Modulation of TGF-*B* in Platelets and Neutrophils by Dietary Lipids

by

Jay M. Adam

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MODULATION OF TGF-B IN PLATELETS AND NEUTROPHILS BY DIETARY LIPIDS

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Abstract

Dietary lipids have been implicated in the pathology and prevention of variety of chronic illnesses including cancer and atherosclerosis. Transforming growth factor-beta (TGF-B) is a multifunctional cytokine that is believed to modulate the key steps in pathology of these chronic illnesses. Platelets and neutrophils are known to play a role in the development of these illnesses and are also known to transport high concentrations of TGF-B throughout the vascular system. Therefore the possibility that dietary lipids modulate the TGF-B content of these cells was investigated in a series of studies. A preliminary study was conducted with weanling Spragure-Dawley rats to assess TGF-B status of platelets as affected by dietary lipids utilising the CCL-64 bioassay. The growth inhibitory effect of platelet lysate, employing the CCL-64 bioassay, was quantified to reflect TGF-B content. Rats were put on four different high fat diets and one low fat diet for 6 weeks. Diets were composed of 18% test fat and 5% soy oil, by weight. Beef tallow (HFB), fish oil (HFF), corn oil (HFC), and olive oil (HFO) were used for the high fat diets and a low fat soy oil (LFS) diet was included with 5% soy oil. When TGF-B equivalent of platelet lysate was measured, significantly more TGF-B equivalent was found in the HFB platelet lysate than the HFC or HFF platelets ($p < 0.05$). Upon acidification, to activate latent TGF-B, HFC platelets were found to have significantly more TGF-B than LFS ($p < 0.05$). When the modulation of growth factor status was assessed in eight month old rats, proliferation of CCL-64 cells increased in response to platelet lysate from all groups. The stimulatory affect of platelet lysate was in the order of HFF>HFO>HFB>HFC>LFS. Acidification of lysate resulted in a marked reduction of

the stimulatory potential of platelet lysate in all treatment groups, however the results followed the same order, as noted for nonacidified samples. Supplementary studies were conducted with the neutrophils and plasma of the older rats to assess if the effect noted in platelets was an isolated phenomenon and the effects could be found in the general circulation. The TGF-*B* of neutrophil samples was significantly higher in HFO samples than in HFB and LFS samples ($p<0.05$). Western blot analysis demonstrated that HFB had the highest concentration of TGF-*B*1 in platelets, HFO the highest in neutrophils and LFS and HFF the lowest in plasma. These findings demonstrate that dietary lipids modulate the growth factor content of platelets and neutrophils. Future research is needed to describe these effects in greater detail and demonstrate how these changes affect disease pathology and the ability of platelets to secrete or store TGF-*B* under a variety of dietary manipulations or during different disease states.

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LIST OF ABBREVIATIONS

ACD	acid-citrate dextrose
AIDS	acquired immune deficiency syndrome
ADP	adenosine diphosphate
AA	arachidonic acid
BCP	bovine calf plasma
CPM1	counts per minute
DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid
ET-1	endothlin-1
EGF	epidermal growth factor
ECM	extracellular matrix
HDL	high density lipoprotein
HFB	high fat beef tallow
HFC	high fat corn oil
HFF	high fat fish oil
HFO	high fat olive oil
HIV	human immuno-deficiency virus
IDDM	insulin dependent diabetes mellitus
Kda	kilodalton
LAP	latency associated peptide
LFS	low fat soy oil
LOA	linoleic acid
LA	alpha-linolenic acid
LDL	low density lipoprotein
MUFA	monounsaturated fatty acid
PBM	peripheral blood monocytes
PBS	phosphate-buffered saline
PDGF	platelet derived growth factor
PUFA	polyunsaturated fatty acid
PGI_2	prostacyclin$_2$
SFA	saturated fatty acid
SMC	smooth muscle cells
SMP	skim milk powder
RBC	red blood cells
RNA	ribonucleic acid
TSP-1	thrombospondin-1
TxA_2/A_3	thromboxane A_2/A_3
TGF-B	transforming growth factor-beta
RI	transforming growth factor-beta receptor I
RII	transforming growth factor-beta receptor II
RIII	transforming growth factor-beta receptor III
VSMC	vascular smooth muscle cells

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Chapter 1.   INTRODUCTION

Lipids and chronic illnesses

Dietary lipids have been implicated in the pathogenesis of a number of chronic illnesses while in other instances dietary lipids prevent certain diseases. These illnesses include diabetes, cardiovascular diseases and cancer. It is apparent that all these diseases are multifactorial in nature and their genesis involves interaction among a variety of biological responses occurring in a number of tissues. Localized pathological lesions identified as atheromatous plaque or precancerous or cancerous growth, also occur as a result of aberrant environmental conditions and involve alterations occurring at the genotypic and phenotypic levels. The environment surrounding the early lesions, profoundly affects their development. The circulatory system is a vehicle which continuously bath vital organs and provide them with important nutrients, as well as bioregulators such as hormones and growth factors. Cellular constituents of the vascular pool include platelets, red blood cells (RBC) and white blood cells. The half life of these cells in the circulatory system varies from hours to days and are influenced by physiological states. Both platelets and neutrophils are formed in the bone marrow from stem cells and their functional capabilities are affected by their hosts' nutritional and physiological status. One example in which the nutritional status of a host alters functional capability of these organelles includes, the modulation of eicosanoid metabolism by dietary lipids.

Platelets and neutrophils contain a variety of cytokines and growth factors with important implications to the genesis of a number of chronic illnesses. The main objective of the present research was to determine if fatty acid composition of dietary

lipids, varying in fatty acid composition, would affect platelet growth factor status of adult rats. Of the growth factors contained in platelets, transforming growth factor-Beta (TGF-*B*) is of very high concentration. In addition the over expression of TGF-*B* has been demonstrated to be important in the pathogenesis of many illnesses. For these reasons TGF-*B* was chosen as the target growth factor for this thesis. Additional studies were conducted with the neutrophils and plasma of the same animals to assess if their TGF-*B* status would be modulated in a fashion similar to platelets.

A limited effort has been invested to understand how effective a diet can be in altering the amount of growth factors, or other cellular constituents, in platelets and neutrophils that in turn may affect their functionality.

n-3 and n-6 fatty acids are known to differ from each other in ability to influence a number of chronic illnesses such as diabetes, atherosclerosis and cancer. TGF-*B* has a pathogenic role in the development of these diseases. We hypothesize that dietary lipids that contain different combinations of fatty acids will differ from each other in altering the level of TGF-*B* in platelets, neutrophils and the circulation.

Chapter 2   LITERATURE REVIEW

A. Lipids and Modulation of Cell and Tissue Function

Dietary lipids, their level and composition have been implicated with the pathogenesis and prevention of a variety of chronic illnesses such as cardiovascular disease, cancer and diabetes. The mechanisms through which dietary lipids exert their effects remain unknown. However, several physiological responses that are altered by dietary lipids have been described. A brief description of the potential mechanisms follows.

Dietary lipids are macronutrients consumed primarily for the purpose of supplying energy. Beyond supplying energy, lipids are the fundamental structural component of biological membranes that allow the cell to control its own permeability (Yeagle, 1989). Lipids also function as components of second messaging systems via their metabolism to eicosanoids. Dietary lipids may modulate cell function by altering membrane composition or by being metabolized into different forms of eicosanoids. Lipids have also been demonstrated to modulate gene expression in cells that are directly involved with fatty acid metabolism.

The potential of membrane fatty acids to be modified by dietary fatty acids is organ specific. The heart, liver and testes are the most sensitive and the brain and retina the most resistant to dietary change. Different organs of the body have preferences for different kinds of fatty acids. The majority of the polyunsaturated fatty acids (PUFA) in heart and liver membranes are in the n-6 form. In contrast, the membranes of the cerebral

cortex, sperm and the photoreceptor cells of the retina contain higher proportions of their PUFA in the n-3 form (Murphy, 1990).

It is generally accepted that the fatty acid composition of biological membranes is not constant, but rather it changes responding to its environment. New fatty acids from the diet are incorporated into membrane lipids via de novo membrane phospholipid synthesis and acyl group turnover in membrane phospholipids (Clandinin, et al., 1991). With the lipid composition of the membrane altered, the function of the membrane's proteins may also be altered, tempering certain functions of the cell. Protein concentration of most cellular membranes is so high that the amount of surface area occupied by proteins is just as high as the areas occupied by lipids (Yeagle, 1989). Fatty acids are an integral component of membranes as they are required to provide a specific microenvironment around hydrophobic regions of membrane proteins (Clandinin, 1994). There are a variety of lipid components of membranes that could have an affect on protein function. Examples include cholesterol content, phospholipid head group composition, asymmetry of phospholipid distribution in the membrane, and phospholipid fatty tail composition. Due to the variety of lipid components of membranes it is very difficult to ascertain which components may control the function of a protein in question (Clandinin, 1985).

Dietary lipids also modify protein function through covalent binding between membrane proteins and fatty acids (Clandinin et al., 1994). Due to the relatively low polarity exhibited by membrane proteins they have a high affinity for lipids. These membrane proteins that are covalently bound to membrane lipids are tightly bound (Marinetti and Cattieu 1982). Even after isolation, various membrane proteins are found

to have lipids tightly bound to them. Glycophorin from human erythrocytes, and cytochrome oxidase from mitochondria are two examples of proteins that are bound in this fashion (Yeagle, 1989).

Fatty acids with a large number of double bonds, can not be grouped as tightly together as fatty acids with a low number of double bonds. Therefore, biological membranes with a larger proportion of SFA in their phospholipids are more rigid than membranes with lower proportions of SFA. As fatty acids with a different chemical structure are incorporated into a membrane, the function of that membrane may actually change. The membrane's fluidity is somewhat dependent on its fatty acid composition. A more fluid membrane, constructed of a higher proportion of unsaturated fatty acids, theoretically may allow greater flexibility around proteins or easier transfer of proteins in or out of a cell (Murphy, 1990).

An example of a membrane protein that can be modulated by changing dietary fatty acid composition is adenylate cyclase. Adenylate cyclase is involved in transmembrane signaling, and is therefore a key regulator of cell function. Increased adenylate cyclase activity occurs when rats are fed fish oil instead of corn or coconut oil, indicating that the activity of adenylate cyclase is dependent on the level of n-3 PUFA (Alam & Alam, 1988). Whether this increase in activity is a result of increased membrane fluidity around the proteins or due to increased adenylate cyclase synthesis has not been demonstrated. Adenylate cyclase activity in cardiac membranes also may be reduced when a diet deficient in essential fatty acids is fed to rats. When the animal is refed essential fatty acids, membrane fatty acid composition returns to normal, but adenylate cyclase activity continues to be partially decreased (Murphy, 1990). Therefore,

there is more to modulation of membrane protein function by dietary fatty acids than simply the membrane composition. Dietary lipid composition may have an impact on cell function for an extended period of time.

A change in dietary fat intake may also alter the fatty acid composition of a cell's nuclear membrane. Theoretically this may in turn alter nuclear function by changing receptor mediated stimulation of transport of ribo nucleic Acid (RNA) out of the nuclear envelope through nuclear pore complexes. Control of gene expression may occur at this point by regulating the flow of RNA or gene products into the cytoplasm (Clandinin et al., 1991).

A large portion of the research on the impact of different dietary fatty acids on cell or tissue function has been conducted with hepatic tissue. Addition of linoleic acid (LOA) (18:2n-6) to fat free diets has been demonstrated to decrease the activity of enzymes involved in fatty acid synthesis, while addition of palmitic acid or oleic acid have no effect. It is believed that PUFA can exert this effect by changing the gene expression of these enzymes (Clarke and Jump, 1994). A variety of different studies on the effect of different fatty acids on hepatic tissue have demonstrated an alteration in gene expression of enzymes involved in fatty acid synthesis (Clandinin et al., 1991; Clarke and Jump, 1994; Clarke and Jump, 1996).

Fatty acids have also been observed to exert control over gene expression in adipocytes. Animals on diets high in SFA have been found to have three times the number of retroperitoneal adipocytes in comparison to animals on high PUFA diets (MacDougald and Lane, 1995). *In vitro* studies have also been conducted demonstrating increased gene expression of fatty acid binding protein by preadipocyte cell lines in

response to incubation with long chain fatty acids in comparison to medium or short chain fatty acids (Distel et al. 1992). Increased proportion of PUFA in the diet has also been associated with increased binding of insulin to adipocytes in a diabetic animal model. Adipocytes from animals fed the high PUFA diets had greater ability to transport glucose than those from animals on lower PUFA diets (Clandinin et al., 1985).

Activated oncogenes, in model cell culture systems, have been demonstrated to be modulated by different fatty acids. The affect of a c-H-ras oncogene on a fibroblast cell line was found to be modulated by different fatty acids in the medium. SFA were found to increase the number of transformed foci obtained, while unsaturated fatty acids had little effect on the action of this oncogene (Hsiao et al., 1990).

Dietary lipids also modulate cell function through their metabolism into mediators of cell function known as eicosanoids. The three families of eicosanoids are the leukotrienes, thromboxanes and prostaglandins. Prostaglandins and thromboxanes are products of the cyclooxygenase pathway, while leukotrienes are products of the lipoxygenase pathway. Thromboxanes are primarily synthesized by platelets, prostacyclins (a class of prostaglandins) are primarily synthesized by the vascular endothelium and leukotrienes are primarily synthesized by leukocytes. Prostaglandins and thromboxanes exhibit a wide range of physiological functions including the control of blood pressure, diuresis, platelet aggregation, smooth muscle contraction, and other effects on the immune and nervous systems. Leukotrienes affect the function of a variety of different tissues including; respiratory, vascular, and intestinal smooth muscle cells by altering their ability to contract. Through these functions leukotrienes serve as mediators in asthma, inflammatory reactions, and myocardial infarction (Hunt and Groff, 1990).

Platelets do not store eicosanoids, but rather synthesize them in response to various stimuli, such as thrombin or adenosine diphosphate (ADP). The synthesis of eicosanoids requires free fatty acids such as arachidonic acid (AA) (20:4n-6) or eicosapentaenoic acid (EPA) (20:5n-3). A resting platelet does not contain free AA or EPA. AA is stored as phospholipids in both a platelets plasma and internal membranes, and is released in response to a stimulus (Roth, 1986). Therefore when membranes contain higher proportions of AA, more AA can be released, which can then be converted to eicosanoids of the 2-series. If the membrane contained lower proportions of AA and a higher amount of EPA, when the membrane was stimulated to release fatty acids, more EPA may be released. With n-3 rather than n-6 fatty acids acting as substrates for the cyclooxygenase pathway eicosanoids of the 3-series, that have different characteristics, are produced. For example thromboxane A_2 (TxA$_2$), metabolized from n-6 fatty acids, has much stronger pro-coagulant properties than Thromboxane A_3 (TxA$_3$), which is metabolized from n-3 fatty acids (Hunt and Groff, 1990). Therefore, membrane fatty acid composition can have an affect on cell function by supplying different substrates for metabolic reactions. Nutritional modulation of platelet function will be discussed later in the section **Modulation of Platelet Function by Nutrition.**

Besides altering platelet composition, n-3 fatty acids have also been demonstrated to affect disease processes. n-3 fatty acids are thought to inhibit experimental colon cancer by altering the release of prostaglandins within the colorectal mucosa, as demonstrated in animal models. Prostaglandin E_2 levels have been shown to decrease in the colonic mucosa during fish oil supplementation. Low doses of EPA and docosahexaenoic acid (DHA), given for 30 days or longer to patients who had undergone

clearing polypectomies for colorectal polyps, significantly reduced proliferation in subjects who had abnormal baseline proliferation patterns (Anti et al., 1994).

DHA has been demonstrated to inhibit vascular cell adhesion molecule-1 *in vitro*, a leukocyte adhesion molecule. Its expression can lead to atherosclerotic lesion progression and potential moderation of its expression could decrease disease progression. Use of aspirin or indomethacin, inhibitors of cyclooxygenase, did not alter the effect of DHA, therefore the effect does not seem to depend on DHA being converted to eicosanoids (Terano et al., 1996). This may mean that DHA is altering the gene expression of vascular cell adhesion molecule-1 at the nuclear level.

B. Growth Factors

A growth factor is a regulatory substance that transmits a biological signal involved in the regulation of cell growth, differentiation and nutrient metabolism. Cells from a wide variety of tissues produce growth factors, which then act on nearby cells or even act on the same cell that produced it. Many growth factors circulate in the blood and act on cells far from their place of origin. During circulation, growth factors become involved in physiological processes including; inflammation, immune reactions and wound repair. Growth factors have also been implicated in the development of common diseases including atherosclerosis, cancer and autoimmune diseases. Some tumour cells are able to produce and utilize growth factors on their own (Pimentel, 1994). Examples of growth factors are epidermal growth factor (EGF), platelet derived growth factor

(PDGF) and TGF-*B*. These growth factors travel outside of cells until they are picked up by a receptor or degraded.

The regulatory actions of growth factors are performed on cells via a wide variety of intracellular mediators. The growth factor's function is to bind to its specific receptor on a cell membrane. The receptor then passes the message along to a regulatory protein, such as a G protein, for further communication to second messengers within the cell. When the message is communicated to a cell's nuclease, DNA synthesis can be affected by up regulating or down regulating expression of a specific gene. This process of communication of a message between different regulatory proteins to a cell's nuclease is known as signal transduction and is the manner in which a cell regulates its functions.

For example, a growth factor binding to its receptor may result in phosphorylation of the receptor, leading further to the activation of second messengers such as phospholipase C in phosphatidyl inositol metabolism. Signal transduction can function in the control of mitosis, and a growth factor binding to its receptor on the membrane could be a controlling step in the control of this phenomenon. It has been proposed that quiescent cells are initially brought to competence, from the G0 to G1 phase, by specific growth factors. Cells subsequently become committed to DNA synthesis via growth factor progression. Due to the control that growth factors have over cell function an imbalance between positive and negative feedback mechanisms can result in an abnormal cellular response (Ross et al., 1993).

An abundance of research is being conducted on the functions of TGF-*B* and TGF-*B* is the focus of the present investigation. TGF-*B* has been implicated in the pathology of multiple diseases including cancer (Young et al., 1996; Markowitz et al.,

1995), atherosclerosis (Grainger and Metcalfe, 1995) and autoimmune disease (Fernandes, 1994). A large portion of the research on this growth factor involves modulation of TGF-*B* in a manner that could slow or reverse the progression of certain disease states.

C. TGF-*B* and its Biology

TGF-*B* is a 25 kilodalton (Kda) peptide composed of two polypeptide chains that are joined together by disulphide bonds. It is widely distributed throughout normal and transformed cells and most organs and tissues. In most cases TGF-*B* inhibits the growth of normal epithelial cells and stimulates the growth of mesenchymal cells, with some exceptions. For example, TGF-*B* stimulates the growth of endothelial and smooth muscle cells (SMC) for angiogenesis but can inhibit the growth of SMC *in vitro* and possibly *in vivo* under different circumstances. Proliferation of a minority of epithelial tissues, including vaginal epithelium, is actually stimulated by TGF-*B* (Glick et al., 1991).

There are three isoforms of TGF-*B* in mammals, each of them have high sequence homology and share the same functions and receptors. TGF-B exerts its effect on cells by binding to specific receptors located on the cell surface, known as TGF-*B* receptor I (RI), receptor II (RII) and receptor III (RIII). These receptors have a serine/threonine kinase domain, and part of the action of TGF-*B* on the receptor involves phosphorylation of these serine and threonine residues (Pimentel, 1994B). Most mammalian cells express all three receptors, which can bind and be cross linked to all of the TGF-*B* isoforms. The RI, RII, and RIII receptors can be distinguished from each other by their structural and

functional properties. RI and RII have high affinity for TGF-*B*1 and low affinity for TGF-*B*2. RIII has high affinity for TGF-*B*1 and *B*2, but is less common in mammals and is not required for TGF-*B* function (Pimentel, 1994B; Derynck, 1994). TGF-*B* is not cross reactive with receptors other than these main receptors, although it has been observed to bind to CD36 (Khalil, 1997). The pattern of TGF-*B* binding in cos cells was examined by transfecting the cells with one kind of TGF-*B* receptor at a time. Cells were transfected with one form of a TGF-*B* receptor at a time in order to elucidate which form of the receptor was more important in the binding of TGF-*B*. TGF-*B*1 was found not to bind well to either RI or RII when cells were transfected with RI alone. However TGF-*B*1 bound well to RII but not endogenous RI after transfection with RII alone. After cotransfection of RI and RII, TGF-*B*1 will bind well to both receptors. The increased ability of TGF-*B*1 to bind to RII in comparison to RI, combined with the increased binding ability of RI when both receptors were transfected together suggests that the RII may be the primary binding subunit for TGF-*B*1 (Rodriguez et al., 1995). Further research has supported the finding that RII binds TGF-*B* without the requirement of another receptor or detectable accessory protein and the ability of some RI to bind TGF-*B* depends on the coexpression of RII (Miettinen et al., 1994; Derynck, 1994).

Both RI and II must be phosphorylated in order to be able to transmit signals. RII is highly phosphorylated in the absence of TGF-*B* and is constitutively phosphorylated by cellular kinases at multiple sites and by itself at additional sites. RI does not have the ability to phosphorylate itself without the aide of RII, and therefore has an inability to bind TGF-*B* when expressed in the absence of RII. RI recognizes TGF-*B* bound to RII but not free in the medium. Ligand bound RII recruits RI and phosphorylates it on serine

and threonine residues via RIIs kinase activity. RI can then phosphorylate downstream substrates (Wrana et al., 1994).

Theoretically the functional TGF-*B* receptor is a heteromeric complex of RI and RII receptors. In different cell lines TGF-*B* has been found to take different routes of action to have an effect on a cell. TGF-*B* has been shown to induce protein phosphatase 1 activity in keratinocytes. In several epithelial cell lines TGF-*B* appears to activate ras, and to increase mutogen-activated MAP kinase. TGF-*B* has been demonstrated to have different effects on cell function depending on a cells state of development. A cell line may be responsive to TGF-*B* at an early stage in its development but unresponsive at later stages (Derynck, 1994).

The effects of TGF-*B* are mediated through a variety of G protein dependent and independent pathways (Pimentel, 1994B). Other investigations conducted with cell lines have demonstrated that phosphatidylcholine-phospholipase C and protein kinase C are early intermediates in TGF-*B* signal transduction pathways (Halstead et al., 1995).

TGF-*B* has been demonstrated to decrease B cell and T cell activity, antibody function and have an immunosuppressive effect (Puolakkainen et al., 1995; Sosroseno et al., 1995). Increased TGF-*B* secretion from the peripheral blood monocytes (PBMC) of Human Immuno-deficiency Virus (HIV) positive patients has been observed. Subsequent inhibition of B-cell proliferation by TGF-*B* from these same PBMC has been observed to occur *in vitro*. Yet, when B-cells from either HIV+ or healthy donors are incubated with the equal amounts of TGF-*B* they proliferate at the same rate. Therefore, this research demonstrates that TGF-*B* may be a key mediator of the suppression of B-cell function

that occurs in acquired immune deficiency syndrome (AIDS) patients (Kekow et al., 1991). An HIV associated glycoprotein, HIV gp160, that is expressed on the surface of HIV and HIV infected cells has been demonstrated to induce production of TGF-*B* by PBMC *in vitro* (Hu et al., 1996). This research supports the previous work in that it demonstrates increased production of TGF-*B* in response to HIV infection.

Theoretically, immunosuppression by TGF-*B* may allow tumours an increased chance of survival by inhibiting the immune system's ability to recognize or destroy tumour cells. Tumours from head and neck cancers, that were surgically removed, release TGF-*B* in culture. Of interest, is that tumours that were of a metastic variety released almost twice as much TGF-*B* as did primary tumours. This research demonstrates that tumour cells are capable of contributing to the immune suppression of cancer patients (Young et al., 1996). Perhaps the increased ability of these tumours to produce TGF-*B*, and subsequently suppress the immune system, is a characteristic that allowed them a greater chance of metastasizing successfully.

Other research supports the possibility that TGF-*B* released from tumour cells inhibits the proliferation of CD4 and CD8 T-cells and natural killer cell function (Sosroseno, 1995). Many glioma cell lines release TGF-*B* in its mature active form, in contrast to most untransformed cells that release it in an inactive form. Many malignant glioma patients suffer from immunosuppression induced by the disease and the release of TGF-*B* from their tumours may be a key mediator of this symptom (Weller et al., 1995).

Biological state of TGF-B

TGF-*B* exists in both an active state and an inactive latent state. Latent TGF-*B* is complexed to a series of proteins, known collectively as the latency associated peptide (LAP), that blocks the active site of the TGF-*B* molecule from binding to a receptor. Activation of TGF-*B* is a complex process that involves either cleaving the LAP protein off of the active TGF-*B* molecule or changing the configuration of the LAP so that the active sight on the TGF-*B* molecule can bind to a receptor. Different proteins and enzymes known to be involved in this process include thrombospondin-1 (TSP-1), trypsin and plasmin (Grainger and Metcalfe, 1995). Thrombospondins are glycoproteins that are present in connective tissue, the alpha granules of platelets, wound fluid and embryonic tissue.

The importance of protease's like plasmin in TGF-*B* function has been demonstrated by using aprotinin, a protease inhibitor which suppresses plasmin activity. Aprotinin has been demonstrated to abolish increases in the proportion of active TGF-*B* produced by mesangial cells in response to phorbol dibutyrate (Studer et al., 1995).

TSP-1 activates latent TGF-*B* by binding to the molecule and reconfiguring it so that the active sight is available for binding to a receptor. (Schultz-Cherry et al., 1994; Khalil, 1997). Activation of TGF-*B* by TSP-1 or plasmin is not necessarily a straightforward reaction. *In vivo* research has demonstrated that TSP-1 can not activate TGF-*B* in solution without the presence of macrophages. In this model TSP-1 binds latent TGF-*B*, then they are both bound to the macrophage by CD36. Plasmin then cleaves off the LAP protein (Khalil, 1997). TGF-*B* can also be activated by heat, alkaline or acid *in vitro*. When it is activated in this fashion the LAP protein is usually not

removed but merely denatured enough so that the active sight on the TGF-*B* molecule can bind to its receptor (Grainger and Metcalfe, 1995).

D. Genesis of Platelets and Their Importance in Health and Disease

Platelets are small disc shaped cells with an average diameter of 2-4 *u*m. In humans they circulate 9-12 days, and about 30% are sequestered in the microvascular or in the spleen as functional reserves after their release from bone marrow. Platelets gradually diminish in size later in their life span. The average person has about 250,000 (150,000 to 400,000) platelets per *u*l of blood. Platelets are not cells in the traditional definition of the term, because they are actually small packets of cytoplasm that are nipped off from the cytoplasm of large mother cells in the bone marrow known as megakaryocytes. The megakaryocytes are derived from cells in the bone known as stem cells. Other blood cells such as RBC and monocytes are derived from these same stem cells. The progression of stem cells to megakaryocytes and platelets is inhibited by TGF-*B* and other alpha granule proteins (Gewirtz & Schick, 1994).

The growth factors carried by platelets are also produced in the megakaryocytes in the bone marrow. This has been investigated with PDGF, a growth factor that is also carried in platelets like TGF-*B*. PDGF production in megakaryocytes is demonstrated to be associated with the PDGF content of platelets. Research (Haen, P.J., 1995) has demonstrated that the activity of PDGF found in megakaryocytes is proportional to the total number of megakaryocytes examined and the PDGF activity of platelet homogenates. This suggests that the PDGF found in platelets is derived from the

megakaryocytes. TGF-*B*1 mRNA has also been identified in mature megakaryocytes (Gewirtz & Schick, 1994). If the PDGF in platelets was there during platelet development it is possible that the other growth factors stored in the alpha-granules, including TGF-*B* are also derived from megakaryocytes. This is significant as it suggests platelet genesis in megakaryocytes as a possible site where modulation of growth factor content of platelets could occur.

Since platelets are produced from megakaryocytes the function of the megakaryocyte is important to platelet function. Megakaryocytes have the ability to synthesize fatty acids, yet fatty acid composition of megakaryocytes has been demonstrated to be dependent on the fatty acid composition of the diet. If megakaryocyte function is dependent on its fatty acid composition, then potentially platelet function may also be dependent on megakaryocyte composition. Previously it has been demonstrated that fatty acid composition of the diet, affects megakaryocyte and platelet function (Schick et al., 1993). This study involved feeding guinea pigs diets that contained the majority of their lipid as either SFA, monounsaturated fatty acid (MUFA) or n-3 PUFA. Fatty acid composition of megakaryocytes and platelets, to some extent, mimicked what was available from the diet. Significant differences were found in the oleic acid, palmitic acid, AA and n-3 fatty acid content of both megakaryocytes and platelets. Megakaryocytes from animals fed n-3 fatty acids were significantly larger in diameter compared to those from animals on a control diet ($p < 0.05$). Thromboxane synthesis of platelets was inhibited in animals fed the n-3 diet compared to the control group. Therefore platelet physiology and genesis are in part modulated by diet and may be dependent on megakaryocyte structure.

Platelets contain glycogen, mitochondria and other organelles such as alpha-granules, dense bodies and a dense tubular system that functions in prostaglandin synthesis. The alpha-granules and dense bodies store serotonin and catecholamines. There are 20-200 alpha-granules in a platelet, which contain TSP, TGF-B, other growth factors and enzymes. During their time in circulation, platelets are known to decrease in size and slowly release some of their alpha-granules (Corash, et al., 1984). TGF-B is up to 100 times more concentrated in platelets than in other non-neoplastic tissues (Assoian et al., 1983; Haen, 1995; Pittiglio and Sacher, 1987).

Therefore it is possible that dietary lipids modulate the TGF-B content of platelets. Dietary lipids theoretically could have an affect on the phenotype of platelets resulting in alteration of TGF-B content. TGF-B content of platelets may be dependent on the amount of the growth factor that is synthesized by the megakaryocytes before the formation of platelets. The content may also be affected by the manner in which TGF-B is packaged into the platelets during their formation. If the ability of a platelet to secrete alpha-granules during circulation was modulated by diet then the content of TGF-B would be subsequently modulated (Figure 1).

The primary function of platelets is to maintain the integrity of the endothelial lining of blood vessels. Upon injury to the endothelium of the vessel wall, by trauma or immune reaction, collagen fibers of the subendothelial vessel wall are exposed. Platelets adhere and cover the exposed subendothelium and undergo morphological changes. When receptors on platelets bind collagen, thrombin, ADP or epinephrine, the release of ADP and eicosanoids such as TxA_2 is triggered. ADP and TxA_2 cause the platelets to aggregate and form a plug. Platelets then undergo further degranulation, releasing

Figure 1: Theoretical mechanism by which dietary lipids may modulate TGF-*B* concentration in platelets. Lipids are metabolized or incorporated into megakaryocytes and alter their phenotype. The megakaryocytes synthesize platelets that in turn have a varying phenotype, with respect to TGF-*B* content or ability to retain or pick up TGF-*B*.

growth factors and other components of their granules. These growth factors may then function in the control of SMC proliferation. This overall process of platelet adhesion, aggregation and degranulation is commonly referred to as clotting (Grouse, 1982; Haen, 1995).

E. Platelets and Disease Processes

Development of atherosclerotic lesions

Platelets modulate atherosclerosis through clot formation as a part of their function in maintaining the structure of the vessel wall. The formation of the platelet clot itself is an early key step in the development of an atherosclerotic lesion. After an injury to the vessel wall and subsequent reinforcement with platelets, repair of the injury to the vessel wall begins to occur. During this healing process lesion formation begins when SMC migrate from the tunica media into the intima of the vessel wall under the endothelium. These early lesions are termed diffuse intimal thickenings and are composed almost entirely of SMC and extracellular matrix (ECM). This thickening of the arterial intima is a common feature of all forms of experimental atherosclerosis (Moore, 1985).

As a person ages these early lesions can increase in size via further migration of SMC or proliferation of the SMC occupying the intima. Monocytes and macrophages infiltrate the intima, at these sites, from the general circulation and accumulate. Blood lipids can be endocytosed, by the macrophages and intimal SMC, via a scavenger receptor. There is some evidence that thickening of the arterial intimal wall precedes the

deposition of lipids. Experimental models do not demonstrate increased lipid deposits in the vessel wall until a neo-intima has been formed by SMC that migrate from the arterial media and subsequently proliferate in the intima (Moore, 1985). Over a long period of time, the lipid concentration of the accumulated macrophages, now known as foam cells, increases and the cells eventually die. Lipid from the cytoplasm of the foam cell contributes to the necrotic core of the lesion. The intima may also become calcified which has been associated with instability of the lesion.

After a period of many years the atherosclerotic lesion, or plaque, increases in size until blood flow through the vessel is eventually restricted (Kovanen et al., 1986). The plaque also may rupture, releasing the decayed macrophages and lipids into the lumen of the vessel. This triggers a massive coagulation of platelets that form a blood clot over the ruptured lesion, this growing blood clot is referred to as a thrombus. The thrombus can grow rapidly over a few hours until blood flow through the vessel is completely blocked, causing an occlusive thrombus that results in debilitation of the tissue that derives blood from the vessel. If the vessel is a coronary artery a myocardial infarction may occur (Figure 2: A,B,C,).

Two factors will determine the likelihood of an occlusive thrombus forming (Kovanen, 1986). The first factor is the presence of atherosclerotic plaque. The second factor is the stability of the existing plaque structure, a weaker structure will contribute to the probability of the plaque rupturing. Theoretically it would be possible to reduce the frequency of myocardial infarction by slowing the formation of a lesion, or by increasing the stability of the plaque on the lesion thus preventing rupture and thrombus formation. This is the basis for platelet and growth factor involvement in atherosclerosis.

Figure 2. The three phases of atherosclerosis development.

Figure 2A. When an injury occurs to the endothelial lining and the intima and VSMC are exposed, platelets coagulate and begin to cover the exposed area (Kovanen, 1986).

Figure 2B. Monocytes, macrophages, T cells and lipoproteins then migrate below the endothelium, into contact with SMC. These SMC then may change their gene expression and migrate to the endothelium of the blood vessel and an atherosclerotic lesion develops. These immune cells then die and contribute to the necrotic core of the lesion (Kovanen, 1986).

Figure 2C. When the plaque covering the lesion ruptures the contents of the lesion are released into the lumen of the vessel. The contents activate the platelets in circulation causing them to coagulate and cover the ruptured lesion. This clot may grow until the entire vessel is blocked forming an occlusive thrombus that can cause a myocardial infarction (Kovanen, 1986).

Macropage/
monocyte   lipoproteins   Media

Intima   Endothelial   Platelets   Macrophage
          cells

Platelets

SMC   Macrophages

Platelets   Thrombus   Macrophages

Altered SMC

The vascular smooth muscle cells (VSMC), which compose the intima of the developing atherosclerotic lesion have been demonstrated to be different from the VSMC that are part of the normal tunica media. They differ in that VSMC of the intima differentiate less and have different patterns of gene expression. Intimal cells have been demonstrated to express genes for osteopontin and matrix Gla protein, while medial cells express genes for SM-22 alpha and calponin. When medial VSMC are cultured *in vitro* they undergo changes and after a period of time more closely resemble the intimal VSMC. It has been proposed that this transition is a pre-requisite for subsequent proliferation of the VSMC. It is possible that the cause of these changes to the cultured VSMC are a result of the same changes that cause migration of VSMC into the space between the endothelial cells and internal elastic lamina when a lesion is developing *in vivo* (Grainger and Metcalfe, 1995).

Platelets from diabetics

Platelets from either insulin dependent diabetes mellitus (IDDM) or hypertensive patients have been demonstrated to have higher growth promoting potential, for VSMC, than platelets from healthy controls. This higher potential to stimulate growth was found to be normalized by intensive insulin therapy in the patients with IDDM (Mikhail et al., 1993). No significant correlation was found between increased growth promoting ability of platelets and plasma glucose, haemoglobin or cholesterol (Sugimoto, et al., 1987). Therefore the growth promoting compounds derived from the platelets of diabetics are a property that may be intrinsic to these platelets and relate directly to a component of the pathology of diabetes.

It is known that diabetics are at a higher risk for the development of atherosclerosis than the rest of the population. If platelet growth promoting ability is a significant factor in lesion progression then the higher growth promoting ability of platelets of diabetics could be a factor in their higher risk for this disease. Another characteristic of diabetes patients that could cause an increased risk for atherosclerosis is their higher platelet production of TxA_2 and reduced vascular production of prostacyclin$_2$ (PGI_2) (Mikhail et al., 1993). TxA_2 is a strong stimulator of platelet aggregation and vasoconstriction, while PGI_2 is an inhibitor of platelet aggregation and a vasodilator. It is believed that these two eicosanoids compete with each other at sites of vascular injury (Figure 3 A & B).

The reaction of rats to de-endothelialization of the aorta was examined in both diabetic and control animals (Winocour et al., 1993). Rate of platelet accumulation, turnover and intimal thickening on the de-endotheliazed aorta were all examined. In the short term after de-endothelialization there was no difference in the net accumulation of platelets between the two groups. Six or seven days after de-endothelialization the rate of platelet accumulation was higher in diabetic rats than controls. Intimal thickening was greater in aorta from diabetic rats than control rats at both 15 and 28 days after injury. The thicker intima of diabetic rats was accompanied by a greater number of layers of SMC. For some reason the aorta of diabetic rats is able to attract platelets over a longer period of time than controls. This continued interaction of platelets with the aorta may be what causes the increased migration of SMC and the thicker neointima.

Figure 3: A & B. TxA_2 versus the action of PGI_2. A) When PGI_2, released from the endothelial cells is dominant, platelets are prevented from adhering together. B) When TxA_2 released from platelets overpowers the PGI_2 the ability of the platelets to adhere to one another is increased.

Endothelium

Platelets

Arterial Wall

PGI_2

TxA_2

Endothelium

Platelets

This research demonstrates that diabetes can alter platelet function in a manner that could predispose a patient to the further development of disease. This is significant to the study of the relationship between nutrition and platelet function as both the control and development of diabetes are closely related to diet. Also of particular interest is that both growth promoting ability and TxA_2 production of platelets is increased with diabetes. It is well established that TxA_2 production is modulated by the presence of n-3 or n-6 fatty acids in the diet (Hunt and Groff, 1990). Therefore, both diet and diabetes can modulate platelet TxA_2 production and diabetes also modulates platelet growth stimulating potential. The possibility that diet also modulates growth stimulating potential, through a mechanism similar in fashion to how diabetes modulates platelets, is a concept that merits investigation.

This alteration of growth factor status of the platelets of diabetics has been further studied with different cell lines (Hamet et al., 1985). Mouse 3T3 fibroblasts, rat VSMC, and human WI-38 fibroblasts have all been demonstrated to proliferate more in response to the supernatant from diabetic platelets than normal platelets. Possible explanations for this phenomenon have been proposed. It is possible that the different growth factor status of diabetic platelets is a result of different rates of enzymatic degradation of the growth factors in diabetics. Another possibility could involve release of growth factors into the blood or collection of growth factors from the blood by platelets during circulation. Increased release of alpha-granules from platelets of diabetics has been described providing support for this theory (Hamet et al., 1985).

F. Platelets, Disease Processes and the Role of TGF-*B*

Growth factor control of atherosclerotic lesion pathology

In vivo TGF-*B* and PDGF are released from platelets upon degranulation at sites of injury and affect mitosis of connective tissue cells. When PDGF is incubated with VSMC *in vitro*, their proliferation increases. This same exposure *in vivo* may be an important component of the proliferation of these cells during atherogenesis (Stiles, 1983). With respect to inhibition of proliferation, TGF-*B* is the best characterized growth inhibitor of VSMC (Assoian and Sporn, 1986). With a living culture of SMC, proliferation is stimulated by the addition of EGF. Addition of TGF-*B* to the same culture then inhibits proliferation of cells to control levels (Assoian and Sporn, 1986).

PDGF is another growth factor demonstrated to have importance in the regulation of disease processes (Ross et al., 1993; Martyre et al., 1991). Research suggests that PDGF has an important role in the initiation and progression of atherosclerotic lesions through activated macrophages and its expression by endothelial cells.

It is possible that control of VSMC growth in response to platelet degranulation may not be due to either TGF-*B* or PDGF alone. Rather, an overall interaction of platelet peptides, that have the intrinsic ability to limit as well as stimulate mitosis, may take place. The final effect of these growth factors on proliferation may depend on the overall potency of positive or negative growth modulators.

TGF-*B* could also be involved with the progression of atherosclerosis through mechanisms other than direct affect on SMC proliferation. TGF-*B* has been shown to upregulate production of ECM by VSMC in culture, particularly type IV collagen. Large

doses of TGF-*B* may stimulate lesion development by over production of the ECM (Reinhold et al., 1995). When VSMC progress into the intima, as part of the progression of atherosclerosis, they increase production of collagen, particularly type I and osteopontin, compared to VSMC in the media. Osteopontin has been shown to act as a potent chemoattractant for macrophages and SMC. If TGF-*B* also upregulated the production of osteopontin at the same time it upregulates type IV collagen it could further the progression of atherosclerotic plaque by attracting more macrophages and SMC (Gong and Pitas, 1995; Grainger and Metcalfe, 1995).

Endothelin-1 (ET-1) is a potent vasoconstrictor peptide found in the supernatant of cultured porcine aortic endothelial cells. TGF-*B*1 has been shown to induce the expression of ET-1 messages and increases ET-1 release in endothelial cells. Other platelet derived substances including: ADP, serotonin, TxA_2 and PDGF have failed to have an effect on ET-1 mRNA levels in endothelial cells. This suggests that vasoconstriction associated with thrombus formation could potentially be exaggerated by TGF-*B*1 via ET-1 production by endothelial cells (Kurihara et al., 1989; Murata et al., 1995). The association between TGF-*B*1, with vasoconstriction would then be mediated by the effects of TGF-*B*1 on ET-1 and is another possible mechanism through which TGF-B may aggravate the affects of atherosclerotic lesions.

Several studies have shown that when atherosclerotic lesions develop, whether they be from animal models or human autopsy specimens, TGF-*B* is upregulated in the intimal cells of the vessel wall. The role of this TGF-*B* has been the subject of much speculation. Some argue (Grainger and Metcalfe, 1995) that TGF-*B* is responsible for promoting intimal proliferation, while others feel that this argument is contrary to the

predominant role of TGF-*B* as a growth inhibitor of VSMC *in vitro*. Other circumstantial evidence in support of the protective role for TGF-*B* includes lower plasma levels of both total TGF-*B* and active TGF-*B* in patients with atherosclerosis. PDGF and bFGF have both been demonstrated to increase latent TGF-*B* production by VSMC, which may account for higher levels of TGF-*B* expression in areas where proliferation is occurring in the developing intima. Another possibility is that removal of VSMC from the ECM present in the artery wall and their subsequent dispersal in the intima is sufficient to trigger de-differentiation, and the TGF-*B* is a by product of this. TGF-*B* present in the intima or the plasma may play an important role in reducing macrophage adherence and preventing endothelial damage during lesion initiation and monocyte recruitment as the lesion progresses to become clinically significant (Grainger and Metcalfe, 1995). Therefore, the actual role of TGF-*B* with respect to the pathology of atherosclerosis is not clearly defined at this point. Since TGF-*B* is present in several sites in the disease development its actions merit further investigation.

TGF-B and tumour development

TGF-*B* helps to keep proliferation of cells in balance so a tissue or organ can function in equilibrium. TGF-*B* typically inhibits growth of normal differentiated cells of ectodermal origin while stimulating growth of cells derived from the mesoderm. Loss of this regulation is believed to contribute to tumour development. Certain epithelial cells that have preneoplastic characteristics are not inhibited by TGF-*B*, suggesting that these cells may have lost their ability to respond to TGF-*B* and therefore have a higher probability of developing cancer. Three possibilities could explain this loss of response

to TGF-*B* by cells from tissues that have a higher probability of developing cancer: cells could have totally lost expression of TGF-*B* receptors, tumour cells may only express the nonsignalling forms of the TGF-*B* receptors or the post receptor signal transduction pathway for TGF-*B* could be defective (MacKay et al., 1995). Therefore, in an organ or tissue where regulation by TGF-*B* has been lost, a higher concentration of TGF-*B* may not inhibit tumour growth. An example where TGF-*B* appears to control normal tissue function yet has negative affects during the progression of cancer occurs in the colon.

In the colon, TGF-*B* modulates cell growth, development and differentiation so that the cells stop dividing. TGF-*B* appears to inhibit proliferation of enterocytes as they leave the intestinal crypt and move to the villus tip. Immunohistochemical staining for TGF-*B* reveals higher concentration of TGF-*B* at the top of the crypt compared to the bottom (Glick et al., 1991). When cells are at the bottom of the crypt they proliferate at a faster rate, as they reach the villus tip their proliferation slows and they differentiate. These changes in cell cycle may possibly be controlled by the higher concentration of TGF-*B* at the top of the crypt that forces the cells to stop proliferating and differentiate. TGF-*B* has also been demonstrated to inhibit the growth of colon carcinoma cell lines including FET and CBS. Due to the inhibition of colon cancer cell line proliferation and regulation of enterocyte proliferation it is believed that TGF-*B* has a protective role against the development of colon cancer (Sun et al., 1994).

While it has been demonstrated that TGF-*B* can inhibit the growth of most well differentiated and some moderately differentiated human colon carcinomas, poorly differentiated colon cancer cells are not inhibited by TGF-*B*. This indicates that the stage of differentiation of the cancer is related to its responsiveness to TGF-B. It has also been

found that giving high amounts of TGF-*B*, stimulated DNA synthesis in poorly differentiated colon cancer cells (MacKay et al., 1995). Some colon carcinoma cell lines, including U9A, have even been shown to display autocrine growth in response to TGF-*B*1. Colorectal cancers that express higher levels of TGF-*B*1 have been found to have a very high probability of progression to metastases. Therefore during progression of human colon tumours, TGF-*B*1 may function as a stimulator of tumour growth or alter the environment in favour of the tumour. The reason for these affects may be that the more aggressive colon cancer cells use a different TGF-*B* signaling pathway than that used by less aggressive colon cancer cells (Huang et al., 1995). This research indicates that TGF-*B* can actually increase the chance of a tumour developing in a situation where cells are susceptible, instead of acting solely as an inhibitor of tumour development in the colon.

Research conducted on humans with colorectal cancer has found that plasma TGF-*B* is significantly higher in patients with colorectal cancer, and their plasma TGF-*B* decreases following curative surgical resection. The TGF-*B* mRNA in the colorectal cancer tissue of these patients was found to be 2.5 times more abundant than normal colonic tissue (Tsushima et al., 1996). This research provides further evidence that TGF-*B* can have different functions or purposes in the pathology of a disease depending on the situation or the stage of disease progression.

G. Modulation of Platelet Function by Nutrition

Platelets have a high turnover rate in both humans and rats, with an average lifespan of 9 to 12 days in humans and 5 to 6 days in rats. Due to this high rate of turnover, platelet composition consists of nutrients most recently consumed. Their dependence on available nutrients for construction could leave their function sensitive to nutritional modulation. It is believed that at least part of the essentiality of PUFAs resides in their requirement for a direct modulation of membrane protein function. Many different studies conducted with humans or animals have demonstrated that modulation of dietary fat can lead to changes in the fatty acid composition of platelet membrane phospholipids as well as modify the platelets' function.

To demonstrate that alteration of dietary lipids can affect platelet function, a study (Nelson et al., 1997) was conducted where the diet of human subjects was supplemented with 1.7 g/day AA, with a cross over design, for 50 days on each treatment. The proportion of AA in platelet phospholipids was significantly higher after supplementation with AA, in comparison to when the subjects were not given additional AA supplements. Interestingly, blood coagulation or platelet aggregation properties were not found to differ between the two treatment groups, with any of the methods used, with the exception of prothrombin time (a measure of blood coagulation), that was significantly lower in the AA supplemented group.

Another study demonstrated that the proportion of EPA (20:5n-3) and DHA (22:6n-3) in human platelets can be modulated regardless of the source from which these fatty acids are derived. This was demonstrated when subjects consumed either fish as a

meal, whole fish oil or DHA in a capsule. Those on a fish diet consumed an average of 0.38 g EPA and 0.67 g docosahexaenoic acid (DHA) per day. Subjects who consumed whole fish oil received 1.33g of EPA and 0.95g of DHA per day. Subjects that consumed DHA in triglyceride form in a capsule consumed 1.68g per day. A positive relationship was shown between the total n-3 PUFA and EPA and DHA consumed and the increase in total n-3 PUFA and EPA and DHA in all lipid fractions analyzed. DHA supplementation alone caused an increase in the proportion of EPA making up platelet membranes, while EPA was only present in the diet at 0.09g/day. This increase in EPA is most likely caused by retroconversion of DHA to EPA and then incorporation of the EPA into platelets. This likely takes place in the peroxisomes by delta-4 enoyl reductase and delta-3 and delta-2 enoyl CoA isomerase enzymes (Vidgren et al., 1997). This is an important example of membrane biology as it demonstrates a change in platelet structure that is not directly related to the addition of a fatty acid to a diet.

The effects of alpha-linolenic acid (LA) (18:3n-3) versus EPA and DHA were compared with respect to their impact on haemostatic factors (Freese and Mutanen, 1997). Each lipid was fed as a proportion of total energy consumed, at 1g/200 kcal of calculated energy expenditure. This is a much larger addition of experimental fatty acids than in the previous studies discussed. The subjects were fed the experimental lipids for four weeks. Significantly different proportions of fatty acids in the platelet membranes were observed between the treatment groups. Significant changes were also observed in total cholesterol and triacylglycerides, which decreased with the fish oil treatment and but not with the LA treatment. High density lipoprotein (HDL) cholesterol, bleeding time, as well as fibrinogen, AT-III, factor VIIc (measurements of coagulation factors) and PAI-1

(measurement of plasminogen activator) were all measured and no significant differences were found between the two treatments. This indicates that the chain length of fatty acids consumed does not affect the function of the platelet.

Manipulation of the phospholipid composition of rat platelets by dietary lipids has also been demonstrated. Rats were put on one of four different diets for four weeks. Diets included 5% olive oil, 5% sunflower oil, 25% olive oil and a 25% sunflower oil, with all lipids proportioned by weight. The concentration of oleic acid in platelets was found to be 75% higher in rats fed the 25% olive oil diet, than those fed the 25% sunflower oil diet. The rats fed sunflower oil had two to three times higher concentration of LOA compared to those fed olive oil. The concentration of long chain (>18 carbons) n-6 fatty acids was significantly higher (p<0.05), in platelets, when rats were fed the 5% sunflower diet compared to the 5% olive oil diet. This difference was not found when the oils were fed at a higher concentration. The concentration of long chain n-3 fatty acids was significantly higher (p<0.05) when rats were fed the 25% olive oil diet compared to the 25% sunflower oil diet, this difference was not evident when rats were fed the lower concentration of fats (Navarro et al., 1994).

Modulation of eicosanoid production by dietary lipid composition has been demonstrated under a variety of conditions in both humans and experimental animals. Studies with male Sprague Dawley rats have demonstrated approximately 50% reduction in platelet TxA_2 production in response to a diet containing high proportions of n-3 fatty acids compared to a diet containing a high proportion of n-6 fatty acids (Ikeda et al, 1996). Decreased plasma concentration of thromboxane and decreased thromboxane synthesis by platelets of pigs in response to a diet high in n-3 fatty acids has also been

observed (Schick et al., 1993 & Murray et al., 1993). In contrast, serum TxA_2 increases in response to AA, a n-6 fatty acid, in male Sprague Dawley rats (Sanigorski et al., 1996). Decreased urinary thromboxane B_2, a stable metabolite of TxA_2, is observed with dietary supplementation of n-3 fatty acids in adult human males (Nordoy et al., 1994). These studies all demonstrate that n-6 fatty acids increase and n-3 fatty acids inhibit production of TxA_2, a key platelet eicosanoid.

Dietary components other than lipids have been demonstrated to have an effect on platelet function. Supplementation of coenzyme Q10, an electron carrier in oxidative phosphorylation and a free radical scavenger, has been demonstrated to improve clinical outcome in patients with chronic heart failure and ischemic disease. Free radicals are known to promote platelet activation and aggregation, therefore it is possible that coenzyme Q10 improves outcome in these patients in part via decreasing platelet aggregation. A diminished percentage of large platelets are found when human subjects are fed 200 mg of coenzyme Q10 per day, in comparison to a baseline population. It has previously been shown that large platelets are more active than smaller platelets. Other studies have demonstrated that dietary supplementation with either vitamin E, B-carotene, or selenium reduced platelet aggregability and decreased the serum concentration of such proaggregatory substances as B-thromboglobulin and thromboxane in humans (Serebruany et al., 1997). This research that has demonstrated that anti-oxidants have an affect on platelet function is justification for not adding antioxidants to experimental diets above what is recommended by excepted protocols (Reeves et al, 1993).

A study was undertaken to investigate the effect of a diet low in folic acid on platelet and macrophage activity. Rats were fed experimental diets with 250 ug/kg folic

acid or 750 *ug*/kg folic acid for six weeks. Homocysteine level of plasma was four fold higher in rats fed a folic acid deficient diet. Serum homocysteine levels that are above normal have been reported to be a risk factor for atherosclerosis among non-homocysteinuric subjects with vascular disorders. Macrophages from deficient rats had a significantly higher ($p<0.01$) tissue factor activity than control rats, where tissue factor is an indicator of procoagulant activity in macrophages. Platelets from folic acid deficient rats had significantly higher rates of aggregation in response to thrombin and ADP ($p<0.0025$ and $p<0.0002$, respectively), as well as significantly higher rates of AA uptake ($p<0.01$). Cyclooxygenase products, including thromboxane B_2, 12-hydroxyheptadecatrienoic acid, 12-hydroxyeicosatetraenoic acid were significantly higher in platelets of folic acid deficient rats. Platelet samples from folic acid deficient rats contained significantly more conjugated dienes, thiobarbituric acid reactive substances and lipid peroxides than controls. The authors thought that the increased lipid oxidation products could have been the cause of the platelet hyperactivity (Durand et al., 1996). If the findings of this study were indeed an effect of lipid oxidation and the lipid oxidation rate of platelets is actually altered when their fatty acid composition is changed then it is possible that fatty acids could affect platelet function via their different oxidation rates.

The effect of a diet, that was high in both cholesterol and beef tallow, on platelet and serum growth factors was studied in a porcine model. Piglets were fed either a regular pig chow feed or the same diet with an additional 2% cholesterol and 20% beef tallow for 4 months. Samples were brought to equal platelet concentration for analysis. PDGF and TGF-B1 concentration were assessed using an I^{125} radio immunoassay.

Samples that were analyzed for TGF-B1 concentration were acidified before the assay to activate the latent TGF-B1. Platelet count was higher and mean platelet volume was significantly lower in the high cholesterol/beef tallow group. Overall platelet mass was similar for both groups (Garcia-Bolao et al., 1996). This finding suggests that platelet count may not be an appropriate method for comparing samples, whereas protein concentration could be an appropriate method.

The mean concentration of TGF-B1 was 42% higher in the serum of control animals compared to the high cholesterol/beef tallow group, but was not found to be statistically significant. This may however be a physiologically significant difference, even if the difference in the means is not statistically significant. No other differences were observed with respect to TGF-B1 or PDGF concentration in platelet lysate or serum. When Swiss-3T3 and VSMC were incubated with either platelet lysate or serum no statistically significant difference in proliferation index was found between the two treatment groups (Garcia-Bolao et al., 1996). Although the differences were not found to be statistically significant (p>0.05), the proliferation rate of both cell types was higher with the high cholesterol serum samples compared to the control samples. This effect could have been caused by the previously stated decrease in TGF-B1 concentration in cholesterol serum samples.

It is known that dietary fatty acids can modify the TxA$_2$ activation of platelets. Lipids can potentially modulate signal transduction by inducing structural changes in platelet membrane composition. To examine this mechanism Wistar rats were put on one of three different high fat diets or one low fat diet. Diets included: high fat hydrogenated coconut oil, high fat sunflower oil, high fat fish oil and a low fat diet with 5% energy as

sunflower oil. Fatty acid composition of platelet phospholipids was measured. The only platelets that contained a detectable amount of n-3 fatty acids were from fish oil fed rats, which contained 15% of their total fatty acids as n-3. Platelets from coconut oil fed rats aggregated significantly less in response to thrombin than platelets from the other treatment groups. Since an increase in intracellular calcium is an early event in the signal transduction pathway, this was measured as an indicator of dietary affect on signal transduction. The ADP evoked rise in intracellular calcium was not influenced by diet treatment. Thrombin evoked a relatively small change in intracellular calcium of the platelets from the coconut oil group compared to the other treatments. It is known that phospholipase C is stimulated by thrombin and is upstream of the calcium response in signal transduction. While at the same time, degree of platelet response to ADP, at best a weak activator of phospholipase C, was not modified by diet. Platelet response to thrombin, including both aggregation and intracellular Ca concentration, was found to be different between the diet groups. This indicates that phospholipase C activity is a step in signal transduction that is modulated by dietary fat (Heemskerk et al., 1995).

H. Influence of Nutrition on TGF-B Status

A study was undertaken to investigate the effects of n-3 or n-6 long chain fatty acids on the progression of autoimmune disease in weanling, lupus prone, B/W mice. Two different diets that contained 10% of their mass as lipid were provided for 6.5 months. One diet contained lipid from corn oil, which contains a large proportion of n-6 fatty acids, and the other from fish oil, which contains a large proportion of n-3 fatty

acids. By using western blot analysis it was demonstrated that the TGF-B1 protein concentration of the spleen was significantly higher when mice were fed the fish oil diet in comparison to the corn oil diet. The average life span of rats on the same diet was 267 days for mice on the corn oil diet and 402 days for mice on the fish oil diet. In this disease model it was hypothesized that the increased concentration of TGF-B1 would have an immunosuppressive effect, as described previously in the section **TGF-B and its biology**. This decrease in immune system function would slow down the progression of the autoimmune disease and lengthen the life of the rats (Fernandes et al., 1994).

TGF-B expression in rats, as measured by immunohistochemistry, can be controlled by dietary retinoic acid in vitamin A deficient rats. A group of vitamin A deficient rats were given an oral dose of all trans-retinoic acid, a second group was not given the oral dose and a control group received weekly oral doses of retinyl acetate. Before the dose of retinoic acid the epidermis, small intestine, colon and bronchial epithelium were devoid of any expression of TGF-B, while the vaginal epithelium expressed a higher than normal amount of TGF-B. Within 24 to 48 hours of the dose of all trans-retinoic acid, significant changes in the binding of TGF-B antibodies were observed in all tissues examined. The dose of retinoic acid increased the expression of TGF-B in all tissues except vaginal epithelium where it decreased. This is interesting, as TGF-B is known to stimulate the proliferation of vaginal epithelial cells, while it inhibits the proliferation of the others (Glick et al., 1991). These results demonstrate that TGF-B expression can be regulated by a single dietary component in a variety of tissues. Although the dietary manipulation was more extreme than just changing the fatty acid

source this research combined with the previous research sited proves that TGF-*B* metabolism can be regulated by diet.

Ethanol ingestion has been demonstrated to increase hepatic TGF-*B* mRNA, as measured by RT-PCR, in rats. Ingestion of thromboxane inhibitors decreased TGF-*B* mRNA by 50% in these rats, in comparison to rats that consumed ethanol without thromboxane inhibitors. Plasma concentration of TGF-*B*, as measured by a radio immunoassay, was found to increase from less than 3ng/L to over 20ng/L with ethanol ingestion. When thromboxane inhibitors were ingested, plasma TGF-*B* decreased significantly ($p < 0.01$). Plasma thromboxane B_2 levels have been found to positively correlate with the severity of pathological liver injury. The alterations in hepatic TGF-*B* observed with ethanol and thromboxane inhibitor ingestion indicate a possible link between thromboxanes, TGF-*B* and liver fibrosis (Nanji, 1997). n-6 fatty acids are metabolized by cyclooxygenase to 2-series thromboxane, therefore thromboxane is a possible link between liver fibrosis and dietary fat.

Other connections between n-6 metabolites and TGF-*B* metabolism have been observed in the HL-60 leukemia cell line. When inhibitors of the eicosanoid synthesis enzyme, 5-lipoxygenase, were added to culture, TGF-*B* induced differentiation of HL-60 cells was enhanced. Effects were accentuated when cells were incubated with retinoic acid prior to the experiment. This study demonstrated that the level of 5-lipoxygenase activity or leukotriene concentration might modulate the action of TGF-*B* (Kozubik et al., 1997). Eicosanoid and TGF-*B* metabolism have also been shown to be interwined in both mesangial and osteoblastic cell lines (Pricci et al., 1996; Klein-Nulend et al., 1996).

These investigations demonstrate that connections between eicosanoid and TGF-*B* metabolism may occur in a wide variety of organs or systems throughout the body.

There are very few examples of modulation of TGF-*B* or other growth factors by dietary lipids in the literature. One study was conducted on the PDGF of PBMs. Like TGF-*B*, PDGF is stored in the alpha-granules of platelets and can be found in many different organs and organelles. Therefore the modulation of PDGF by dietary lipids is pertinent to this review. PDGF mRNA expression, as quantified by 3n-PCR, was measured in PBMs from human subjects who received 7g/day of fish oil concentrate, containing 54.7% EPA, 28.7% DHA and 12.3% other n-3 fatty acids. PDGF mRNA was found to be decreased to 32% of baseline after 1 week and stayed at this level for 6 weeks. Long chain n-3 fatty acid composition of PBMs increased dramatically between baseline and week 1, but then reverted to levels closer to baseline after 6 weeks. The fact that fatty acid levels began to return to normal after 6 weeks, while PDGF mRNA did not, indicates that the gene expression of this growth factor is regulated by more than membrane fatty acid composition (Kaminski et al., 1993).

Chapter 3. MATERIALS AND METHODS

A. Animals

Male Sprague-Dawley rats, that were purchased from *Charles River* (Montreal, Canada) were used in all studies. Rats were housed in stainless steel cages with wire mesh bottoms in groups no larger than three per cage. A 12 hour light:dark cycle was maintained throughout the study. Food and water were provided ad libitum. The guidelines of the Canadian Council on Animal Care were adhered to throughout the studies. Weanling rats were put on experimental diets for 8 weeks and eight month old rats were on experimental diets for 6 weeks.

B. Diets

For all studies animal were acclimatized for a minimum of two weeks on laboratory chow. This diet is made from ground corn, meat and bone meal, soybean meal, wheat middlings, ground wheat, ground oats, dehydrated alfalfa meal, dried milk product, brewers dried yeast, dried molasses, animal fat (with preservatives), iodized salt, dicalcium phosphate, and vitamin and mineral premixes. All experimental diets were based on the AIN-93G diet with some alterations (Reeves et al., 1993) as follows: animals on a low fat soy oil (LFS) diet (Table 1) received 5% instead of 7% soy oil by weight, which was replaced with corn starch. This change was justified as the purpose of our low fat diet was to compare the effect of a diet with a low amount of fat against those with high amounts and is still considered to be nutritionally adequate (Reeves et al., 1993). The purpose of the 7% soy oil in the AIN-93G diet was to guarantee that the

animals had an ample source of all essential fatty acids. The low fat diet was included in each study to determine if the level of fat itself is an important variable. The four high fat diets were 23% total fat by weight, and the amount of carbohydrate added as corn starch and dextrose was reduced to accommodate the extra fat (Table 2). Proportions of casein (protein), mineral mix (Table 3), vitamin mix (Table 4) and cellulose (Harlan Teklad, Madison, Wisconsin) were increased in the high fat diets so that the animals on this diet would consume comparable amounts of these nutrients as those on the low fat diet. The level of 23% fatty acid was used for the high fat diets, to make the results comparable to research in the literature (Bird et al., 1996).

Soy oil (Vita Health, Winnipeg MB), high fat beef tallow (HFB) (Maple Leaf Foods, Winnipeg, MB), high fat corn oil (HFC) (Mazola, Etobicoke, ONT) and high fat olive oil (HFO) (Primo, Etobicoke, ONT) were stored at 4°C. High fat fish oil (HFF) was the standard Manhaden blend and was a gift from *Zapata Protein* (Reedville, Virginia) and stored at -30°C, until fed to rats, to reduce oxidation (Table 5, 6).

C.   Body Weight

Body weight of animals were recorded at the start of each experiment and at two week intervals throughout the studies.

D.   Isolation of Plasma, Platelets and Neutrophils From Whole Rat Blood

Isolation of Plasma. All animals were terminated by CO_2 asphyxiation and diaphragm separation. Blood was drawn from a heart puncture with a 12 inch multiple sample luer adapter (Becton Dickinson, Franklin Lakes, New Jersey) and collected into

Table 1: Low fat diet composition

Ingredient	% Composition
Casein	20.0
Corn Starch	52.0
Dextrose	13.0
Soybean oil	5.0
Cellulose	5.0
Mineral Mix AIN-93G	3.5
Vitamin Mix AIN-93	1.0
Choline Bitartrate	0.2
L-cysteine	0.3

4 kcal/g, 11.7% energy as fat

Table 2: High fat diet composition

Ingredient	% Composition
Casein	23.0
Corn Starch	33.75
Dextrose	8.52
Soybean oil	5.0
Test fat	18.0
Cellulose	5.9
Mineral Mix AIN-93G	4.11
Vitamin Mix AIN-93	1.18
Choline Bitartrate	0.24
L-cysteine	0.3

5 kcal/g, 44% energy as fat

Table 3: Composition of mineral mix, AIN-93G

Ingredient	g/kg
Calcium Carbonate	357.0
Potasium Phosphate, monobasic	196.0
Potasium Citrate, monohydrate	70.78
Sodium Chloride	74.0
Potasium Sulfate	46.6
Magnesium Oxide	24.3
Ferric Oxide	6.06
Zinc Carbonate	1.65
Manganous Carbonate	0.63
Cupric Carbonate	0.31
Potasium Iodate	0.01
Sodium Selenate	0.010
Ammonium Paramolybdate	0.008
Sodium Meta-silicate	1.45
Chromium Potassium Sulfate	0.275
Lithium Chloride	0.017
Boric acid	0.081
Sodium Fluoride	0.064
Nickel Carbonate, hydroxide	0.032
Ammonium Vanadate	0.007
Sucrose, finely ground	220.72

Supplies the recommended concentration of mineral elements for AIN-93G diet, when mineral mix is used at 35g/kg of diet (Reeves et al., 1993).

Table 4: Composition of vitamin mix, AIN-93-VX

Ingredient	g/kg
Nicotinic Acid	3.0
Calcium Pantothenate	1.6
Pyridoxine HCl	0.7
Thiamin HCl	0.6
Riboflavin	0.6
Folic Acid	0.2
D-Biotin	0.02
Vitamin B_{12} (0.1% in mannitol)	2.5
DL-Alpha Tocopherol Acetate (500 IU/g)	15.0
Vitamin A Palmitate (500,000 IU/g)	0.8
Vitamin D3 (cholecalciferol, 500,000 IU/g)	0.2
Vitamin K (phylloquine)	0.075
Sucrose, finely ground	974.7

Supplies the recommended concentrations of vitamins for AIN-93G diet, when vitamin mix is used at 10g/kg of diet. Choline is listed separately (table 1 & 2) (Reeves et al., 1993).

Table 5: % Fatty acid composition of experimental lipids

Fatty acid	Beef tallow	Corn oil	Fish oil	Olive oil	Soy oil
14:0	3.1	ND	10.5	ND	0.1
16:0	23.0	10.1	16.1	7.8	10.4
16:1	2.2	ND	12.7	ND	0.4
18:0	19.4	1.7	2.9	2.6	4.0
18:1n-9	38.6	26.5	9.0	74.2	23.7
18:2n-6	3.4	59.2	1.1	11.1	52.2
18:3n-3	1.0	0.8	1.0	0.5	7.0
20:5n-3	ND	ND	16.4	ND	ND
22:5n-3	ND	ND	3.0	ND	0.2
22:6n-3	ND	ND	8.0	ND	0.1

Fatty acid composition measured by gas chromatography

Table 6: % Fatty acid composition of experimental diets

Fatty Acid	HFB	HFC	HFF	HFO	LFS
14:0	2.4	ND	8.2	ND	0.1
16:0	20.6	10.6	14.8	8.7	10.4
16:1	1.8	0.1	9.9	0.1	0.4
18:0	16.0	2.2	3.2	2.9	4.0
18:1n-9	34.9	25.5	12.2	62.7	23.8
18:2n-6	14.3	57.8	12.3	20.3	52.2
18:3n-3	2.6	2.4	2.3	2.2	7.0
20:5n-3	ND	ND	12.8	ND	ND
22:5n-3	ND	ND	2.3	ND	0.2
22:6n-3	ND	ND	6.2	ND	0.1

Fatty acid composition measured by gas chromatography

20ml red top vacutainers (Becton Dickinson, Franklin Lakes, New Jersey) containing 3.2 ml acid-citrate dextrose (ACD). Blood was then transferred to 50 ml conical bottom polypropylene tubes and 25% of total volume was added as 0.9% NaCl solution. Samples were centrifuged at 300 X gravity (G) for 20 minutes at 19°C. The upper platelet rich plasma was drawn off down to 1/8 to 1/4 of a inch above the RBC level to avoid drawing up neutrophils. The remaining blood that contained the RBC and neutrophils was stored on ice until neutrophil isolation. Platelet rich plasma was centrifuged at 800 X G for 15 minutes at 19°C (Heemskerk, et al., 1989). Platelet poor plasma was drawn off the pellet and frozen at -80°C.

Isolation of Platelets. Platelet pellet was resuspended in 5 ml phosphate buffered saline (PBS) and 1 ml ACD. Samples were centrifuged at 800 X G for 15 minutes at 19°C. Supernatant was drawn off and platelet pellet was resuspended in 1 ml 1 X PBS before being stored at -80°C in a siliconized 1.5 ml eppendorf (Heemskerk, et al., 1989). Prior to storage, 1 unit of each of leupeptin, pepstatin and aprotinin was added to each sample to inhibit protease enzymes.

Isolation of Neutrophils. Five ml of 6% Dextran T-500 (Pharmacia Biotech, Uppsala, Sweden) in 0.9% NaCl was added to the RBC, neutrophils and remaining lower plasma, 0.9% NaCl was added to 40 ml and the sample was gently resuspended. Samples sat undisturbed for 45 minutes at ambient temperature (21°C) then supernatant containing neutrophils was drawn off and stored on ice. The original sample sat an additional 45 minutes before the supernatant (containing neutrophils) was again drawn off and mixed with the previous supernatant. Neutrophil solutions were then centrifuged at 300 X G for 12 minutes at 19°C. The pellets were then resuspended in 1ml 0.9% NaCl before an

additional 7 ml of 0.9% NaCl was added. Resuspended samples were then gently layered on top of 3 ml Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) in a 15 ml conical bottom tube and centrifuged at 750 X G for 25 minutes at 19°C. Supernatant was drawn off to 0.5cm above the pellet and then the pellet was resuspended in the small amount of liquid remaining and stored on ice. Five ml of 1°C double distilled H_2O was added to the sample and resuspended for 45 seconds, to osmotically lyse RBC. Immediately following, 5 ml of 1°C 1.8% NaCl was added and resuspended. The sample was centrifuged at 300 X G for 6 minutes at 0-4°C, then supernatant was removed to the pellet. If the pellet still had visible RBC, 1 ml ACD was added and osmotic lysis steps were repeated. Neutrophils were then resuspended in 1 ml ice cold 1 X PBS and stored at -80°C in a sterile, siliconized 1.5 ml eppendorf (Mahadevappa & Powell, 1989). Prior to storage, 1 unit each of leupeptin, pepstatin and aprotinin were added to every 1 ml sample.

Neutrophil Extraction. Neutrophil samples were frozen at -80°C to lyse cells. Samples were thawed and centrifuged at 200 X G to condense membranes. Remaining lysate was drawn off of the condensed membrane and transferred to separate silconized eppendorfs for storage at -80°C.

E. Quantification of TGF-*B* Equivalent With CCL-64 Mink Lung Cell Bioassay

Throughout the bioassay, all steps up to the radioactive stages were preformed under a sterile flowhood. CCL-64 mink lung cells (American Type Culture Collection) were stored at -80°C until needed for use in the bioassay. Cells were thawed and plated in sterile culture plates with 25 ml of alpha-Minimum Essential Medium (MEM) (Gibco-

BRL, Grand Island, NY) + 10% silver medium (ICN) and grown at 37°C and 4.8% CO_2.

Cultured cells proliferated until cell population had increased to yield enough cells to

conduct the bioassay. Care was taken to ensure that the culture did not become

confluent. CCL-64 cells were removed from the culture by drawing off medium then

washing plate with 10 ml of citrate saline solution at 37°C. Four to six ml of trypsin-

EDTA (Gibco-BRL, Grand Island, NY) was added to the plate and then incubated 5-7

minutes (37°C, 4.8% CO_2) to detach live cells from the surface. The solution of cells was

then drawn off and put into a sterile conical bottom tube. Ten ml of 0.2% bovine calf

plasma (BCP) (National Biological Laboratory, Dugald, MB) /alpha-MEM solution was

used to wash off the plate and added to the cells in the tube. This served a dual purpose

of washing the remaining CCL-64 cells off the culture plate and neutralizing the trypsin-

EDTA. The solution was centrifuged at 4°C for 8 minutes at 160 X G. Supernatant was

drawn off and cells were then resuspend in remaining liquid and made up to 10 ml with

0.2% BCP solution and resuspended. Live cells were counted manually using a

haematology counter taking care to only count live cells. Live cells were distinguished

from dead cells by their morphological appearance. The solution was diluted with 0.2%

BCP so that the final concentration of cells would be 9 X 10^4 cells/ml. 500 ul of cell

solution was added to each well in a 24 well sterile culture plate, then incubated at 37°C,

4.8% CO_2 for three hours.

To assess TGF-*B* activity, a standard curve was created using TGF-*B*1 isolated

from porcine platelets (R & D, Minneapolis MN). TGF-*B*1 was in a 25 ul solution at 12

pg/ul. A serial dilution was created by diluting the TGF-*B* with 175 ul 0.2% BCP, then

transferring 100 ul of this solution to a sterile siliconized eppendorf containing 100 ul

0.2% BCP and repeating this for 11 intervals. Eighty ul of these solutions were transferred to each of the first 12 wells of the culture plate. A baseline value for non-acidified samples was created by adding 80 ul of 0.2% BCP to the next six wells. The baseline value for acidified samples was also determined. To acidify 0.2% BCP, 15 ul of 1.8 M HCl was added to 600 ul of 0.2% BCP and resuspended. This sample was subsequently neutralized by adding 15ul of a 5N NaOH:Hepes (5:2) solution (solution D). Eighty ul of this acidified solution was added to wells 19 to 24. Three hundred and twenty ul of 0.2% BCP was added to each well when young rat samples were analyzed and 120 ul of 0.2% BCP and 200 ul PBS were added to each well when old rat samples were analyzed. Platelet lysates from young and eight-month-old rats were added to the cultures at different volumes. For young rats 280 ul of platelet lysate at a concentration of 0.322 ug/ul was added to each well in duplicate. Then 140 ul of platelet lysate was acidified with 4 ul of 1.8 M HCl and then neutralized with 4 ul of solution D. Seventy ul of acidified platelet lysate was added to each well in duplicate. In the case of eight-month-old rat samples 100 ul, 50 ul, and 25 ul of 0.322 ug/ul platelet lysate were added to three cultures respectively. Then 175 ul of platelet lysate was acidified with 5 ul of 1.8 M HCl and then neutralized with 5 ul of solution D. 100 ul, 50 ul, and 25 ul of this acidified sample were added to the three cultures respectively. 100 ul, 150 ul, or 175 ul of PBS were added to each well so that total volume added would be equal for all cultures.

Neutrophil samples. Fifty ul and 25 ul of 0.28 ug/ul neutrophil lysate were added to three consecutive cultures. 75 ul of neutrophil extract was acidified with 3 ul of 1.8 M HCl and then neutralized with 3 ul of solution D. 50 ul and 25 ul of this acidified sample were added to three consecutive cultures. 150 ul, or 175 ul, of PBS were added to

each well so that total volume added would be equal for all cultures. All wells were brought to 900 ul total with 0.2% BCP. All cultures were then incubated overnight at 37°C, 4.8% CO_2.

Radioactive stages. 0.5 uCi diluted in 50 ul of 0.2% BCP was added to each well. Iodine[125] deoxyuridine (I^{125}) was used as the labeling agent. Samples were then incubated at 37°C, 4.8% CO_2 for three hours. After incubation with I^{125}, 1 ml of methanol/acetic acid (3:1) was added to each well to fix the cells. The samples were then allowed to incubate for one hour at ambient temperature and atmosphere. After one hour the supernatant was drawn off and all wells were washed twice with 80% methanol to eliminate the acetic acid. The plates were then allowed to dry for 15 minutes. 1 ml of 1 M NaOH was added to each well to lyse the cells and left to stand for 30 minutes. The solution was then resuspended and 800 ul were used to assess counts per one-minute (CPM1) using a gamma counter (model 1282 Compugamma).

Data Analysis:

Young rat platelets and neutrophil samples. TGF-*B* is a reflection of growth inhibition, extrapolated from a standard curve that was created with known concentrations of TGF-*B*. CPM1 values were converted to TGF-*B* equivalent using the standard curve (figure 4). Duplicate samples of each platelet and neutrophil sample were averaged together.

Eight-month-old rat platelets. CPM1 was converted to a constant proliferation index so that results from different cultures of the CCL-64 cells could be compared. A baseline value was determined with the mean CPM1 from the wells that had no lysate or
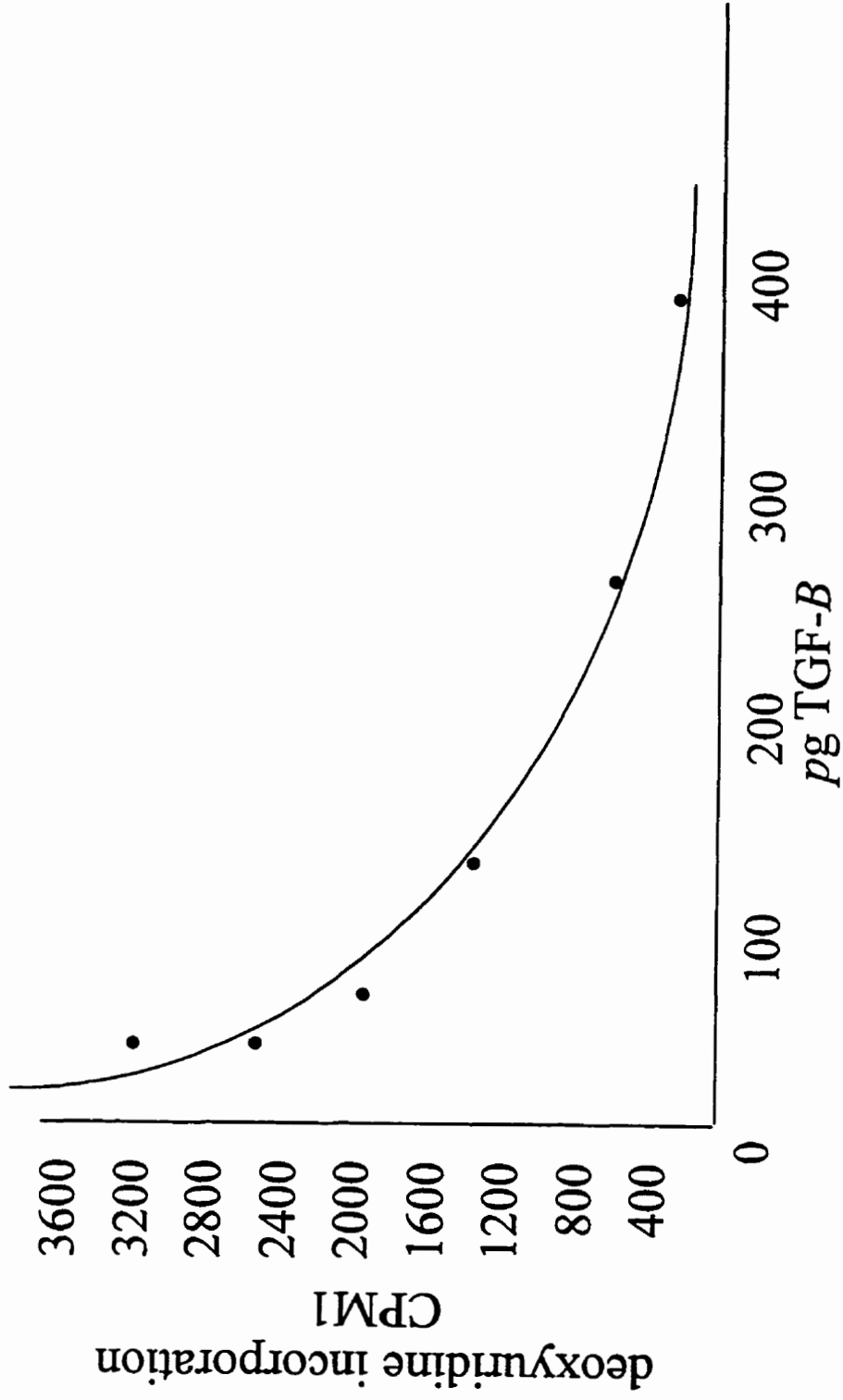
Figure 4: TGF-*B* standard curve using CCL-64 cells

TGF-*B*1 added to them. Sample CPM1 values were then divided by their corresponding mean baseline value. The product was then multiplied by 100 to give the proliferation index of the sample tested in the pertaining well. A sample that had a proliferation index of 100 would therefore have proliferated at the same rate as if no lysate or TGF-*B*1 had been added to it at all.

Results from all three different concentrations of platelet lysate were grouped together to assess an overall effect. In order to assess the overall effect, the result from each sample assessed was divided by the mean proliferation index of the LFS group with the corresponding concentration of platelet lysate. The product was then grouped with the results from the other concentrations.

F.   Western Immunoblot methods

Western immunodetection of TGF-*B*1 was conducted with platelet lysate. Twenty *u*g of platelet protein was diluted to equal concentration with 1 X PBS, then diluted with 33% volume of 6 X SDS sample buffer containing *B*-mercaptaethanol. A Low Molecular Weight (LMW) standard (Gibco BRL) was used to assess the molecular weight of the immunodetection bands. Samples were heated at 90°C for three minutes. Twenty *u*g of sample protein and LMW standard were loaded in a 5% acrylamide stacking gel. Electrophoresis was performed on a 4cm, 15% acrylamide tris-glycine gel for protein separation. Electrophoresis was run at 170 volts until the dye front ran to the bottom of the gel. Transfer of proteins from gel to membrane was performed in 20% methanol transfer buffer, at 100 volts for 2 and 1/2 hours at 4°C. 0.45 micron nitrocellulose hybond-C (Amersham Life Sciences, Arlinton Heights, IL) membrane was

soaked in H_2O for 1 hour and transfer buffer for 10 minutes prior to transfer. The membrane was blocked with 5% skim milk powder (SMP) in TBS-T for 1 hour at ambient temperature. The membrane was incubated with primary antibody (TGF-B1, catalogue # sc-146, Santa Cruz Biotechnology Inc., Sant Cruz, CA,) at 1:100 dilution in 5% SMP/TBS-T for 15 hours at 4°C. The membrane was washed 5 times, with TBS-T, for 7 minutes at ambient temperature. The membrane was incubated with secondary antibody, Igg Horse Radish Peroxidase (Santa Cruz Biotechnology Inc.) at 1:1000 dilution in 5% SMP/TBS-T at ambient temperature for 1 hour. Luminescence was conducted using an enhanced chemilluminescence (Amersham Life Science) after the membrane was washed 5 times, with TBS-T, for 7 minutes at ambient temperature. Film (Kodak scientific imaging film) was exposed to illuminating membrane for 5 minutes. Densitometric analysis was performed with Image Processing and Analysis, 1.49 on a Macintosh IIci (Rasband, 1992). The densitometric totals were adjusted to compensate for differences in band intensity that could occur between samples run on different gels. Totals were adjusted with a constant value that was established by measuring the difference in density between the same sample that was repeated on different gels.

Detection of TGF-B1 in plasma samples was conducted with 40 ug of plasma protein. All other conditions were the same.

Western Blot analysis for TGF-B1 in neutrophil lysate samples was performed by loading 10ug of neutrophil protein and incubating for ten minutes in B-mercaptaethanol. All other conditions were constant.

TGF-*B*1 antibody was an affinity-purified rabbit polyclonal antibody raised against a peptide corresponding to amino acids 328-352 mapping at the carboxy terminus of the precursor form of human TGF-*B*1.

G.   Protein Analysis

Young rats.   Protein concentration was assessed using a Milton Roy Spectronic 3000 Array. The standard curve was created with bovine serum albumin. All standard samples were analyzed in duplicate. All platelet samples were analyzed in duplicate.

Eight-month-old rats. Protein concentration was assessed using a Spectra Max 340 (Molecular Devices, Sunnyvale, CA). The standard curve was created with bovine serum albumin. All standard samples were analyzed in duplicate. All samples were analyzed in duplicate.

Chapter 4.

EFFECT OF DIETARY FATTY ACID COMPOSITION ON LEVELS OF TGF-*B* IN PLATELET LYSATE OF MALE SPRAGUE-DAWLEY RATS

1. <u>Introduction</u>

Risk of the development of chronic diseases including: diabetes, cancer and atherosclerosis has been demonstrated to be affected by diet. Dietary components, especially dietary lipids have been associated with the modulation of growth factor status (Fernandes et al., 1994; Glick et al., 1991; Kaminski et al., 1993). Platelets are known to play a role in the pathology of atherosclerosis and potentially play a role in the development of some cancers. The precise mechanisms of growth factor development that are modulated by dietary lipids are unknown, although several possibilities exist. Fatty acids could affect the growth factor status of platelets by altering the fatty acid composition of the platelets or megakaryocytes and in turn the gene expression of the growth factors before the platelets are produced. With different fatty acid composition the membrane permeability of the platelets could be changed, altering the platelet's ability to retain its growth factors. The concentration of various receptors on the platelets could also be altered, causing a change in the rate at which platelets potentially pick up growth factors from the serum.

Chronic diseases usually do not begin to exhibit themselves until a person is of an advanced age. However it is likely that disease progression begins many years prior to the appearance of actual symptoms. The stage in life in which growth factor status has its

largest effect on the progression of these diseases is unknown. The effect could be part of the early initiation of disease, as occurs when an injury is first sustained in a blood vessel wall, where growth factors assisting in repair over stimulate proliferation of VSMC. Growth factors can also exert effects later in disease progression, by contributing to the build up of plaque to the point that it bursts, acting as stimulators of tumour cell proliferation or inhibiting immune function.

TGF-*B* is a growth factor that has been associated with the pathology of a number of chronic illnesses (Grainger and Metcalfe, 1995; Mikhail et al., 1993; Young et al., 1996; Markowitz et al., 1995). If TGF-*B* does have an effect on disease progression then it is possible that its metabolism may respond differently to diet at different points during the lifespan. Therefore the assessment of the effect of diet on growth factor status is important both earlier and later in life. The concentration of TGF-*B* is very high in platelets (Assoian et al., 1983) and an unsuccessful attempt has been made to demonstrate that its status can be affected by diet (Garcia-Bolao et al., 1996). The objective of these studies was to determine the effect of fatty acid composition on levels of TGF-*B* in platelet lysate of male Sprague-Dawley rats.

2.   Materials and Methods

Animals.   Male weanling Sprague-Dawley rats were used to conduct a preliminary study, after being allowed two weeks to acclimatize after shipping. Eight-month-old Sprague-Dawley rats were used for the second phase of the study. This age was considered appropriate because they had stopped growing, thus confounding variables associated with growth were avoided.

Study design. Rats were randomly assigned to one of the following diets: HFB, HFC, HFF, HFO and LFS. All high fat diets contained 18% experimental fat and 5% soy oil, while the low fat diet contained only 5% soy oil. Weanling animals remained on diets for eight weeks, while older rats were on diets for six weeks, which are considered to be long enough time periods for an intervention in dietary lipids to have an effect. All rats were terminated by CO_2 asphyxiation. Platelets were isolated, diluted in 1 X PBS and samples and stored in siliconized eppendorfs at $-80^\circ C$.

CCL-64 mink lung cell bioassay for TGF-B equivalent, conducted with platelets from weanling rats. In keeping with the fact that total platelet lysate contained a mixture of growth factors we quantified the growth inhibitory effect of TGF-*B* equivalent because the growth inhibitory response may not represent the actual level of TGF-*B* in the lysate. To assess TGF-*B* equivalent a standard curve was created using TGF-*B*1 purified from porcine platelets. Platelet TGF-*B* concentration was indirectly measured by incubating platelet lysate with cultures of CCL-64 mink lung cells. CCL-64 cells are known to express a high concentration of TGF-*B* receptors, making them highly sensitive to exogenous TGF-*B* in culture (Danielpour et al., 1989). Ninety *u*g of platelet lysate from each sample was incubated with the cells and used to measure TGF-*B* concentration. 22.5 *u*g of platelet lysate that had been acidified and neutralized was also incubated with the cells to determine the concentration of total TGF-*B* including both active and latent forms. All samples were analyzed in duplicate. 0.5 *u*Ci of I^{125} deoxyuridine was used as the labelling agent and CPM1 was assessed using a 1282 Compugamma gamma counter.

Assessment of the TGF-B equivalent in platelet lysates of eight-month-old male Sprague-Dawley rats by the CCL-64 bioassay. Platelet lysate was incubated with CCL-64 cells with the intention of assessing TGF-*B* equivalent. Eight *u*g, 16 *u*g and 32 *u*g of platelet lysate protein from each sample were used to conduct each assay. With increasing sample concentration CPM1, as measured by deoxyuridine incorporation, increased in virtually all samples measured. Proliferation of cells was found to respond to platelet lysate in a consistent manner, therefore measurement of the proliferation index of CCL-64 cells incubated with platelet lysate was used to assess the overall concentration of growth factors or growth effectors present in platelet lysate. Equal amounts of sample lysate were acidified to assess the affect of activating the latent TGF-*B* present.

Modulation of TGF-B1 in platelets of male rats as measured by western blot.

Platelet samples were heated for three minutes at 90°C with SDS buffer containing *B*-mercaptaethanol. Twenty *u*g of platelet sample were separated by electrophoresis on a 15% acrylamide tris-glycine gel. Platelet proteins were transferred to nitrocellulose membranes for 2.5 hours. Membranes were incubated with TGF-*B*1 antibody for 16 hours at 4°C and secondary antibody for 1 hour at room temperature. Iluminesence was carried out with ECL and film was exposed to membrane for 5 minutes at room temperature. Densitometric analysis was conducted on visible protein bands. TGF-*B*1 from platelet proteins were found at 12 Kda, as *B*-mercaptaethanol degraded the disulphide bonds of the active TGF-*B*1, splitting the 24 Kda molecule in half.

Statistical Analysis. Statistical analysis of the data was performed using the statistical analysis software (SAS). Version 6.12, for microcomputers (Cary, NC).

ANOVA in combination with Duncan's Multiple Range test was used to separate treatment means for significant differences. Differences were regarded as significant at $p<0.05$.

3. Results and Discussion

Body weight. Dietary intervention had a significant impact on the weight gain of both the weanling and the eight-month-old rats. Weanling HFC and HFO groups gained significantly more weight than the LFS group ($p<0.05$) (Table 7). Eight-month-old rats on the LFS diet were found to gain significantly less weight than those on any of the high fat diets ($p<0.05$) (Table 8). These findings were expected for two reasons. Firstly, the LFS animals consumed a diet that supplies 18% less energy than the high fat diets provide on a weight basis. Secondly, dietary fat is more easily converted to adipose tissue than dietary carbohydrate or protein.

Assessment of the TGF-B equivalent, conducted with platelets from weanling rats. Fatty acid source had a significant effect on the concentration of TGF-*B* in the platelet lysate of weanling rats (Table 9). Active TGF-*B* was found to be the most highly concentrated in the platelets of rats fed a HFB diet, with a mean equivalent TGF-*B* concentration of 3.24 pg/*u*g of platelet protein. In other words the highest amount of inhibition occurred in response to the HFB samples. TGF-*B* in platelet lysate from rats was significantly lower in HFC and HFF platelet lysate than HFB samples with mean equivalents of 0.71 and 1.28 pg/*u*g respectively ($p<0.05$). HFO and LFS samples were not significantly different from any of the other samples with mean equivalents of 2.24 and 2.38 pg/*u*g respectively (Table 9).

Table 7: Body weight (g) of weanling rats upon diet intervention and termination

	HFB	HFC	HFF	HFO	LFS
Initial weight	139(2.7)[b]	135(4.2)[ab]	138(3.4)[b]	136(4.0)[ab]	125(5.3)[a]
End weight	532(12)[ab]	562(28)[b]	541(26)[b]	561(14)[b]	486(13)[a]
Change	393(10)[ab]	427(25)[b]	404(25)[ab]	425(15)[b]	362(10)[a]

N=5, SEM in parenthesis, superscript denotes significant difference between the groups in each growth assessment category, in each row, at $p<0.05$

Table 8: Body weight (g) of eight month old rats upon diet intervention and termination

	HFB	HFC	HFF	HFO	LFS
Initial weight	729(23)^a	738(18)^a	764(30)^a	657(21)^b	698(23)^{ab}
End weight	839(20)^a	862(35)^a	869(51)^a	785(28)^{ab}	721(22)^b
Change	110(9)^a	123(32)^a	105(22)^a	129(12)^a	23(6)^b

N=6, SEM in parenthesis, superscript denotes significant difference between the groups in each growth assessment category, in each row, at p<0.05

Table 9: Growth modulating effect of platelet lysate from young rats, expressed as TGF-*B* equivalent

	HFB	HFC	HFF	HFO	LFS
Active	$3.24(0.60)^a$	$0.71(0.66)^b$	$1.28(0.64)^b$	$2.24(0.56)^{ab}$	$2.38(0.54)^{ab}$
Total	$8.0(1.60)^{ab}$	$14.2(1.71)^a$	$8.9(3.35)^{ab}$	$9.5(1.11)^{ab}$	$7.0(2.1)^b$
Active /Total	$0.43(0.10)^a$	$0.06(0.05)^b$	$0.19(0.05)^b$	$0.27(0.08)^{ab}$	$0.46(0.11)^a$

N=5, All values *pg/ug* platelet protein, SEM in parenthesis, superscript denotes significant difference between groups in each row at $p<0.05$

The total TGF-B equivalent that represented both latent and active TGF-B showed a different trend (Table 9). Following acid activation of TGF-B, HFC samples had the highest total mean equivalent of TGF-B with 14.2 pg/ug. HFO, HFF, HFB and LFS had lower equivalents of 9.5, 8.9, 8.0 and 7.0 pg/ug respectively. The only treatment group that had significantly less TGF-B than the HFC group samples was the LFS group ($p<0.05$) (Table 9).

The equivalent of active TGF-B was divided by the total TGF-B in order to assess the proportion of total TGF-B that was in the active form before acidification. LFS samples were found to have the largest proportion in the active form with 46% active before acid treatment. HFB, HFO, HFF and HFC had lower proportions in descending order. Significant differences in mean active proportion of TGF-B equivalent were found, with LFS and HFB samples containing a significantly higher proportion in the active form than HFF and HFC ($p<0.05$) (Table 9).

This study demonstrated that dietary lipids modulated the growth factor status of platelet lysate. The presence of beef tallow in the diet resulted in the highest concentration of active TGF-B in platelet samples, significantly ($p<0.05$) more than rats fed a HFF or a HFC diet. These results could be viewed as unexpected as it has been proposed that platelet TGF-B has a protective effect against atherosclerosis while beef consumption has been found to be associated with increased rates of atherosclerosis (Fraser et al., 1994; Kushi et al., 1995; Snowdon et al., 1984). The result that consumption of beef tallow leads to higher concentrations of TGF-B in platelets could be explained logically as part of the pathology of atherosclerosis through a variety of different theories.

Among the high fat groups the HFB samples had both the highest amount of TGF-*B* in an active form and the lowest amount of total TGF-*B* after acid activation. This increase could be due to changes in the platelet membrane properties that resulted in slow release of alpha-granules. One possibility is that the total amount of TGF-*B* present in the platelets is the controlling variable in the effect of this growth factor on VSMC or other vascular tissues, as a part of the pathology of atherosclerosis. It is known that TGF-*B* functions as a promoter of angiogenesis, possibly by promoting angiogenesis it also quickens the progression of atherosclerosis. Another explanation for higher concentrations of TGF-*B* acting to hasten the progression of atherosclerotic lesions involves TGF-*B* functioning as a growth stimulator of VSMC or vascular epithelial cells after lesion progression has already begun. Other examples of this kind of response to TGF-*B* have been demonstrated in colon cancer progression. With colonic tissue, TGF-*B* is believed to inhibit the proliferation of normal colonic epithelial tissue, as well as the proliferation of many colon cancer cell lines, yet has been shown to have high rates of expression in colonic tumours and be elevated in patients with colon cancer (Tsusima et al., 1996). Therefore when a phenotype of a cell or tissue changes, the manner in which it may respond to TGF-*B*1 also may change. A third possible explanation is that other growth factors, such as PDGF, may out compete TGF-*B* in the regulation of proliferation in a person that consumes a diet high in beef tallow.

The HFC diet resulted in twice as much total TGF-*B* equivalent in comparison to the HFB or LFS diets but kept the vast majority of it in an inactive latent state. Without knowing the ability of a tissue or organ to activate this TGF-*B* *in vivo* it is difficult to say what effect this high concentration of TGF-*B* would have on the function of a tissue.

From one view point this TGF-*B* could have very little impact on function due to the low proportion in an active state, while it is also possible that a large portion of this TGF-*B* could be easily activated in a system allowing it to have a large impact on function. If the large proportion of TGF-*B* was easily activated it may inhibit the over proliferation of VSMC that is part of atherosclerotic lesion development as in the model described. From a completely opposite viewpoint a higher amount of functional TGF-*B* might function as a growth stimulator of a lesion once its progression has already begun.

With respect to LFS fed animals it was found that they had the lowest concentration of total TGF-*B* in their platelets. As it is reasonably well established that a low fat diet is associated with decreased incidence of atherosclerosis and some forms of cancer (Reddy, 1995; Fraser, 1994; Potter, 1997), and there is a low concentration of TGF-*B* in LFS platelets, it is a distinct possibility that a low platelet concentration of TGF-*B* is a positive condition that protects against disease development. When the complexity of disease pathology is taken into account it is not surprising that the function of TGF-*B* does not fit into a straight forward theory involving a lower risk of disease with a higher concentration of TGF-*B*.

Epidemiological studies that demonstrate the positive effects of a low fat diet are naturally conducted with humans. Rats are not known to spontaneously develop atherosclerosis or cancer. Therefore the finding that a low concentration of TGF-*B* exists when a animal consumes a low fat diet may not be comparable to what occurs in humans. Platelet TGF-*B* in humans may respond in a similar or opposite manner, in response to diet, than it does in rats. Platelet genesis and function is similar in both humans and rats, therefore some similarity in growth factor metabolism may also exist. Whether a

human's response to diet occurs in the same manner in which it does in rats or in a different manner is unknown.

Assessment of the TGF-B equivalent in platelet lysates of eight-month-old male Sprague-Dawley rats by the CCL-64 bioassay. Addition of platelet lysate to cultures of CCL-64 cells stimulated proliferation of cells, in a dose dependent manner, in all samples tested. This was a surprising result because the proliferation of these cells is known to be decreased by mediums that contain TGF-*B* (Danielpour et al., 1989; Khalil et al., 1993; Luzhe et al., 1994; Miettinen et al., 1994). The total platelet lysate of these samples contained enough growth stimulators to overcome the TGF-*B* in the platelets of older Sprague-Dawley rats. Assessment of the platelet lysates for their ability to modulate the growth of CCL-64 mink lung cells was conducted to determine the overall proportion of positive and negative growth modulators in these samples and the following results were found.

Platelet lysate from rats fed the HFF diet solicited significantly more proliferation than lysate from HFC and LFS fed rats when 8 *u*g and 32 *u*g of sample protein was used and significantly more than lysate from HFC, LFS and HFB fed rats with 16ug of protein (Table 10).

After acidification of platelet lysate the proliferation of CCL-64 cells, in response to all samples, decreased in comparison to non acidified duplicate samples. Lysate from HFF fed rats continued to solicit significantly more proliferation than lysate from LFS fed rats with all concentrations of protein measured and significantly more than lysate from HFC fed rats when 8 *u*g and 16*u*g of protein were measured. The samples from

Table 10: Proliferation of CCL-64 cells in response to platelet lysate, expressed as a percentage of baseline

platelet protein	HFB	HFC	HFF	HFO	LFS
32ug	143(4.8)^{ab}	117(18.0)^b	175(20.2)^a	161(15.6)^{ab}	118(10.4)^b
16ug	111(5.4)^{bc}	97(14.6)^c	148(8.2)^a	134(13.7)^{ab}	90(7.0)^c
8ug	92(6.2)^{ab}	80(10.0)^b	120(12.0)^a	104(14.2)^{ab}	78(8.0)^b

N=5, SEM in parenthesis, superscript denotes significant difference between the groups in each row representing different protein concentrations at $p < 0.05$

HFO fed rats were found to solicit significantly more proliferation than HFC samples and 8 ug (Table 11).

After comparing non-acidified samples against the mean of the LFS samples and grouping the results from all three concentrations together, proliferation in response to HFF samples was found to be significantly higher than HFO samples ($p<0.05$). HFO was significantly higher than HFB, and in turn HFB was significantly higher than HFC and LFS samples ($p<0.05$) (Table 12, Figure 5). When acidified samples were compared against the mean of the acidified LFS group significant differences were found between the treatments. The HFF and HFO groups were significantly higher than the HFC and LFS groups. While the HFF group was also significantly higher than all groups except HFO ($p<0.05$) (Table 12, Figure 6).

When proliferation index after acidification was subtracted from proliferation index before acidification the HFF group was found to have undergone the largest change (table 13). The acidification process sharply decreased the proliferation index of all samples and therefore appeared to activate the latent TGF-B in all of the samples. As the change in proliferation index of the HFF group was the largest, in all three concentrations of lysate measured, it could be assumed that this group also had more latent TGF-B than any other group. The change in proliferation index was significantly more in the HFF samples then the HFC samples when the 32 ug portion was measured ($p<0.05$) (Table 13).

Table 11: Proliferation of CCL-64 cells in response to acidified platelet lysate, expressed as a percentage of baseline

platelet protein	HFB	HFC	HFF	HFO	LFS
32ug	78(10.8)[ab]	76(20.8)[ab]	108(18.8)[a]	100(13.0)[ab]	54(6.6)[b]
16ug	53(9.8)[ab]	45(14.1)[b]	80(12.6)[a]	73(17.1)[ab]	37(5.8)[b]
8ug	37(10.6)[ab]	23(6.0)[b]	50(8.8)[a]	50(13.8)[a]	20(3.3)[b]

N=5, SEM in parenthesis, superscript denotes significant difference between the groups in each row representing different protein concentrations at $p<0.05$

Table 12: Proliferation of CCL-64 cells in response to platelet lysate, with all concentrations combined

	HFB	HFC	HFF	HFO	LFS
non-acid	1.21(0.03)^c	1.03(0.08)^d	1.60(0.06)^a	1.40(0.08)^b	1.00(0.05)^d
acid	1.57(0.21)^{bc}	1.23(0.19)^{cd}	2.41(0.20)^a	2.08(0.28)^{ab}	1.00(0.08)^d

N=15, SEM in parenthesis, superscript denotes significant difference between the groups in each row at $p<0.05$

Figure 5: Proliferation of cells in response to platelet lysate, with all concentrations combined, as affected by dietary lipids.

Figure 6: Proliferation of cells in response to acidified platelet lysate, with all concentrations combined, as affected by dietary lipids.

Table 13: Change in proliferation of CCL-64 cells after acidification of platelet lysate as compared to the baseline value

platelet protein	HFB	HFC	HFF	HFO	LFS
32ug	64(8.6)^{ab}	42(6.0)^{b}	94(28.6)^{a}	68(2.2)^{ab}	64(12.2)^{ab}
16ug	58(6.0)	52(2.6)	68(5.4)	61(6.1)	54(3.9)
8ug	56(7.6)	58(9.6)	70(9.6)	62(8.2)	58(6.6)

N=5, SEM in parenthesis, superscript denotes significant difference between the groups in each row representing different protein concentrations at $p < 0.05$

This study demonstrated that dietary fatty acid composition has an effect on the growth factor status of platelets. The HFF diet appeared to modulate the concentration of growth factors and growth effectors in platelets resulting in a combination that was a strong stimulator of growth. The fatty acids in fish oil have the following characteristics: 31% of their total fatty acids have a chain length 20 carbons or longer, with a double bond in the n-3 position, and a large degree of unsaturation with many fatty acids having 4 or 5 double bonds, and less than 2% of their fatty acids in the n-6 position. The fatty acids in olive oil have 74% of their fatty acids with one double bond at the n-9 position, less than 1% at the n-3 position, 11% at the n-6 position and a maximum chain length of 18 carbons. Corn oil has 59% of its fatty acids with a double bond at the n-6 position, with less than 1% n-3 and 26% n-9 (Table 5).

Given that olive oil and fish oil have few physical similarities, in terms of fatty acid composition, their similar effect on CCL-64 proliferation is surprising. Perhaps the similar affect of the n-3 and n-9 fatty acids is not due to the presence of these fatty acids themselves but rather the absence of n-6 fatty acids. n-6 fatty acids can be metabolized to AA, a key fatty acid in many different physiological functions, that has been demonstrated to increase platelet coagulation via its metabolism into TxA_2 (Hunt & Groff, 1990; Sanigorski et al., 1996). AA is metabolized to 2-series prostaglandins and thromboxanes by cyclooxygenase and 4-series leukotrienes by lipoxygenase. HFC contained a large amount of n-6 fatty acids and therefore would have been able to produce more AA. Perhaps the presence of more AA had an indirect effect on the factors that controlled proliferation, which may be a reason that HFC samples did not stimulate proliferation to the degree that HFF or HFO did. Beef tallow used in this study contained

less than 4% n-6 fatty acids and a large amount of more highly saturated fatty acids. Therefore beef tallow had only a small proportion of fatty acids that could have possibly been metabolized to AA, as mammals can not insert a double bond beyond the ninth carbon from the carboxyl end of a fatty acid. It is difficult to base the stimulation or inhibition seen in response to these samples solely on the ability to produce AA, as animals fed beef tallow would not have been provided with extra n-6 fatty acids, and would not likely be able to produce as much AA as the HFC animals could, yet their proliferation index was still similar. Nevertheless each fatty acid source may have their affect via different mechanisms, therefore the discussion of one logical explanation for all treatment groups may not be appropriate.

The results of this study are significant in that they demonstrate an association between dietary fatty acid composition and growth factors. Potentially this association could be part of the reason for diet having an effect on the development of chronic illness.

Effect of age of the animal and dietary lipids on growth modulating ability of platelet lysates. The approach, utilizing CCL-64 cells, for analyzing platelet lysate from animals of the different age groups was slightly different. The main difference was with respect to the amount of lysate used. For instance, 89 ug of platelet lysate was used when young rat samples were analyzed and 8 to 32 ug of platelet lysate was used when older rat samples were analyzed. As stated previously, platelet lysates from older rats stimulated proliferation while platelet lysate from young rat samples inhibited proliferation. A lower amount of lysate had to be used with the older rats samples, because of the stimulatory affect that it had on proliferation, to keep proliferation index

close to baseline. With proliferation index close to baseline, the possibility of proliferation reaching a maximum threshold was decreased.

The age of an animal was found to have an effect on the growth modulating ability of platelets from male rats. Differences were observed in the manner in which platelet lysate modulated the growth of CCL-64 cells. When the proliferation of cells was observed after incubation with non-acidified platelet lysate from young rats, platelet lysate was found to inhibit proliferation, when compared to baseline, in the majority of samples. Nine samples deviated from this trend. The samples which did not respond in a normal manner were distributed among all the treatment groups. Incubation of CCL-64 cells with platelet lysate from older animals consistently stimulated proliferation in a dose dependent manner (Table 10). It could be assumed that if the same amount of platelet lysate was used when analyzing older rat samples, as young rat samples, the proliferation index would have been even higher because proliferation index increased in a dose dependent manner. Similar amounts of platelet lysate from younger (22 ug) and older (8-32 ug) animals were analyzed when samples were acidified. Proliferation index was found to be similar, with the vast majority of samples soliciting proliferation below the rate of baseline in samples from either age group.

This data suggests that platelets from older rats are physiologically different from platelets of younger rats. It would appear that a shift in proportion of growth factors occurs from more inhibitory growth factors, when a rat is less than 3 months of age, to more stimulatory growth factors when a rat is over eight months of age. However this trend could not be decerned after samples had been acidified. These findings demonstrate that the choice of the age of animals is an important variable in an

experiment. While it is likely that the pathology of chronic disease begins when a person is young, it does not normally exhibit it self until that person is older. This may have a relationship to the different growth factor profile found in the older animals. A limited amount of research has investigated the effect of age on physiological parameters in conjunction with nutritional variables. The findings of the present research provide impetus for research in this area in the future.

Modulation of TGF-B1 in platelets of male rats as measured by western blot.

Our experience with the CCL-64 cell bioassay clearly indicated that this bioassay was an excellent system to assess and compare overall growth modulating ability of platelet lysates. However, this bioassay was not specific for TGF-*B*. Western immunoblots were conducted for TGF-*B*1 to assess if the concentration of this growth factor is modulated by diet in a similar fashion to what is demonstrated with the CCL-64 analysis. Analysis was conducted for the TGF-*B*1 isoform because it is known to be highly concentrated in platelets and is the most abundant form of TGF-*B*1 found in mammals (Reilly et al., 1993; Martyre et al., 1991). TGF-*B*1 that was quantified with western blots were conducted with the 12 Kda weight of TGF-*B*1 (Figure 7).

Dietary intervention did not appear to have a large impact on TGF-*B*1 level in platelets when measured by western blot. HFB samples reacted with TGF-*B*1 antibody significantly more than HFC samples ($p < 0.05$). HFF, HFO and LFS samples all had similar amounts of reactivity that was not significantly different from any other groups (Table 14).

Figure 7: Sample western blot conducted with platelet lysate.

Table 14: Concentration of TGF-B1, represented by 12 Kda protein, in platelet lysate of eight month old rats as determined by western immunoblot

HFB	HFC	HFF	HFO	LFS
10500(1388)[a]	3538(306)[b]	5380(2118)[ab]	5517(2236)[ab]	7639(3155)[ab]

N=4, all values in pixels as determined by densitometric analysis, SEM in parenthesis, superscript denotes significant difference between groups at p<0.05

These results are somewhat surprising for two main reasons. Differences in proliferation index of the CCL-64 cells were found among most treatment groups, however differences in western results were not as easily distinguishable (Figure 5, 6, Table 14). Secondly, the western TGF-B1 results do not correlate with the CCL-64 results. For example, the HFC samples had a very low concentration of TGF-B1 according to the value derived by western immunoblot, but exerted a more noticeable inhibitory effect on the growth of CCL-64 cells. A low rate of proliferation of CCL-64 cells would be expected in an environment where a large concentration of TGF-B was present. A reason for the lack of correlation between the two methods involves the sensitivity of the CCL-64 cells to all isoforms on TGF-B, and not just TGF-B1. If the proportion of TGF-B that was in the B1 isoform was decreased in the HFC or LFS diets, this could explain the lack of correlation between the two methods. Another possibility is that the HFC lysate could have contained a lower amount of stimulating growth factors. The inconsistency between the western blot analysis and the CCL-64 analysis does not refute the effectiveness of either method as the two methods have fundamental differences. Firstly the CCL-64 analysis was conducted with acidified lysate and non-acidified or untreated lysate. The untreated samples were analyzed to assess the affect of the lysate while it was still in a state closest to how it would originally be found *in vivo*. The acidification assessed the total TGF-B equivalent present in the lysate. The lysate examined with the western blot was treated differently in that it was not examined in either an untreated state, or an acidified state, but it was examined after incubation for three minutes with B-mercaptaethanol. During this treatment some of the bonds joining TGF-B1 to the LAP protein may have been denatured by an effect of either the heat or

the *B*-mercaptaethanol. Incubation at 80°C for ten minutes has been demonstrated to biologically activate a significant portion of TGF-*B* from rat platelets (Murata et al., 1995; Studer et al., 1995). Therefore the amount of TGF-*B*1 quantified may include some that had been released from the LAP binding protein.

Examination of the ECL picture clearly showed that a large amount of antibody bonded to the area at the top of each lane on the acrylamide gel (Figure 7). This most likely is due to the antibody reacting to TGF-*B*1 while it is still bound to the heavy LAP protein. This indicates that western blot was not able to quantify all of the TGF-*B*1 in each sample. Therefore the western blot analysis quantified an amount of TGF-*B*1 that was neither in a completely untreated state, or an entirely activated state.

A second fundamental difference between the two approaches involves the higher specificity of western blotting. The antibody used in western blotting reacts specifically with TGF-*B*1, and the only area on the membrane analyzed for antibody binding is a specific area based on the known weight of TGF-*B*1. As has been discussed above, the proliferation of the CCL-64 cells is affected by factors other than the concentration of TGF-*B* in the medium. For example the concentration of TGF-*B*1 in HFC samples was significantly lower than that in HFB samples according to western blot, while the proliferation of cells in response to HFC lysate was also low. It may be assumed that growth stimulating factors are not present in the HFC samples in large amounts, thus allowing TGF-*B* to be more effective in inhibiting the proliferation of cells.

Platelet lysate from different treatment groups exerted variable growth responses in CCL-64 cells. Platelets appear to contain positive as well as negative growth factors.

The physiological significance of these findings remains to be evaluated in the experimental models of chronic diseases.

Chapter 5.

EFFECT OF DIETARY FATTY ACID COMPOSITION ON THE TGF-*B* LEVEL

OF NEUTROPHIL LYSATE OF MALE SPRAGUE-DAWLEY RATS

1.   Introduction

Neutrophils are involved in a variety of biological responses throughout the entire body. The immune system is believed to assist in protection against cancer. Tumours, preneoplastic lesions or mutated cells can be recognized as foreign bodies and attacked or even destroyed by the immune system. Neutrophils and macrophages are related to atherosclerotic lesion development in that they function in the initial inflammation that may cause a vascular injury and infiltrate into the interior of lesions after they have begun developing. Neutrophils are also known to produce and excrete growth factors including TGF-*B* and PDGF that may theoretically function in the progression or inhibition of lesions. These growth factors are known to affect proliferation of vascular endothelial and SMC. Growth factors from neutrophils could be released over the time the neutrophil is in circulation or could be incorporated with the neutrophils into a lesion. Research has demonstrated that growth factor expression from human PBMs can be manipulated by altering dietary fat (Kaminski et al., 1993). TGF-*B* is associated with the progression of atherosclerosis and other chronic diseases, and neutrophils are a known source of TGF-*B* (Capo et al., 1996; Reinhold et al., 1996; Wong et al., 1991). Neutrophil fatty acid composition (Careaga-Houck and Sprecher, 1989; Palombo et al., 1997), eicosanoid production (Fan & Chapkin, 1992; Jackson et al., 1997), and chemotaxis (Sperling et al., 1993) have been demonstrated to be modulated by the

alteration of dietary lipids. The ability of mononuclear cells to synthesize interleukin-1 and tumour necrosis factor are also inhibited by the addition of n-3 fatty acid to a diet (Endres et al., 1989). The main focus of the present research was to use platelets as the target tissue, however it was also important to determine if other cell types in the circulation respond to dietary treatment. Therefore the potential of dietary fat to modulate TGF-B concentration in neutrophils was also investigated as an adjunct to the main study.

2.   Materials and Methods

Animals.   Male eight-month-old Sprague-Dawley rats were used.

Study design. Rats were randomly assigned to one of the following diets: HFF, HFB, HFC, HFO or LFS. Animals remained on diets for six weeks before they were terminated. Neutrophils were isolated and stored at -80°C.

CCL-64 mink lung cell bioassay for TGF-B equivalent. Samples of neutrophil lysate were acidified and neutralized. 14 ug and 7 ug of neutrophil lysate was incubated with cultures of CCL-64 mink lung cells and proliferation index was assessed. TGF-B equivalent was assessed using a standard curve for TGF-B.

Western blot analysis for TGF-B1. Ten ug of sample was heated for 10 minutes at 90°C at 1:1 dilution with SDS buffer containing B-mercaptaethanol. Sample was separated on a 15 % acrylamide tris-glycine gel and proteins were transferred to nitrocellulose membranes for 2.5 hours. Membranes were incubated with TGF-B1 antibody. TGF-B1 was quantified at the 12 Kda molecular weight.

Statistical Analysis. Statistical analysis of the data was performed using the statistical analysis software (SAS). Version 6.12, for microcomputers (Cary, NC). ANOVA in combination with Duncan's Multiple Range test was used to separate treatment means for significant differences. Differences were regarded as significant at $p < 0.05$.

3.   Results and Discussion

CCL-64 mink cell bioassay for TGF-B equivalent. The dietary fatty acid source was found to significantly modulate the inhibition of growth potential by neutrophil lysate when measured as TGF-*B* equivalent (Table 15). TGF-*B* was found to be most highly concentrated in samples from HFO rats with a mean total of 8.3 pg TGF-*B*/ug lysate. This total was significantly higher than samples from HFB and LFS rats that contained 3.4 and 2.7 pg TGF-*B*/ug lysate ($p < 0.05$). The mean total TGF-*B* concentration in HFC and HFF samples were 5.9 and 5.7 pg/ug respectively. Although not considered a statistically significant difference, the mean concentration of TGF-*B* in HFO samples was over 40% higher than HFC or HFF samples, which may be of physiological significance (table 15).

The presence of olive oil in a diet resulted in a high level of TGF-*B* equivalent in neutrophils. Replacement of dietary lipids with MUFA is known to affect blood lipid profiles positively by decreasing blood low density lipoproteins (LDL) and replacement of carbohydrates with MUFA increases blood HDL (Kushi et al., 1995). This association between blood lipoproteins and MUFA is the main reason that olive oil, which contains up to 80% of total

Table 15: Growth modulating effect of neutrophil lysate expressed as TGF-B equivalent

neutrophil protein	HFB	HFC	HFF	HFO	LFS
14ug	3.1(0.6)[b]	4.5(1.8)[ab]	5.3(1.8)[ab]	9.2(2.4)[a]	2.0(0.4)[b]
7ug	3.7(0.8)[a]	7.0(3.1)[a]	6.1(2.4)[a]	7.4(1.4)[a]	3.5(0.5)[a]
mean	3.4(0.5)[b]	5.9(1.9)[ab]	5.7(1.4)[ab]	8.3(1.3)[a]	2.7(0.4)[b]

N=4 for 7 and 14 ug, n=8 for mean, pg TGF-B/ug protein sample lysate, SEM in parenthesis, superscript denotes significant difference between the groups in each row representing different protein concentrations at $p < 0.05$

fatty acids in the MUFA form, is recommended to be consumed to protect against the development of atherosclerosis.

Dietary fat may also have an affect on the pathology of atherosclerosis by modulating the TGF-*B* content of neutrophils. The higher concentration of TGF-*B* from HFO neutrophils could suppress the over proliferation of VSMC or vascular epithelial cells that takes place in the early development of a lesion as discussed above. TGF-*B* also may play a protective role by counteracting the effects of tumour necrosis factor-alpha. Tumour necrosis factor-alpha is a pro-inflammatory cytokine, produced by leukocytes, that can induce endothelial dysfunction and inhibit release of nitric oxide from the endothelium and hence the vasodilation that nitric oxide induces. Since TGF-*B* can oppose the function of tumour necrosis factor-alpha it can help to prevent inflammation or indirectly increase vasodilation (Lefer and Lefer, 1993).

The low concentration of TGF-*B* in HFB samples could explain part of the reason that a high beef diet increases the risk of atherosclerosis. The positive association of beef tallow consumption with atherosclerosis, via an effect on blood lipoprotein composition, is not as easy to explain as what is seen with olive oil. Beef tallow has been assumed to promote atherosclerosis due to its high saturated fat and cholesterol content. However one of the main saturated fatty acid (SFA) in beef tallow is stearic acid, and neither stearic acid or dietary cholesterol have been demonstrated to have a significant negative affect on blood lipoproteins (Kushi et al., 1995). Since beef tallow does not alter blood lipids via stearic acid or its cholesterol content, it must promote the progression of atherosclerosis through an alternate mechanism. If dietary beef tallow alters neutrophil

TGF-*B* in humans in a manner similar to which occurred in the present study in rats, this may be a key connection between dietary beef tallow and atherosclerosis.

LFS samples were found to have the lowest concentration of TGF-*B*. This could be interpreted as an unexpected result and contradictory to the rationale behind the effect of olive oil. A low fat diet is also associated with decreased risk of the development of chronic disease and a more favourable blood lipid profile than a high fat diet. Changes in growth factor status that occur in a high fat environment are possibly more important than when the same changes occur in a low fat environment. For example low TGF-*B* combined with high blood lipids may be conclusive to the development of disease states. High blood lipid levels or their metabolites may function to mediate TGF-*B* effects.

If the theory that TGF-*B* protects against lesion progression is correct, the lower concentration of TGF-*B* in these samples could actually increase the potential of a vascular injury progressing to form an atherosclerotic lesion by allowing cell proliferation to continue unchecked. An alternative possibility is that the vascular endothelium of animals on a low fat diet recognizes TGF-*B* differently than the vascular endothelium of animals on a diet higher in fat. For example, the vascular endothelium may respond to growth factors differently if it were conditioned to lower concentrations of growth factors over time. If this were true it would be an argument against growth factors having an effect on disease progression. A process of adaptation to lower TGF-*B* levels could involve increased gene expression of TGF-*B* receptors in response to low levels of TGF-*B* in circulation or increased expression in response to a low fat diet.

Increased gene expression in response to a low fat diet is conceivable when it is considered that change of gene expression in response to fat concentration of the diet has

been observed in a variety of other cell types. Hepatic fatty acid synthase and S14 enzyme from the liver, have been found to be reduced when a high fat diet is fed in comparison to a low fat diet (Clarke & Jump., 1994). Transcription rates of hepatic fatty acid synthase and S14 mRNA enzyme are reduced in response to a diet containing fish oil in comparison to one consisting of saturated fat (Blake and Clarke, 1990). Gene expression of fatty acid binding protein and adipocyte P2 have also been demonstrated to be modulated by the chain length of fatty acids in cultures of preadipocytes (Distel et al., 1992). These proteins are intimately involved with fat metabolism. Therefore the fact that their gene expression is modulated by dietary fat is not surprising. TGF-*B* metabolism of vascular endothelial tissue does not have a clearly defined relationship to fat metabolism, thus the possibility that the vascular endothelium's ability to respond to TGF-*B* could be modulated by dietary fat would need to be demonstrated.

Alternative explanations that allow for the low concentration of TGF-*B*, found in LFS samples, to serve a protective effect include comparatively decreased levels of opposing stimulating growth factors, or a higher concentration of TGF-*B* existing in the active form. The concentration of TGF-*B* existing in the active form before acid activation was not successfully assessed with the CCL-64 bioassay.

Western immunoblot analysis for TGF-B1. Western immunoblot analysis was conducted with the TGF-*B*1 isoform because it is the most common isoform of TGF-*B* in neutrophil culture supernatants (Capo et al., 1996). TGF-*B*1 that was quantified with western blots were conducted with the 12 Kda weight of TGF-*B*1 (Figure 8). Some
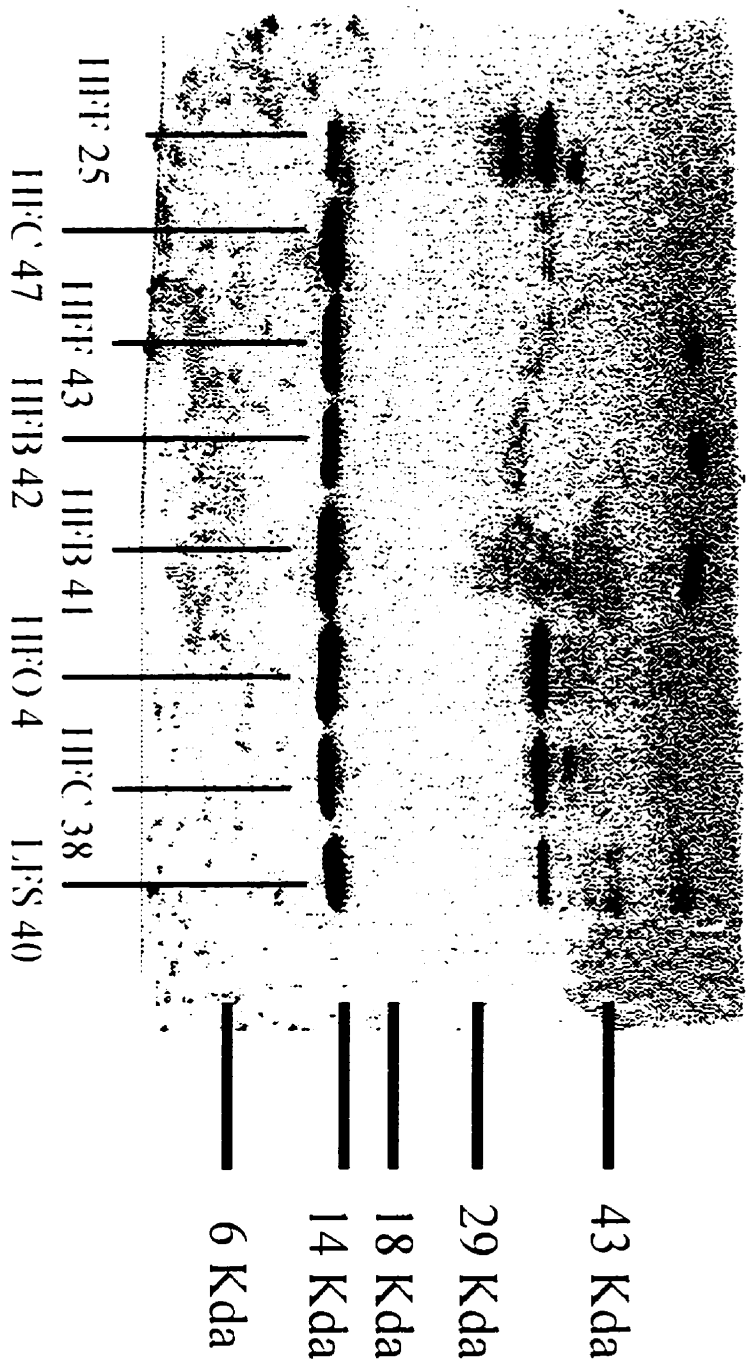
Figure 8: Sample western blot conducted with neutrophil lysate.

TGF-*B*1 was visible at the 39 Kda weight, but was not found consistently and therefore was not analyzed.

According to western immunoblotting, HFO samples had the highest concentration of TGF-*B*1 of all treatments (Table 16). HFO samples had a mean density of 5941 pixels, which was significantly higher than HFB samples which had a mean density of 3351 pixels (p<0.05). Concentration of TGF-*B*1 in HFF, LFS and HFC samples followed HFO in descending order of concentration respectively, and were not significantly different from any treatment group (Table 16).

CCL-64 analysis was conducted with neutrophil samples that had been acidified to elucidate the total amount of active TGF-*B* in each sample. CCL-64 analysis conducted with non acidified neutrophil lysate was inconclusive because a segment of these samples were found to stimulate proliferation in a manner similar to what had occurred with the platelet lysate. However, not all neutrophil samples exerted this effect, therefore it was not possible to assess the concentration of active TGF-*B* in neutrophil lysate with CCL-64 cells.

Acidification of samples is known to activate TGF-*B* that is in a latent form by denaturing the LAP protein. However, acidifying latent TGF-*B* has not been demonstrated to cleave the active portion of TGF-*B* from the LAP protein. It is assumed that the LAP protein is denatured enough to allow the active sight of the TGF-*B* molecule to bind to TGF-*B* receptors on a cell (Khalil, 1997). As the LAP protein is still attached to the TGF-*B* molecule the use of an acrylamide gel for analysis of total TGF-*B* in a sample can not be conducted accurately due to a large portion of total TGF-*B* in each sample still likely being bound to the LAP. Incubation with *B*-mercaptaethanol may

Table 16: Concentration of TGF-B1, represented by 12 Kda protein, in neutrophil lysate as determined by western immunoblot

HFB	HFC	HFF	HFO	LFS
3351(742)[b]	4160(114)[ab]	5522(1303)[ab]	5941(838)[a]	4533(353)[ab]

N=4, all values in pixels as determined by densitometric analysis, SEM in parenthesis, superscript denotes significant difference between groups at $p < 0.05$

cleave some of the TGF-B from the LAP protein, however it is not known what proportion of the total TGF-B present is cleaved from the LAP. Western immunoblot was used in an attempt to assess the amount of TGF-B1 present in the active form and to assess whether the growth factor is modulated by diet in a manner similar to what is demonstrated with the CCL-64 analysis.

Western blot results were supportive of CCL-64 bioassay results in that similar trends were found with both techniques. HFO samples had the highest concentration TGF-B according to both techniques used. HFB samples were found to have significantly less TGF-B than HFO samples with both techniques, while HFF and HFC were intermediate. The only difference found between the two techniques was found in the LFS samples, which had the lowest concentration of TGF-B according to the bioassay but had an intermediate amount of TGF-B according to western blots (Table 15 and 16).

When both sets of results are examined together they indicate that dietary lipid composition may not affect the proportion of total TGF-B found in the active form in neutrophil lysate that has not been acidified. Western blots were conducted on predominantly the unbound portion of TGF-B, while the bioassay was conducted on the total TGF-B in the sample. Therefore, the TGF-B quantified with western blot represented the portion found active in a sample and the bioassay was a reflection of the total TGF-B in the sample. Since results found with both methods demonstrate a similar pattern of TGF-B concentration it may be concluded that dietary lipids did not affect the proportion of total TGF-B in the active form (Table 15 and 16).

It could be argued that differences found between the treatment groups by western blot are not physiologically significant. The difference between the two treatment groups

with the largest discrepancy may not be large enough to have a physiological impact. The density of the HFO group was 77% higher than that of the HFB group. However, the difference in concentration of TGF-B between these two diet groups, according to the bioassay, was 144%. Therefore it may be possible that dietary lipids have an effect only on the amount of total TGF-B found in neutrophils and not the amount found in an active form. If this is true then it means that the neutrophils themselves regulate the amount of active TGF-B that they carry so that it exists in a consistent amount.

While the results from this study demonstrate modulation of the TGF-B quantity in neutrophil samples, the overall effect that neutrophil TGF-B has on the physiology of the various tissues or organs may not be changed. Neutrophils have their own nuclease and their own mRNA for TGF-B as well as their own TGF-B receptors (Capo et al., 1996; Brandes et al., 1991). The mechanisms and factors responsible for TGF-B1 release from a functional neutrophil *in vivo* are not known. TGF-B1 may be released in an active or a latent state. The rate at which a neutrophil activates its TGF-B might even be modulated by dietary fat or membrane composition. Therefore what these results actually mean to a disease process is unknown at this point, but possible to investigate. The possibility that dietary lipids alter the synthesis and release of TGF-B from active neutrophils should be investigated in future studies.

Chapter 6.

EFFECT OF DIETARY FATTY ACIDS ON THE PLASMA CONCENTRATION OF TGF-B1 IN MALE SPRAGUE-DAWLEY RATS, AS MEASURED BY WESTERN IMMUNOBLOT

1. <u>Introduction</u>

Plasma is circulated throughout all tissues and organs of the body and the growth factors that it contains could be derived from platelets, neutrophils or a variety of other organs throughout the body (Puolakkainen et al., 1995; Reilly et al., 1993; Martyre et al., 1994; Capo et al., 1996; Zauli et al., 1993). Higher concentrations of TGF-B could decrease the function of the immune system, affect the proliferation of endothelial and VSMC and increase rates of angiogenesis thereby altering the progression of chronic illnesses (Weller and Fontana, 1995; Kekow et al., 1991; Young et al., 1996; Puolakkainen et al., 1995; Sosroseno et al., 1995).

Analysis of the platelets and neutrophils with the CCL-64 cells demonstrated that dietary fat modulates potential of neutrophil and platelet lysate to affect the growth of CCL-64 cells. As discussed previously TGF-B from platelets and neutrophils may have an impact on disease progression by affecting the proliferation of cells they are in contact with. While platelets and neutrophils come in contact with the vascular wall or other tissues during vascular injury or inflammation, plasma is in contact with tissues constantly over an animal's lifetime. Therefore, the main objective of the present study was to determine if dietary lipids alter the level of TGF-B1, in the medium, that is continuously in contact with the vascular wall. It was also important to determine if

alteration in the TGF-B1 level of the plasma corresponds to the changes occurring in the platelets and neutrophils.

2.   Materials and Methods

Animals.   Male eight-month-old Sprague-Dawley rats were used.

Study design. Rats were randomly assigned to one of the following diets: HFF, HFB, HFC, HFO or LFS. Animals remained on diets for six weeks before they were terminated. Blood was drawn from a heart puncture, centrifuged and platelet rich plasma was drawn off. Plasma was then centrifuged so platelets formed a pellet and plasma was drawn off and stored in siliconized eppendorfs at -80°C.

Western blot analysis for TGF-B1.   Forty *u*g of sample was heated for three minutes at 90°C at 1:1 dilution with SDS buffer containing B-mercaptaethanol. Sample was separated on a 15 % acrylamide tris-glycine gel and proteins were transferred to nitrocellulose membranes for 2.5 hours.   Membranes were incubated with TGF-B1 antibody. TGF-B1 was quantified at the 39 and 43 Kda molecular weight.

Statistical Analysis.   Statistical analysis of the data was performed using the statistical analysis software (SAS). Version 6.12, for microcomputers (Cary, NC). ANOVA in combination with Duncan's Multiple Range test was used to separate treatment means for significant differences. Differences were regarded as significant at $p < 0.05$.

3.   Results and Discussion

Western blot analysis for TGF-B1 in plasma samples. TGF-*B*1 was quantified at the 39 and 43 Kda molecular weight (Figure 9). These were the only visible bands of TGF-*B*1 and were likely TGF-*B*1 complexed to a portion of the LAP protein. Quantifying TGF-*B*1 at these heavier weights was the only way to measure TGF-*B*1 in these samples as a band was not visible at the 12 Kda weight.

Feeding the HFF or LFS diets resulted in a low concentration of TGF-*B*1 in rat plasma according to western blotting. HFF plasma samples had significantly less TGF-*B*1 than HFB and HFO samples (p<0.05), when the 43 Kda band was measured. LFS and HFC had intermediate amounts of TGF-*B*1 compared to the other samples (Table 17).

When the 39 Kda band was quantified, similar trends were found compared to the 43 Kda band. The only treatment that deviated from the trend was the HFC samples that had the highest amount of binding at the 39 Kda band and an intermediate amount at the 43 Kda band. HFF and LFS samples had the lowest amount of TGF-*B*1 at 39 Kda. LFS samples had significantly less TGF-*B*1 than HFB or HFC samples, and HFO samples were intermediate (p<0.05) (Table 17).

When it is considered that their were higher levels of TGF-*B*1, in the plasma of animals fed diets high in fat, with the exception of fish oil and the 43 Kda HFC, the theory that TGF-*B* has a protective effect against atherosclerosis or tumour progression is not logical. Epidemiological evidence has shown that a diet high in fat, particularly beef tallow may increase the rate of tumour progression as well as deaths related to cardiovascular disease (Kushi et al., 1995; Fraser and Shavlik, 1997; Fraser, 1994; Potter, 1997; Snowdon et al., 1984; Statland, 1992). Therefore, due to the higher rate of disease associated with these diets the higher amount of TGF-*B*1 found in the plasma of animals
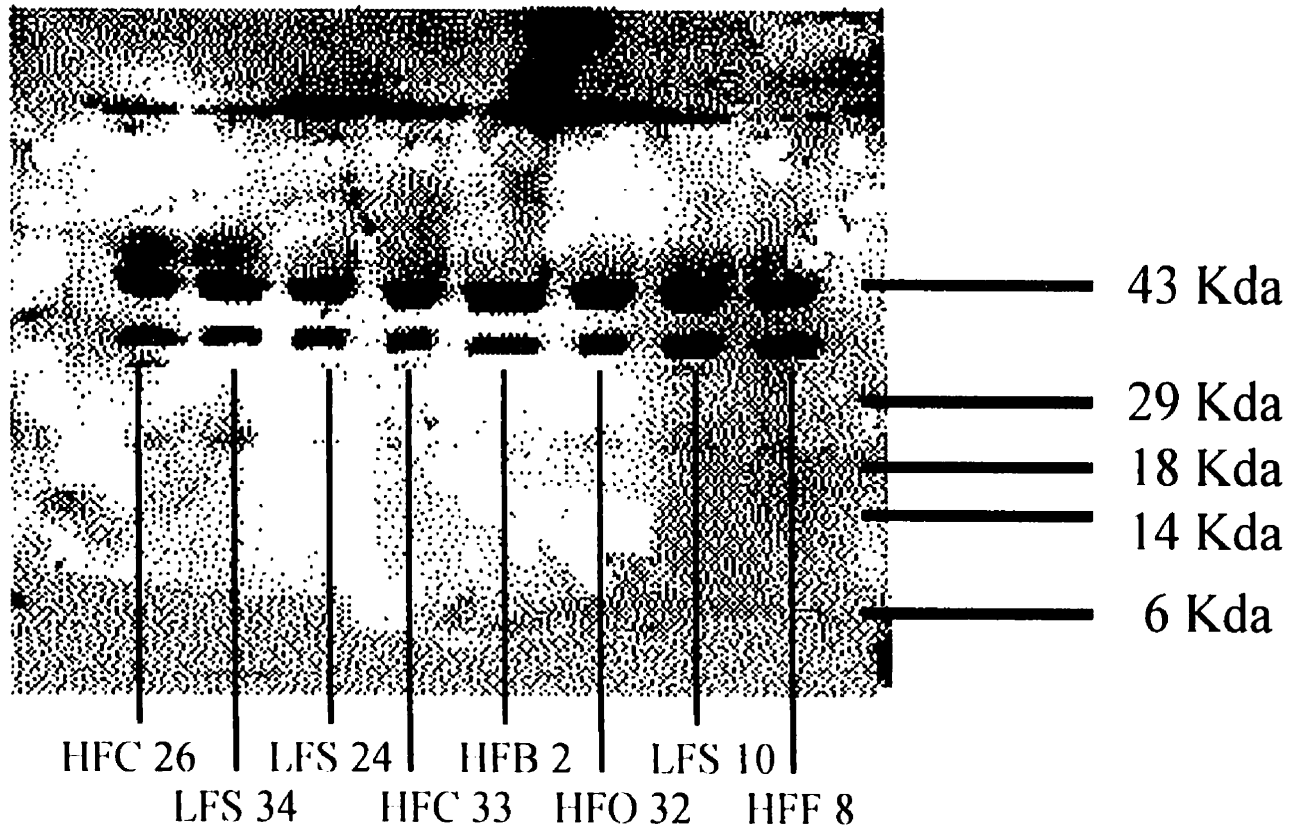
Figure 9: Sample western blot conducted with plasma.

Table 17: Concentration of TGF-B1, represented by 39 and 43 Kda proteins, in plasma as determined by western immunoblot

Wgt.	HFB	HFC	HFF	HFO	LFS
43	4484(603)^a	3194(429)^{ab}	2456(513)^b	4505(553)^a	3347(549)^{ab}
39	4893(419)^a	5412(582)^a	4350(440)^{ab}	4876(430)^{ab}	3663(129)^b

N=6, all values in pixels as determined by densitometric analysis, SEM in parenthesis, superscript denotes significant difference between groups in each row at $p<0.05$

that consumed these diets, could be interpreted as a situation that favours the development of these diseases. The mechanisms through which TGF-B1 may exert diverse effects during the disease process, have been discussed previously.

Higher amounts of TGF-B1 in plasma could favour development of atherosclerotic lesions by upregulating production of ECM and angiogenesis (Reinhold et al., 1995). TGF-B1 has also been demonstrated to function as an immunosuppressive agent, theoretically allowing tumours a better chance of survival (Kekow et al., 1991; Puolakkainen et al., 1995; Sorroseno et al.,1995; Weller and Fontana, 1995). Plasma TGF-B has been found to be elevated in patients with different forms of cancer including: Hodgkins, liver, cervical, breast, prostate and lung cancers (Kong et al., 1998). It is possible that the elevation of TGF-B found in the plasma of these patients plays a role in the progression of these tumours.

As stated previously higher TGF-B1 in circulation could have an immuno-suppressive effect allowing greater opportunity for infection and cytotoxicity. One condition that may occur from increased rate of infection could involve increased chances of developing diabetes by loss of pancreatic B-cell function due to an autoimmune reaction triggered by viral infection (Baek & Yoon, 1991; Szopa et al., 1993). Alteration of growth factor status has already been associated with diabetes (Sugimoto et al., 1987; Winocour., 1992; Winocour et al., 1993). The possibility that diet could modulate the pathology of diabetes via growth factors merits further examination.

An alternative fashion in which higher concentrations of TGF-B1 in the plasma could predispose to lesion progression could be related to the amount of TGF-B1 retained in platelets or neutrophils. For example, it can not be clearly determined where plasma

TGF-*B*1 originates from, but it is known that TGF-*B*1 is highly concentrated in platelets and neutrophils (Assoian et al., 1983; Hu et al., 1996; Capo et al., 1996). Increase in plasma TGF-*B* has been associated with a decrease in platelet count and an increase in alpha-granule secretion by platelets in response to Interleukin-2 therapy. This research indicated that if platelets are prompted to release their alpha-granules, enough TGF-*B* would be released to increase the plasma content of TGF-*B* (Puolakkainen et al., 1995). If high plasma TGF-*B*1 was a result of increased rates of TGF-*B*1 release from either platelets or neutrophils, these organelles could then be carrying lower amounts of TGF-*B*1 throughout the remainder of their time in circulation. When these platelets coagulate at the site of a vascular injury they would have less TGF-*B*1 to release in the immediate environment. This could possibly allow increased proliferation of VSMC and endothelial cells at a sight of injury and an increased possibility of over proliferation of these cells that could lead to a lesion.

The theory that the concentration of plasma TGF-*B*1 is affected by TGF-*B*1 release from platelets is supported by our platelet analysis of LFS samples. LFS fed animals had the lowest concentrations of TGF-*B*1 in their plasma, and most likely contained higher concentrations of TGF-*B*1 in their platelet lysate, as indicated by CCL-64 analysis. Both acidified and non acidified samples of LFS platelet lysate solicited the least proliferation of CCL-64 cells (Table 11 and 12). Possibly the high concentration of TGF-*B*1 in these platelet samples is a result of decreased TGF-*B*1 excretion by platelets that occurs when an animal is on a low fat diet. The amount of TGF-*B*1 in the high fat plasma samples did not correlate with platelet samples, indicating that there must be more involved with plasma TGF-*B*1 concentration than just supply from platelets.

With respect to correlation between neutrophil and plasma samples, neutrophils from HFB fed rats contained a low amount of TGF-*B*, while plasma contained a high amount (Table 15 and 17). TGF-*B* was low in both plasma and neutrophil samples of LFS fed rats, while it was high in the HFC samples of neutrophils, plasma and platelets. The inconsistency of correlation between neutrophil and platelet TGF-*B*, with plasma TGF-*B*, does not allow definite explanation of the connection between dietary lipids and plasma TGF-*B*.

Chapter 7.   GENERAL DISCUSSION AND CONCLUSIONS

The main objective of the present research was to investigate the effect of dietary lipids on platelet TGF-B status. The rational for selecting platelets as the target tissue and TGF-B as the indicator growth factor are as follows. Dietary lipids have previously been shown to influence the physiological responses of platelets in both animals and humans. Platelets and TGF-B are involved in the pathobiology of a number of chronic illnesses and platelets contain an abundance of TGF-B.

In addition to platelets, blood neutrophils and plasma were also assessed for TGF-B status to determine if dietary lipids exert a differential response in a tissue specific manner. Platelets are formed in the megakaryocytes of bone marrow and their phenotype may possibly be established during their development from the stem cells. Platelets are also well equipped with the ability to pickup and release a number of molecules and have a vast array of metabolic capabilities. Platelets lack a nuclease to perform transcriptional activity. In contrast, circulating neutrophils have a nuclease and are capable of gene transcription and synthesis of a number of biomolecules.

To examine the TGF-B status of these cells, two main approaches were taken. Firstly the CCL-64 cell bioassay was used to assess the TGF-B equivalent of platelet and neutrophil lysate. CCL-64 cell proliferation is inhibited by exogenous TGF-B, and these cells are therefore frequently used to measure the level of TGF-B in biological samples. A primary reason for using this technique is that it allows measurement in a living system that allows measurement of biological activity. An additional benefit of the bioassay is that it allows measurement of the proportion of TGF-B in both the active and

inactive/latent forms. A limitation of using a bioassay is that it involves the use of the proliferation index of living cells, which may be influenced by a variety of other biomolecules that may be found in any biological sample.

The second method that was used in the present research to assess TGF-B concentration was the western immunoblot. This technique quantified TGF-B by allowing an antibody to bind to the TGF-B protein and then measuring the amount of antibody bound to a consistent amount of sample. Advantages of this technique are that the antibody used is highly specific and its action is not easily influenced by other biomolecules that may be present in a sample lysate. A second advantage is that the antibody allows quantification of individual isoforms of TGF-B with a very low amount of cross reactivity with other isoforms. A limitation of western blots for TGF-B quantification is that a large proportion of TGF-B may remain bound to the LAP protein and not be found in the active form. Immunoreactivity to TGF-B1 antibody was found to occur at several different molecular weights, causing measurement of total TGF-B1 to be very difficult. Platelet and neutrophil TGF-B1 were examined at the 12 Kda level, the weight of active TGF-B1 after its two peptide chains had been separated by the action of B-mercaptaethanol. Plasma TGF-B1 was quantified at the 39 and 43 Kda weights because this was the only weight where a quantifiable amount was found. The biological significance of 39 Kda and 43 Kda bands remains elusive. Western blot analysis used in this research quantified TGF-B1 successfully in platelet and neutrophil samples and could still be used as a method of measuring TGF-B found in a biological sample in the future.

The findings of this research suggest that lipid source does modulate the growth factor status of platelets and neutrophils. It was also demonstrated that platelet lysate

from weanling and adult rats exerts different effects on the growth of CCL-64 mink lung cells. However lipid source does not appear to have the same affect on the TGF-*B* concentration of neutrophils as it does platelets, where an increase in TGF-*B* concentration in neutrophils does not necessarily coincide with an increase in platelets. This finding is highly significant to the study of the relationship between diet, growth factors and disease, as it means that the relationship is a highly complex one and not simply related by a diet change causing the same increase or decrease in TGF-*B* in all cell types.

These studies have provided fundamental information on the amenability of platelets and neutrophils to respond to dietary lipids. These findings support the hypothesis that diets with different fatty acid composition modulate the growth factor status of platelets. Different combinations of fatty acids that were found to modulate TGF-*B* in this study include; n-6 fatty acids that were a large portion of the HFC diet, n-3 fatty acids that were a large portion of the HFF diet, MUFA that were a large portion of the HFO diet and the low degree of unsaturation that characterized the HFB diet. The findings also provide a strong impetus to further explore the role of nutrients as modulators of the growth factor status of different tissues and the mechanisms by which these modulations impact chronic illnesses. A systemic evaluation of blood constituents with particular respect to platelets and neutrophils for various growth factors is warranted. It is also important to explore the possibility that there are additional lipid metabolites which may also exert growth modulating effects on various tissues and organelles.

Future directions. Analyses of platelets and neutrophils under a variety conditions will be essential to characterize the link between diet and growth factor concentration of platelets and neutrophils. The modulation of platelet growth factors by dietary lipids should be more precisely examined so that the growth factors that change in response to diet can be identified and quantified. The point during the life span of platelets and neutrophils in which modulation of growth factor content takes place should also be determined. The ability of dietary lipids to affect platelet and neutrophil capacity to retain or pick up growth factors during circulation should be examined.

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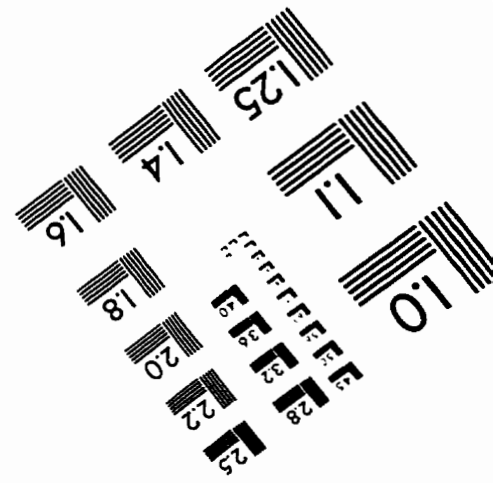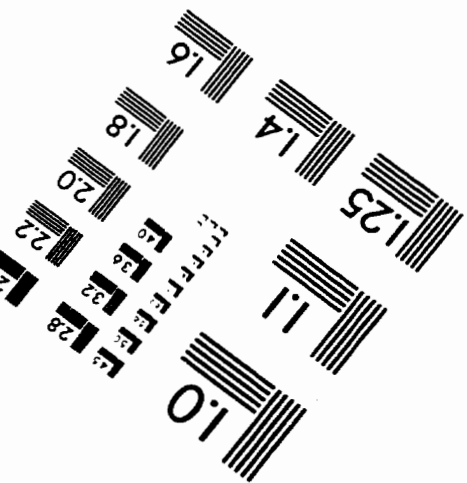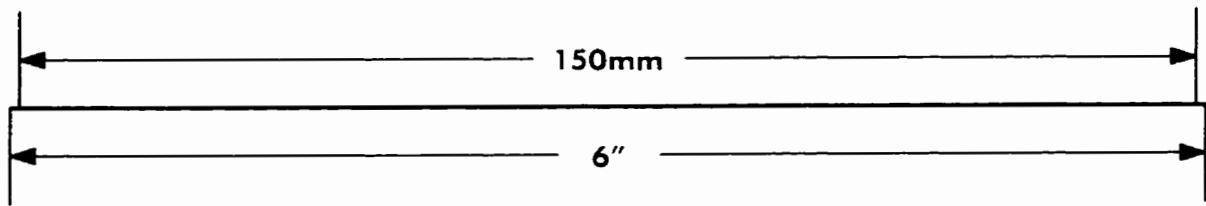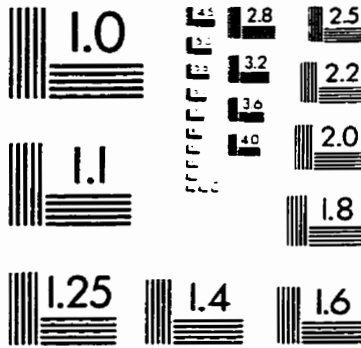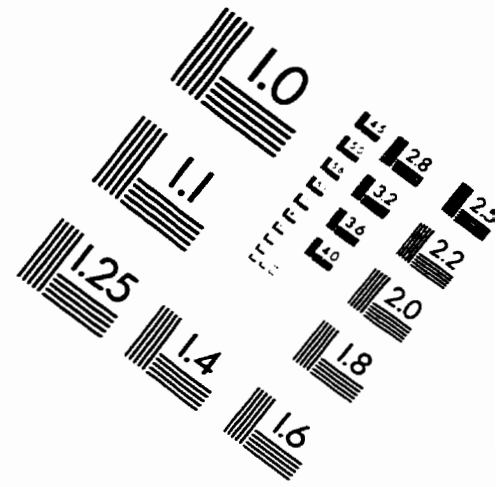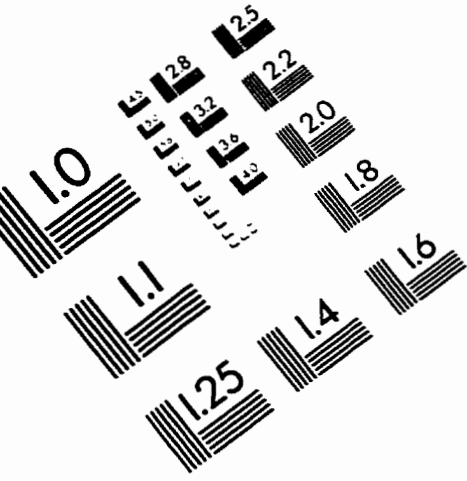
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IMAGE EVALUATION
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