Line MEG-01. *Experimental Hematology*. 28:411-421, 2000.
3. Plant, M., Mroske, C., and Laneuville, O. Regulation of Prostaglandin Endoperoxide H Synthase-1 Gene Expression in the Megakaryocytic Cell Line MEG-01. This paper has been submitted to *Experimental Cell Research*. 2000.

EXTRA-CURRICULAR ACTIVITIES

I am a classically trained pianist. In my spare time I perform, teach, and compose music. I also study Shotokan Karate and maintain a good level of physical fitness. I like to read and listen to most types of music.

REFERENCES

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CONTRIBUTORS

The team who contributed to this project consisted of myself, Dr. Franks, Matthew Plant, and Dr. Laneuville.