THE BIOCHEMISTRY OF ATHEROSCLEROSIS; A POTENTIAL ROLE FOR TRANSGLUTAMINASE

BY

RICKY I. WIEBE

A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the Requirements
for the Degree of

MASTER OF SCIENCE

Department of Biochemistry and Molecular Biology
University of Manitoba
Winnipeg, Manitoba

ISBN 0-315-76988-2

THE BIOCHEMISTRY OF ATHEROSCLEROSIS; A POTENTIAL ROLE FOR TRANSGLUTAMINASE

BY

RICKY I. WIEBE

A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

ACKNOWLEDGEMENTS

The author is grateful for the financial support from the Medical Research Council of Canada, the Manitoba Heart and Stroke Foundation and the Manitoba Health Research Council.

Also greatly appreciated is the technical assistance and the wit and humour of Alan Tarr who provided the encouragement, the laughs and was always there to grab a "'feine".

The author's deepest gratitude goes to Dr. J. M. Bowness. The encouragement, insight, direction, that proved invaluable, were always available. Special thanks go to him as well for the patience he showed in the preparation of this thesis.

TABLE OF CONTENTS

Acknowledgements	i
Table of Contents	ii
List of Tables and Figures	iv
Abbreviations	v
Abstract	vii
I. Literature Review	1
A. Introduction	1
B. Structure of the Normal Artery	3
C. Atherosclerosis: A Definition	5
D. Natural History of Atherosclerosis	6
Diffuse Intimal Thickenings	7
Fatty Streaks or Dots	7
Gelatinous Lesions	9
Fibrous Plaques	10
E. Cell Biology of the Arterial Wall	10
1. Endothelial Cells	12
2. Smooth Muscle Cells	14
3. Macrophages and Monocytes	17
F. Biochemical Components of Atherosclerosis	20
1. Connective Tissue	20
2. Fibrinogen	31
3. Plasma Lipoproteins	35
4. Transglutaminases	37
II. Hypothesis and Rationale	41
III. Experimental	43
A. Transglutaminase-Catalyzed Crosslinking of PIIIP and Fibrinogen	43
1. Introduction	43
2. Methods and Materials	43
3. Results	48
4. Discussion	49

B. Increased Transglutaminase in the Aortas of Cholesterol-Fed Rabbits 51
1. Introduction 51
2. Methods and Materials 51
3. Results 54
4. Discussion 60
C. Binding of PIIIP or Putrescine in Smooth Muscle Cultures 63
1. Introduction 63
2. Methods and Materials 63
3. Results 65
4. Discussion 71
IV. Summary 74
V. References 77

TABLES AND FIGURES

Table 1. Genetically distinct collagens. 22
Table 2. Composition of human serum lipoproteins. 36
Table 3. Precipitation of SDS-insoluble material after incubation with transglutaminase. 47
Table 4. Specific activities of transglutaminases in three layers of normal and atherosclerotic rabbit aortas. 59
Table 5. Total counts in TCA precipitates of confluent SMC layers labeled with ^{3}H-putrescine. 66

Figure 1. Hypothesis. 2
Figure 2. Schematic representation of the normal artery. 4
Figure 3. Schematic representation of a fibrous plaque. 11
Figure 4. Transglutaminase-catalyzed crosslink. 38
Figure 5. SDS-PAGE of fibrinogen and PIIIP preparations. 44
Figure 6. Transglutaminase-catalyzed crosslinking shown by chromatography on Sepharose CL-2B in 0.1% SDS. 46
Figure 7. Transglutaminase activity in three layers of normal and atherosclerotic rabbit aortas. 57
Figure 8. Tissue transglutaminase antigen in three layers of normal and atherosclerotic rabbit aortas. 58
Figure 9. Fluorograph of SDS-PAGE of cell extracts after labeling with ^{3}H-putrescine. 67
Figure 10. SDS-PAGE of lyophilized medium after incubation of ^{125}I-PIIIP with smooth muscle cells. 68
Figure 11. SDS-PAGE of lyophilized medium after incubation of ^{125}I-PIIIP with smooth muscle cells. 69
Figure 12. SDS-PAGE of lyophilized medium after incubation of ^{125}I-PIIIP with smooth muscle cells. 70

ABBREVIATIONS

BSA .....bovine serum albumin

CHD.....coronary heart disease

cpm.....counts per minute

CS.....chondroitin sulfate

Da .....Daltons

DEAE-cellulose.....diethylaminoethyl-cellulose

DIT .....diffuse intimal thickening

DMEM .....Dulbecco's minimum essential medium

dpm.....disintigrations per minute

DS.....dermatan sulfate

DTT .....dithiothreitol

ECM.....extracellular matrix

EDTA.....ethylenediaminetetraacetic acid

e. l. i. s. a. .....enzyme-linked immunosorbent assay

FBS.....fetal bovine serum

FC .....foam cell

FGN .....fibrinogen

Fig. .....figure

FN .....fibronectin

FPA.....fibrinopeptide A

FPB.....fibrinopeptide B

FRA.....fibrinogen-related antigen

FS .....fatty streak

GAG.....glycosaminoglycan

h .....hour

HA.....hyaluronic acid

HDL.....high density lipoprotein

HS.....heparan sulfate

IEL.....internal elastic lamina

LDL.....low density lipoprotein

MDFC.....macrophage-derived foam cell

MGBG.....methylglyoxyl bis-(guanylhydrazone)

min.....minute

MW.....molecular weight

PAGE.....polyacrylamide-gel electrophoresis

PBS.....phosphate buffered saline

PCIII.....procollagen type III

PDGF.....platelet-derived growth factor

PG.....proteoglycan

PGI_2.....prostacyclin

PIIIP.....aminopropeptide of type III collagen

PMSF.....phenylmethylsulfonylfluoride

RER.....rough endoplasmic reticulum

s.....seconds

SDS.....sodium dodecyl sulfate

SE.....standard error

SG.....structural glycoprotein

SMC.....smooth muscle cell

TCA.....trichloroacetic acid

TGase.....transglutaminase

TGF-β.....transforming growth factor–β

VLDL.....very low density lipoprotein

$v_v$myo.....volume fraction of myofilaments

ABSTRACT

A review of the literature on the development of the lesions of atherosclerosis suggests a role for transglutaminase-catalyzed crosslinking of procollagen type III and fibrinogen or low density lipoprotein in the arterial wall.

Bovine type III ^{3}H-procollagen was shown by chromatography under dissociating conditions to form very high molecular weight complexes with excess bovine fibrinogen. Larger complexes of this type formed with fibrinogen or fibrin monomers can be separated by centrifugation and were found to be insoluble upon washing with 1% SDS.

Rabbits were fed for 10-12 weeks on a normal diet or the normal diet supplemented with 1% cholesterol and 6% peanut oil. The aortas of these animals were separated into three layers which were homogenized and extracted. The extracts and the insoluble residues were assayed for transglutaminase activity and tissue transglutaminase antigen. When compared to normal aortas, the inner and middle layers of the aortas with lesions, from cholesterol-fed rabbits, showed higher transglutaminase activities in the buffer-soluble fraction without corresponding increase in antigen. The buffer-insoluble fraction, which contained a lower specific activity transglutaminase than the buffer-soluble, showed higher concentrations of both activity and antigen in the inner and middle layers of the atherosclerotic aortas.

^{3}H-Putrescine incorporation into cultured smooth muscle cells showed the presence of 14 kDa and very high molecular weight labeled compounds by SDS-PAGE after reduction. The amount of labeling was increased when ascorbate or butyrate were added. When smooth muscle cell cultures were incubated with the ^{125}I-aminopropeptide of type III procollagen the medium showed the presence of high molecular weight complexes on SDS-PAGE under reducing conditions. These complexes were not present when the ^{125}I-propeptide was incubated in medium without cells or in medium preconditioned with smooth muscle cells.

The results of these studies are consistent with the hypothesis that transglutaminase, present on the surface of intimal macrophages and smooth muscle cells, catalyzes the formation of crosslinks between procollagen III or its aminopropeptide and fibrinogen or low density lipoprotein in the subendothelium. This process would anchor fibrinogen and low density lipoprotein to the connective tissue and the modification of low density

lipoprotein would cause increased accumulation by macrophages and foam cell formation. The sequestration of the propeptide of procollagen III would inhibit its feedback inhibition of collagen synthesis and cause the increased collagen accumulation found in advanced atherosclerotic lesions.

I. LITERATURE REVIEW

A. Introduction

Cardiovascular disease is the major cause of death in the Western world, and atherosclerosis is the chief cause. Atherosclerosis is a slowly progressive disease which may begin in childhood and does not fully manifest itself until middle age, or later; therefore it is difficult to isolate the initiating events or mechanisms in the development of atherosclerotic lesions. The research in this field over the past 3 decades has produced volumes of information about the cellular and molecular aspects of the vascular wall, providing numerous insights into the causes of the progression of the disease; however, the processes involved in atherogenesis and the relevance and relative importance of various mechanisms in the initiation and progression of lesions of atherosclerosis are still largely unknown.

The predominant theory explaining lesion progression has been put forth by Ross [1]. This theory states that the lesions are due to proliferation of arterial smooth muscle cells (SMC) and progress to mature fibrous plaques as a result of the arteries' response to some endothelial injury associated with various risk factors. The major biochemical risk factors associated with the development of atherosclerotic lesions are high concentrations of fibrinogen (FGN) and low density lipoprotein (LDL) in the plasma and the arterial wall [2]. Clinical trials have shown that reducing circulating levels of LDL-cholesterol are correlated with a reduction in the incidence of cardiovascular disease[109]. Increased production of connective tissue components, specifically collagen, by the arterial SMC in the intima is the hallmark of developing atherosclerotic lesions [134,135], along with intra- and extracellular accumulations of LDL [228,241,244,245,248,253] and extracellular accumulations of FGN [227-235].

It seems quite logical that for effective prevention of a disease, such as atherosclerosis, one of the basic prerequisites is a knowledge of its cause. It is the processes and mechanisms of the development of the complex lesions of

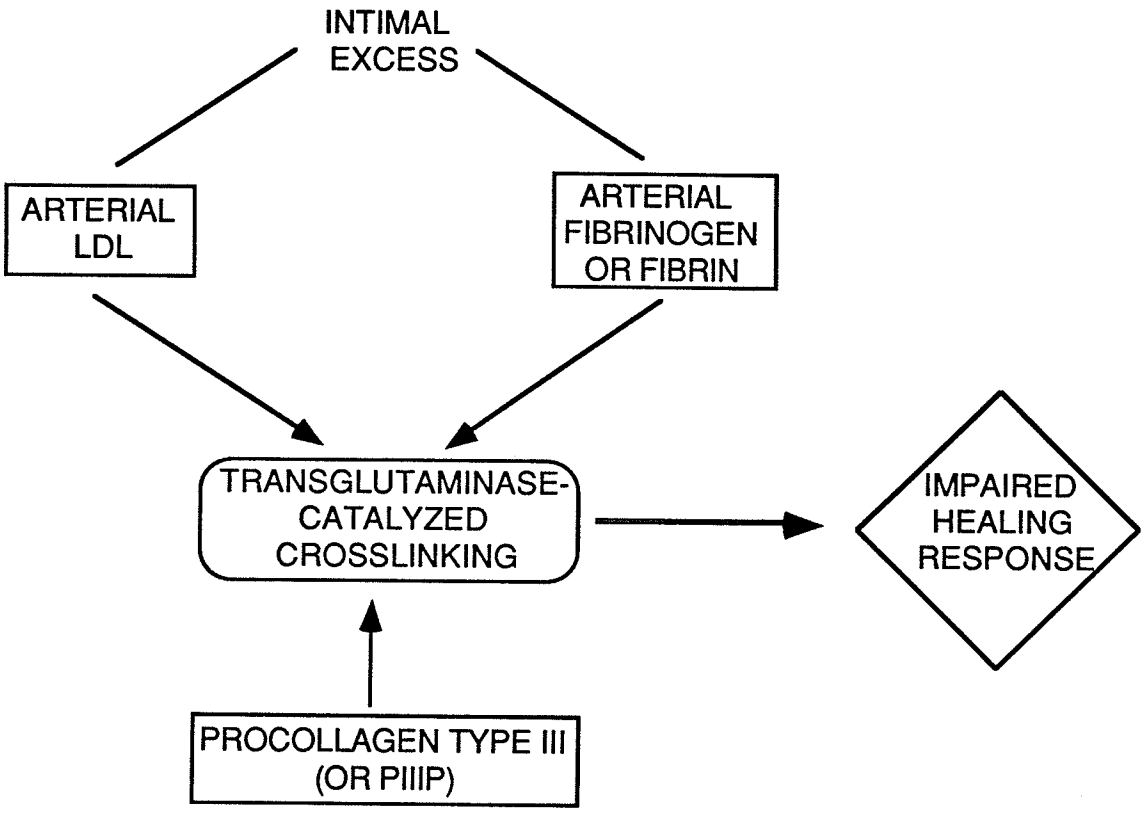


Figure 1. Hypothesis. See discussion in text.

atherosclerosis that are in question. What are the mechanisms by which LDL and FGN are retained within the arterial wall and why are the natural abilities of the artery to clear the intima of these excess plasma proteins impaired? These are the questions that will be addressed in this thesis.

If atherosclerosis is assumed to proceed due to an altered response of the vessel wall to injury, then perhaps connections can be drawn with studies of wounding in skin. Studies have shown that the enzyme transglutaminase (TGase) plays a role in the healing of wounded skin [3,4]. It has also been shown that an early response to wounding of skin is increased production of type III procollagen (PCIII) [163], and that the aminopropeptide of type III procollagen (PIIIP) is a very good substrate for TGase [277]. This thesis will argue that high concentrations of FGN and LDL are bound up in the arterial wall connective tissue matrix to PCIII or PIIIP, produced by proliferating SMC, through a TGase-catalyzed reaction, producing an altered response to injury in the artery (Fig. 1).

B. Structure of the Normal Artery

The arterial wall is not merely a passive tube but a reactive organ. Its major components have a strong structure/function relationship and under normal conditions are in dynamic equilibrium. Any changes in this equilibrium cause remodelling or adaptive changes in the arterial wall. Histologically, the arterial wall has 3 basic layers (Fig. 2) [reviewed in 10]. The innermost layer, adjacent to the arterial lumen is the intima. The intima includes the endothelium, a subendothelial space and the internal elastic lamina (IEL). The media extends from the innermost portion of the IEL to the outermost layer of smooth muscle. The adventitial layer begins at the outer limit of the media and gradually blends with the surrounding tissue.

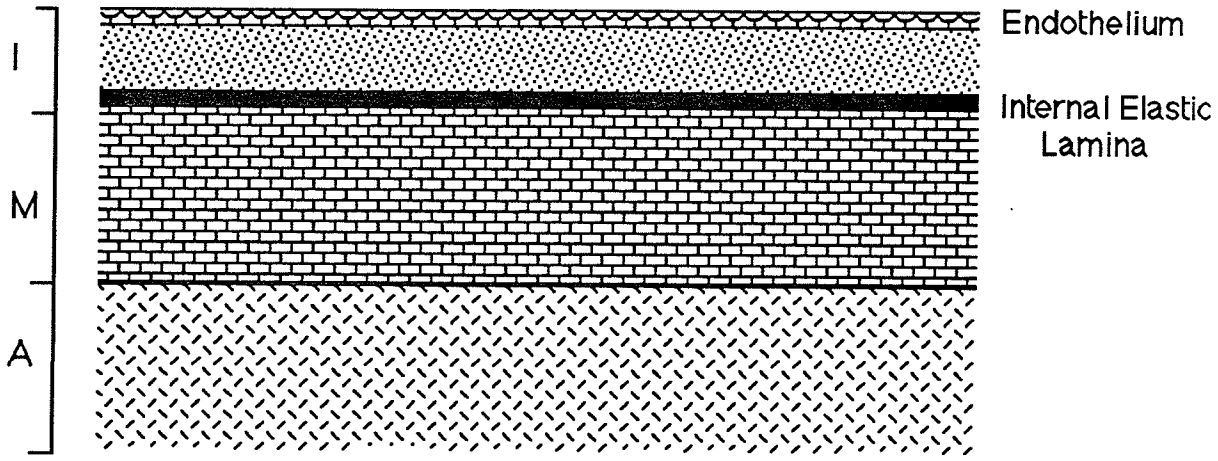


Figure 2. Schematic representation of the normal artery. The normal artery consists of 3 layers: intima (I), media (M) and adventitia (A). The endothelium, a subendothelial space and the internal elastic lamina constitute the intima (see discussion).

INTIMA. The intima is probably the most metabolically active layer in the aorta. The endothelium is a single smooth layer of cells, which was thought for many years to be an impermeable barrier. It is a semi-permeable covering which provides protection for the underlying portions of the vessel wall, maintaining haemostasis. The subendothelium in early life is composed entirely of loose connective tissue, but, with age, medial smooth muscle cells gradually migrate into this region. The IEL is a connective tissue layer, consisting of collagens, proteoglycans and elastin, separating the media from the intima. The IEL serves as a gel-sieve or filter of plasma macromolecules that enter the intima [241], allowing through smaller molecules and only small amounts of large molecules.

MEDIA. The components of this layer are similar in both elastic and muscular arteries. Mammalian arteries contain only SMC in the medial layer. The orientation of the SMC with respect to each other and the artery determine whether the artery is elastic or muscular. Layers of SMC are sandwiched between elastic plates and tissue space containing collagen, constituting the basic structure of elastic arterial media. The connective tissue space between laminae of elastin and their collagen content increase with age.

ADVENTITIA. Most of the arterial adventitia consists of a loose arrangement of collagen, elastic fibres, fibroblasts, blood vessels, lymphatics and nerves. The adventitia is not significantly involved in the process of atherosclerosis and will not be discussed further.

C. Atherosclerosis: A Definition

Atherosclerotic lesions have been observed in humans for centuries [5,6]. Lobstein coined the generic term "arteriosclerosis" to describe scarring and calcification of arteries. In 1904 Marchand first used the term "atherosclerosis" to designate a disease characterized by lipid-rich arterial lesions, intimal thickening, fibrosis and calcification.

Virchow started the debate on the mechanisms of the development of the lesions of atherosclerosis by introducing his imbibition theory in the mid-1800's. He suggested that the atheromatous lesions were due to the imbibition of plasma proteins into the arterial wall. The theories have progressed to the present most widely accepted theory of the response to injury hypothesis [1], which emphasizes the importance of intimal SMC proliferation as the key event in the development of atherosclerotic lesions. Schwartz and colleagues [7] describe atherosclerosis as a "result of a multiplicity of interactive cascades among injurious stimuli and the healing responses of the arterial wall, occurring

concurrently within a hyperlipidemic environment" [p. 23G]. These interactions involve a number of co-factors: the endogenous cells of the arterial wall (endothelial and SMC), elements of the blood (monocytes and platelets), plasma proteins (LDL and FGN) and connective tissue of the arterial wall (collagen, elastin and proteoglycans).

There are, generally, 4 characteristic features of atherosclerotic lesions [reviewed in 1,5-9]: increased infiltration of macrophages/monocytes into the subendothelial space; migration and focal proliferation of arterial SMC in the intima; increased production of extracellular connective tissue elements; and deposition of lipid (from LDL) and FGN/fibrin in the intima accompanied by accumulation of intracellular lipid by intimal macrophages and SMC. Following these conditions, later events include death of intimal SMC and macrophages, calcification, necrosis, ulceration and thrombosis.

D. Natural History of Atherosclerosis

Knowledge about the natural history of atherosclerotic lesions has been difficult to obtain since the lesions often begin in early childhood and progress at varying rates throughout a person's life. The age at which atherogenesis begins also varies among the different arteries, but despite the varying ages of onset the processes of development appear to be the same in all arteries. Most insight into the natural history of these lesions is derived from examination of arteries of persons who have died from causes other than coronary heart disease (CHD) or from experimental animal models. This section will discuss the general progression of the lesions and describe the general morphology of the various lesions.

Diffuse Intimal Thickening (DIT). Also known as the fibromuscuar plaque,this seems to be a part of normal development of the arteries. [10,11] In early life, the intima is composed of lamellar units, as discussed earlier. A musculo-elastic layer, composed of a loose network of elastic fibres and longitudinally oriented SMC, is present in the subendothelial space at birth. Later in life, a second layer, called the elastic-hyperplastic layer, forms medial to the first layer. This layer is composed of elastic fibres, lamellae and a few SMC. These two layers continue to increase in size and by the age of 20 years, the thickness of the intima is about equal to the media. These two layers are joined by a third inner layer, the connective tissue intimal layer, at about the age of 40 years. This connective tissue layer's chief constituents are collagen, glycosaminoglycans (GAG) and some SMC. In animals, such as rat and rabbit, the increased thickness of the intima is due to an increase in the size and number of lamellar units. [10]

These DITs tend to develop in the branching sites of arteries as an adaptive response to blood pressure and vessel size.[12] The DITs seem to be sites of predilection for the formation of atheromatous plaques, but they do not necessarily always form lesions; however, arteries commonly found to be the seat of severe atherosclerosis show prominent degrees of DIT [13].

The majority of the cells in DITs are SMC. Gerrity and co-workers [14] have shown that the SMC in the intima originate in the media and migrate into the subendothelial space. These SMC show higher growth rates and produce more interstitial collagens than those in the media. It is this focal proliferation and synthesis of extracellular matrix that produces the DIT.

Fatty Streak or Dot. These are the first clearly recognizable changes of atherosclerosis to occur. They appear in the intima and inner media as scattered

deposits of lipid in SMC, extracellular spaces and macrophages. These deposits, one type of which is commonly found in children, appear as opaque yellow dots or streaks without staining and are easily visualized with stains such as Sudan IV, and are aptly named fatty streaks (FS) or fatty dots. [15] Many of the SMC and macrophages exist in the FS as cells whose cytoplasms are filled with lipid droplets, mostly cholesteryl esters and free cholesterol. These fat-filled cells are termed macrophage-derived foam cells (MDFC) or SMC-derived foam cells (SMC-FC), because of their appearance when viewed from a microscope.

There are at least 3 different types of FS found in human arteries [reviewed in 15]. The first type occurs largely in childhood and adolescence. It consists of predominantly intracellular lipid, no new connective tissue and no extracellular lipid. The second type is found in young adults and consists of extracellular lipid deposits in areas of few cells, numerous SMC and macrophages and increased extracellular connective tissue. The final type is found mainly in middle aged and elderly persons, consisting of diffuse infiltration of lipid, droplets of plasma material concentrated close to elastic fibres, few cells, no pools of extracellular lipid. It is suggested that the second type of FS may be a precursor to more advanced lesions, but there is no evidence that the third type of lesion undergoes transition.

The cellular events associated with the development of FS have been studied in rabbit [16-19] and non-human primate [20-24] models under various levels of hypercholesterolemia; these yield a similar chain of events. It has been found that the initial events are a special type of inflammatory response. There is monocyte adherence to the endothelium of the arterial wall followed by penetration of the endothelium and migration into the subendothelial space. However, recent evidence suggests that lipid deposition into the subendothelium may occur prior to the adherence and entry of monocytes. [25,26] Monocytes

then differentiate to become macrophages and begin to take up lipid in the form of native or modified LDL, producing the MDFC. The macrophages are joined by migrating SMC from the media and these begin to take up lipid as well. The expanding and maturing FSs increases in thickness and have multiple layers of lipid-laden macrophages and SMC, most commonly by consisting of alternating layers of lipid-laden macrophages and lipid laden SMC sandwiched between layers of macrophages.

There is no hard evidence that the FS progresses to the more advanced fibrous plaque [21]; however, FS are often found at the same sites, in early life, as the SMC-rich fibrous plaques in older persons [27,28]. Also, many lesions in the arteries of young adults are at an intermediate point between FS and fibrous plaques with respect to histologic and biochemical characteristics [28-30].

Gelatinous Lesions. Gelatinous lesions appear visually as translucent, blister-like elevations on the arterial lumen [31-35]. They are loose, focal proliferations of SMC and collagen with high concentrations, about 4 times that in normal arterial wall, of LDL, FGN and other plasma proteins [31-33]. Some gelatinous lesions have large pools of plasma insudate, while other lesions of this type are composed of densely packed fibres and cells. Smith and Staples [33] found that most human gelatinous lesions have high levels of fibrin/FGN and LDL in lipid-rich areas. The lipid in these areas can only be released by incubation with proteases, such as plasmin. Antibody studies have revealed that the FGN/fibrin in gelatinous lesions consists of very fine, diffusely distributed material within the altered intima [36]. Haust [34] has shown that the SMC in the altered intima are not altered from those in the normal intima. There also appears to be a preferential retention of certain proteins in the arterial wall, since proteins such as prothrombin, antithrombin, α_2 macroglobulin, albumin, LDL and FGN are

distributed differently in the gelatinous lesion [33]. Despite the variety of morphologies among individual lesions, they all possess focal edema in the subendothelial area and some necrotic material, suggesting that these lesions may be precursors to the more complicated lesions of atherosclerosis [31-35].

Fibrous Plaques. The fibrous plaques are raised, somewhat rounded lesions with a pale-gray or off-white color and are sometimes called atheromatous or fibrofatty lesions. The plaques exhibit histologic variability among individual plaques, but a typical plaque consists of a fibrous cap, a deeper cell-rich area and a necrotic core (Fig. 3) [37,38]. The fibrous plaque is composed mainly of multiple layers of a special type of SMC, different than that found in normal intima or media, and a few leukocytes and macrophages in dense connective tissue, composed of elastin, collagens and proteoglycans (PG). Underneath the fibrous cap is a cellular area, consisting of a mixture of SMC, macrophages and perhaps a few leukocytes, all surrounded by connective tissue. These cells may contain lipid droplets in their cytoplasms. The deepest part of the lesion is a necrotic core, consisting of necrotic cellular debris, cholesterol crystals, extracellular lipid deposits and calcification.

It is of little doubt that the further development of these lesions leads to the clinical manifestation of the disease. The lesion continues to grow and may rupture the endothelial cell covering, exposing the subendothelium, which may cause thrombosis and occlusion of the artery.

E. Cell Biology of the Arterial Wall

There are essentially 3 different cell types in the arterial wall that are associated with the development of atherosclerosis: endothelial cells, smooth muscle cells and monocytes and macrophages . Macrophages are not original

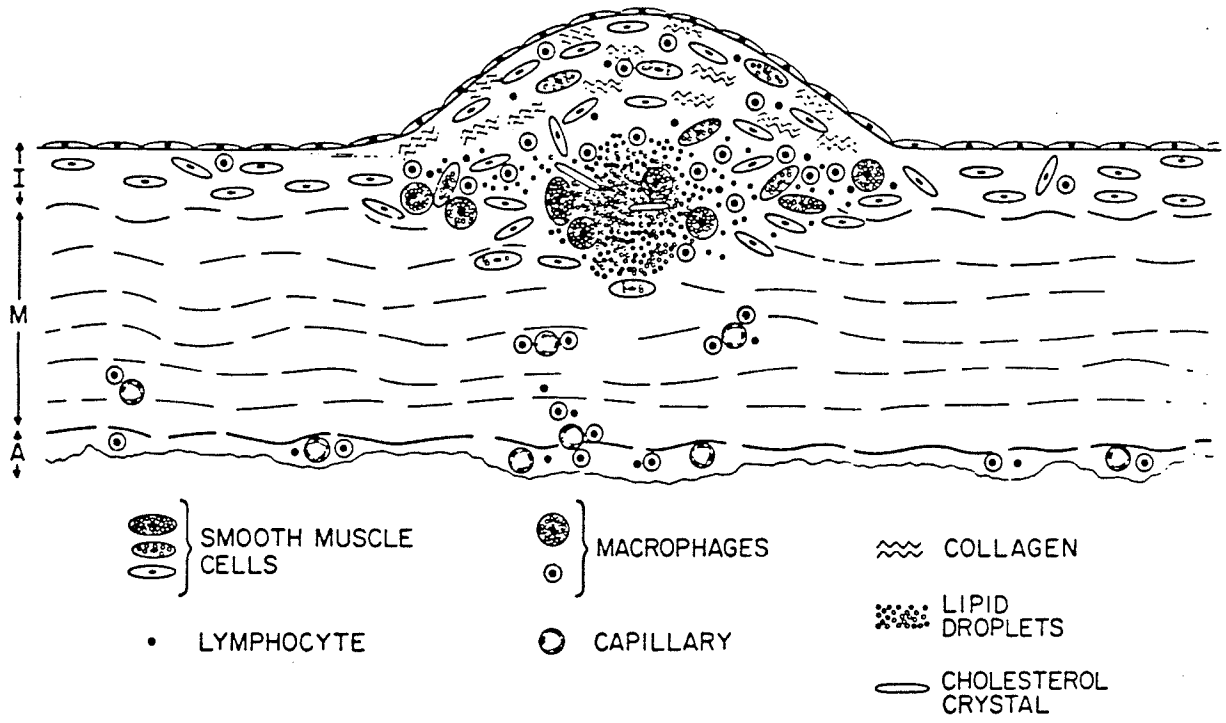


Figure 3. Schematic representation of a cross-section through a fibrous plaque (I, intima; M, media; A adventitia, medial SMC not drawn in). The endothelium in this example is intact. The fibrous cap contains SMC and collagenous bands over a core region abundant in extracellular lipid and foam cells. Adventitial capillaries are gravitating to the core. [from reference 8]

cells of the arterial wall; however they migrate into the arterial intima and soon become an integral part of the development of atheromatous plaque. The key cell type in the development of the lesions of atherosclerosis is the arterial SMC [1] and this will be discussed more than the others.

1. Endothelial Cells

The arterial endothelium is a single layer of polygonal cells lining the entire length of the lumen of blood vessels. Recent research on vascular endothelium has established endothelial cells as active cells which perform a variety of critical functions, including modulating permeability characteristics of tissues, maintaining haemostasis and thromboresistance, synthesizing and metabolizing a variety of biologically active molecules and regulating vascular tone, inflammatory and immune functions and vascular growth [39-41]. Endothelium responds to various stimuli by altering function, metabolism and structure, directly influencing the response to injurious stimuli. The endothelial cells also act to influence both macrophages and SMC by producing vasoactive agents [42,43], growth factors [44-46,50] and growth inhibitors [47,48]. These actions and the regulation of thrombosis are the functions most implicated in the development of the atheromatous plaque and will be discussed in this section.

Most studies on endothelial cells are performed on cultured cells, providing a somewhat limited model of endothelial response to stimuli. Cultured confluent endothelial cells grow in an obligate monolayer, suggesting the importance of cell-cell interaction [53]. Human endothelial cells *in vivo* usually turnover at relatively low rates [51,52]., while cultured endothelial cells have a rate of turnover which is more closely associated with a state of injury *in vivo* than a state of quiescence [52]; therefore, cells grown on plastic, in culture, may be in an abnormal state, or injured state [46,50]. DNA hybridization studies have

shown that cultured bovine aortic and human umbilical-vein endothelial cells have 83x and 10x more mRNA for platelet-derived growth factor (PDGF) than cells *in vivo* [50]. The cultured cells produced PDGF on a continuous basis, while in vivo, cells produce low levels of PDGF [46]. PDGF is a cationic protein (28kD-32kD) which is also stored in the alpha granules of platelets and released upon activation of the platelets [reviewed in 1]. It binds with high affinity to SMC, fibroblasts and other cells derived from mesenchyme, but not endothelial cells. This growth factor is unique amongst other growth factors in that it is not only a mitogen but also a chemoattractant. This property could allow PDGF to attract SMC from the media into the intima.

There may be a link between the release of mitogens such as PDGF by the endothelium and the coagulation pathway. The exposure of endothelial cells *in vitro* to activated Factor X, of the coagulation cascade, or γ-thrombin induces the release of increased amounts of PDGF [54]. This suggests that a perturbation of the endothelium by activation of the coagulation cascade may cause the release of mitogens into the subendothelium.

As mentioned, the endothelium modulates haemostasis and thrombosis. Under conditions of stress, thrombin is generated through the coagulation cascade. The anti-coagulant activity of the endothelium is initiated by the binding of thrombin to the membrane receptor, thrombomodulin, present in unstimulated endothelial cells [55,56]. The binding to thrombomodulin activates Protein C,and in the presence of Protein S, degrades Factors V and VIII, limiting coagulation [57,58]. Activated Protein C also enhances fibrinolytic activity and internalization and subsequent neutralization of thrombin [59]. Fibrinolysis is the ultimate defense mechanism against excessive fibrin formation. The reaction is triggered by the release of plasminogen activators which catalyze the formation of plasmin, a potent serine protease which catalyzes the lysis of fibrin [60].

Another important mitogen produced by endothelial cells is prostacyclin (PGI_2). PGI_2 inhibits platelet aggregation by stimulating adenylate cyclase activity, increasing cAMP levels in platelets [61]. PGI_2 allows platelets to stick to the arterial wall and interact with it while still limiting thrombus formation, since it inhibits platelet aggregation at lower levels than levels needed to inhibit platelet adhesion [62]. Incubation of endothelial cells with high LDL concentrations causes a decrease of PGI_2 production by the cells and incubation with atherogenic LDL also influences PGI_2 synthesis [63]. Also, PGI_2 was less effective in inhibiting platelet aggregation in the presence of increased LDL [64].

Endothelial cells also produce connective tissue components, such as procollagens, GAGs, and fibronectin. In arteries denuded by balloon catheter, SMC proliferation extends only to the region to be recovered by endothelium last, suggesting the endothelium secretes an inhibitor of SMC proliferation into the subendothelium [48]. Cultured endothelial cells produce a heparin-like GAG which inhibits SMC growth.

2. Smooth Muscle Cells

Inflammation and wound healing outside of the artery is often associated with the proliferation of fibroblasts, which deposit collagen and other substances necessary for the remodelling of the tissue. In the intima of the artery, it is the SMC which serve this role. SMC have long been recognized as "multifunctional mesenchyme cells" [89]. SMC are the only cell type present in the media of the mammalian artery and are responsible for maintaining tension, via contraction-relaxation, and arterial integrity, by proliferation and synthesis of extracellular matrix. SMC proliferation is the key event which determines how extensive an atheromatous plaque becomes and whether there are clinical consequences [1]. As mentioned, SMC are found in DIT, fatty streaks and gelatinous lesions. SMC

are the predominant cell type in the fibrous plaques and control the further development of these lesions. SMC in the arterial intima not only possess the ability to proliferate, but can also produce large amounts of connective tissue [65-67] and can accumulate lipid and form foam cells. SMC contain LDL receptors [68,69] and receptors for growth factors, such as PDGF [70] and TGF-β [71].

Early studies have shown that in response to balloon catheter denuding of aortic endothelium there is early proliferation of SMC in the media, followed by migration into the intima and further proliferation [72-74]. The cells that migrate and are highly proliferative contain large amounts of rough endoplasmic reticulum (RER) free ribosomes and mitochondria up to 3 weeks post injury [75,76]. When the endothelial layer is reestablished, the SMC of the neointima appear to be normal; ^{3}H-thymidine levels, in labelled SMC, reach a maximum level shortly after denudation and return to baseline several weeks later.

Several studies have shown that SMC exist in at least two different phenotypes, a "contractile" phenotype at one extreme and a "synthetic" phenotype at the other [77-80]. In fact Campbell and Campbell [80] suggest that SMC exist in a spectrum of phenotypes in the arterial wall, since Bjorkerud and Ekroth [81] found 2 distinct subpopulations of cells in cultures of SMC from human arteries: a group of low adhesive cells and a group of high adhesive cells. They suggested these high adhesive cells were involved in the repair and remodelling of the artery. There is a gradient in cell morphology from normal SMC of the media to cells with greater differentiation closer to the site of injury. The cells at the site of injury undergo mitosis at a much higher rate than those in the media. It appears that this change of phenotype from contractile to synthetic is associated with some type of injury. Macrophages, also associated with the process of wound healing and atherogenesis, have been shown to stimulate

increased proliferation of contractile SMC in culture and stimulate a change to synthetic phenotype [88].

These two distinct phenotypes have been studied extensively and express functional, biochemical and cytoskeletal differences. Differences are evident between the SMC of normal media and those of the atheromatous plaques and the DITs in regions of atherosclerotic plaques [80,82,83]. These differences have been studied and documented [77-80]. It is known that SMC in culture remain in a contractile phenotype if the cells are seeded in such a high concentration that they reach confluency by day 1; however, once the cells undergo at least 5 cell doublings, they change, irreversibly, to a synthetic phenotype. The contractile phenotype is not induced to proliferate in response to serum factors. One of the first observations of the two phenotypes was the large amounts of RER, free ribosomes and Golgi apparatus present in synthetic cells. The synthetic SMC also have a lower volume fraction of myofilaments (low $v_V$myo), compared to contractile cells (high $v_V$myo). There are also changes in actin, vimentin and desmin content per cell and a switch from α-actin to β-actin predominance associated with the change from contractile to synthetic [79,84]. Labelling cells of both phenotypes with ^{3}H-proline shows a 400% increase in collagen labelling in the synthetic phenotype as compared to the contractile phenotype [77]. The synthetic SMC produce up to 30x more collagen than the contractile in culture [85]. This is to be expected, since synthetic cells have more synthetic organelles than contractile and less structural proteins. The two phenotypes also differ in their ability to degrade lipid. Campbell and co-workers [86] have shown that ^{125}I-LDL is degraded by synthetic SMC at one-fifth the rate of contractile in cultured pig and rabbit aortic SMC cells. Synthetic phenotype cells accumulate 7x more lipid than contractile cells in the presence of elevated β-VLDL (β-migrating very low density lipoprotein) [86,87]. These differences are

due to the increase of the specific activity of acid cholesteryl esterase and acyl-CoA cholesterol acyltransferase upon phenotypic change to synthetic SMC.

Ross [1] implicates the activity of growth factors and other mitogens as the major cause of SMC migration and proliferation in the arterial wall. The major mitogen implicated is PDGF from endothelial cells, macrophages and SMC themselves [90-93]. Hosang and Rouge [95] have recently shown that not only do SMC possess PDGF receptors, but they express at least 2 different receptors for PDGF and they secrete a form of PDGF. PDGF's effect on SMC includes stimulation of DNA synthesis and proliferation [90,91,97,98] and chemotaxis [93,96,98,99]. Other factors thought to be involved in the development of atherosclerosis are TGF-β and endothelin [97,100-107]. TGF-β is released by activated platelets, macrophages and endothelial cells [101-103] and causes an increase in the synthesis and secretion of connective tissue by SMC [104]. TGF-β is also chemotactic for SMC ,but TGF-β inhibits migration of cultured rat aortic SMC in the presence of PDGF [104]. Endothelin is a mitogen for SMC, secreted by the endothelium, which stimulates the proliferation of SMC [97,106,107].

3. Monocytes and Macrophages

Experimental evidence, in animal models [16-24], shows that one of earliest visible cellular interactions in the development of atherosclerosis is the attachment of monocytes, from the plasma, to endothelial cells. Peripheral blood monocytes have been shown to preferentially adhere to injured or regenerating endothelial cells [110,121]. Hansson and co-workers [112] have shown that IgG binds to endothelium in areas with a predilection to the development of atheromatous plaques. The presence of IgG on the surface of the endothelium promotes the recruitment and subsequent activation and differentiation of blood monocytes. Another possible mechanism of recruitment of monocytes to the

subendothelium is the presence of chemotactic agents, such as a peptide produced by SMC and endothelial cells [116,119]. SMC and endothelial cells secrete a peptide into the culture medium that is chemotactic to macrophages Modified LDL particles, which accumulate in the arterial wall, have also been shown to be chemotactic for macrophages [117].

In wound healing, the macrophage is believed to behave not only as a scavenger of dead material but also as the cell which, by its secretions, stimulates the ingrowth of fibroblasts and endothelial sprouts for repair of the wound [113]. Leibovich and Ross [114] have shown that the inhibition of macrophages in wound repair causes a decrease in the proliferation of fibroblasts and synthesis of connective tissue. Activated macrophages secrete mitogens and chemotactic substances for fibroblasts, endothelial cells and SMC, including PDGF, TNF and TGF-β; they also secrete enzymes and proteins such as collagenases, elastases, lipases, fibronectin (FN) and PG [108,115]. Macrophages, through secretion of various toxic substances, such as superoxide anions, lysosomal hydrolases and oxidized lipids, are able to injure neighboring cells. The actions of macrophages in the process of atherosclerosis are farther reaching than simply scavenging excess LDL and FGN from the arterial wall.

As monocytes infiltrate into the arterial intima, they almost immediately differentiate into macrophages. The most prominent abnormal structures in the earliest lesion are the large numbers of lipid-filled macrophages. The lipid-filled macrophages, or MDFC, are formed by ingestion of excessive amounts of native or modified LDL that infiltrate the arterial wall. Some monocytes from early lipid-rich lesions migrate back into the blood stream, suggesting that migration into the subendothelial space and ingestion of excess macromolecules is a normal response [118]. Lesions in which cell immigration equals cell emigration appear to be stable and progress no further. In hypercholesterolemic monkeys, pigs and

rabbits, it is the macrophages' normal function as scavengers that contributes to the development of the early lesions of atherosclerosis [120].

Macrophages can accumulate lipids by several mechanisms [125]. Macrophages can internalize lipoproteins via receptors for both native and modified LDL [122]. The receptor for native LDL is down regulated by cellular cholesterol levels and is, therefore, not responsible for the excess lipid accumulation associated with FC formation. The receptor for modified LDL, commonly called the scavenger receptor, is not down regulated and has been characterized [123,124]. Cross competition studies suggest that macrophages express several scavenger receptors [126,127]. The scavenger receptor recognizes several lipoproteins, including β-VLDL,normal VLDL and chylomicron remnants, to produce lipid accumulation in macrophages. Lipoproteins can also aggregate with other lipoprotein particles or interact with plaque components (for example, connective tissue components) and be internalized by phagocytosis. Unesterified cholesterol can associate with molecules that are ligands for receptors expressed by macrophages and be internalized. Cholesteryl ester droplets derived from lysed FC or denatured LDL are internalized by phagocytosis.

Monocytes respond to a number of endogenous chemotactic substances including collagen and its constituent chains, fibronectin and elastin-derived peptides [128-130]. It is suggested that macrophages slide along collagen fibrils, using their scavenger receptors to take up any lipoproteins that stick to the collagen. They also produce leukotriene-β4, the most potent chemoattractant known, as a product of the lipoxygenase system.

F. Biochemical Components of the Atherosclerotic Lesion

There are many factors associated with the development of the atherosclerotic lesion. The cellular and morphological characteristics have been discussed. To understand the mechanisms of atherogenesis one must look more closely at the biochemical aspects of the disease. The key players in atherogenesis are the connective tissue components, FGN and lipoproteins. TGase is also reported to play a role [232,268,269].

1. Connective Tissue

Associated with the proliferation of SMC in the arterial intima is the increased synthesis of extracellular matrix, most notably procollagens. The extracellular matrix of the arterial wall is made up mostly of collagen, elastin, PG and structural glycoproteins. This section will discuss each of these, with special attention to collagen, and their putative role in atherogenesis.

COLLAGEN. Collagen, the major constituent of the arterial extracellular matrix, has been studied extensively and recently, has been thoroughly reviewed [131-133]. There are presently at least 14 different types of collagen that have been identified. The separation of the various types of collagen has depended on the physical-chemical properties, the differential solubility in neutral salt solutions and dilute acid, the susceptibility to proteolytic enzymes and the structural differences of the various collagen types. A recent partial classification of the types of collagen can be found in Table 1.

Collagens are distinguished from other proteins by their relative resistance to most proteases, their susceptibility to the collagenases and by the characteristic structural repeating sequence Gly-X-Y, where proline and hydroxyproline fill X and Y frequently. The sequence Gly-Pro-(OH)Pro occurs in

10% of the molecular sequence. Collagen comprises 20% of the dry weight of large arteries and up to 50% dry weight in smaller vessels. The amount of collagen in the arterial intima increases over 10x during atherogenesis.

The collagens of the arterial wall have been studied and reviewed recently [134,135]. This discussion will be limited to the more abundant collagen types in the arterial wall, types I and III.

The fibrous collagen molecule (types I-III) consists of a rigid triple helix, formed from 3 left-handed helical chains. The molecule, therefore, is a hybrid of two like chains and one different chain or, three chains which are identical. These individual chains are termed α-chains (see Table 1). Several years ago, it was shown that there are precursors to the mature collagen molecule called procollagens, which could be separated from collagen and isolated by precipitating with ammonium sulphate, then ethanol, followed by elution from DEAE-cellulose by an NaCl gradient in 2M urea [136]. Other separation techniques exploit the differential solubility in neutral salt solutions and weak acetic acid, the susceptibility to proteolytic cleavage and the structural differences of various collagens.

The ability to separate and isolate the different collagen types, has allowed the study of the biosynthesis of the collagens. The steps in the biosynthesis of collagen have been extensively reviewed [131-133,137]. The biosynthesis is begun in the same manner as most other secretory proteins [138], with an hydrophobic signal peptide which is fed through the RER membrane and is subsequently cleaved by a signal peptidase.

As translation continues, and peptide appears in the lumen of the RER, hydroxylation of certain proline and lysine residues takes place. These hydroxylations are catalyzed by three separate enzymes, prolyl 4-hydroxylase,

Table 1. Genetically Distinct Collagens*

Type I	$[\alpha1(I)]_2\alpha2(I)$ $[\alpha1(I)]_3$	Most tissues except cartilage; major component of bone, tendon, skin and dentin.
Type II	$[\alpha1(II)]_3$	Cartilaginous tissue.
Type III	$[\alpha1(III)]_3$	Found together with type I; relatively high amounts in blood vessels, skin and gut.
Type IV	$[\alpha1(IV)]_2\alpha2(IV)$	Major component of basement membrane.
Type V	$[\alpha1(V)]_2\alpha2(V)$ $[\alpha1(V)]_3$ $[\alpha3(V)]_3$ $\alpha1(V)\alpha2(V)\alpha3(V)$	Minor component of most tissues except cartilage.
Type VI	$[\alpha1(IV)]_2\alpha2(VI)$	Minor component in most tissues.
Type VII	$[\alpha1(VII)]_3$	Amniotic membrane and skin; associated with all stratified epithelia.
Type VIII	$[\alpha1(VIII)]_3$	Variety of cells, especially endothelial cells.
Type IX	$\alpha1(IX)\alpha2(IX)\alpha3(IX)$	Cartilaginous tissues.
Type X		Cartilage hypertrophic cells.

*[from reference 242]

prolyl 3-hydroxylase and lysyl hydroxylase, producing 4-hydroxyproline, 3-hydroxyproline and hydroxylysine almost exclusively in collagen molecules [139,140]. These enzymes require ascorbic acid, α-ketoglutarate, and ferrous ion , as well as a specific amino acid sequence to catalyse the reactions. Triple-helical formation prevents the hydroxylation reactions from occuring. The function of the 4-hydroxyproline residues appears to be to stabilize the collagen triple helix under physiologic conditions. The role of the 3-hydroxyproline residues is uncertain. The function of the hydroxylysine residues is two-fold, they act as sites of attachment for carbohydrate units and stabilize the intermolecular collagen crosslinks [reviewed in 133,148].

Once the hydroxylysine residues are formed, further modifications of the collagen peptide chains can occur through the action of two specific glycosyltransferases, located in the RER and the smooth ER of the cell [141]. The propeptides of the procollagen molecules contain asparagine-linked carbohydrate units [142].

The propeptides of types I and III procollagens contain intra-chain disulfide bonds. The aminopropeptide of type III procollagen (PIIIP) and its C-terminal propeptide differ from those of other collagens, in that they have inter-chain disulfide linkages, as well as intra-chain linkages [143-146]. The triple-helix can form once the C-terminal propeptide has been translated, with the inter-chain disulfide bonds in the C-terminus most likely providing the nucleation sites for helix formation [133,147].

Once secreted into the extracellular space, the procollagen molecules are converted proteolytically to collagen, and the collagen molecules assemble into fibres in the matrix [133]. The conversion is catalyzed by two types of enzyme, procollagen N-proteinase and procollagen C-proteinase. The actual number of procollagen proteinases is large, since the N-proteinase is collagen type-

specific. This is obvious from the difference of isolated aminopropeptides from type I and III collagens [144,149]. There appears to be no obligatory sequence for the removal of the two propeptides of type I collagen [150,155]. However, the N-terminal propeptides of type III procollagen are removed very slowly [151,152,154,155]. In fact, many tissues contain significant amounts of partially processed PCIII with a complete N-terminal propeptide and no C-terminal propeptide [150,152,154,155]. The PIIIP molecules were found to be bound to the collagen fibrils, suggesting this peptide may regulate the growth of the fibril by steric hinderance. PIIIP may act as a linkage between fully mature collagen fibrils and other components of the ECM. Wiestner and colleagues [153] have also determined that the presence of free aminopropeptides of both PCI and PCIII specifically inhibit collagen biosynthesis in cultured fibroblasts, suggesting a feedback inhibition mechanism for collagen biosynthesis.

The ability to isolate specific types of collagen and establish the mechanisms of the processing of collagens, has allowed an approach to the characterization, localization and metabolism of each type. This work, to date is not complete; however, the amino acid sequences and structures are known for PCI and PCIII [144,156].

Changes in the ratio of synthesis of types I and III collagens occur during changes of metabolic activity in tissues during embryonic development, wound healing and fibrotic diseases [163]. There are some alterations in collagen metabolism in atherosclerosis demonstrated by changes in the proportion of type I:type III collagen synthesis [157-162]. The proportions of type I and type III collagen are well defined in the normal human aorta: 60% type I and 30% type III [158-160]; however, the ratios present in the human atherosclerotic aorta are not well established. There appear to be variances in the proportions of collagen types as the plaque ages. Some authors [159,161,162] have found increased

type III collagen levels in recent developing atherosclerotic plaques. The deposition of increasing amounts of type I collagen seems to be associated with with the later stage of the disease [162].

The collagens of the blood vessels are largely insoluble in neutral salt or weak acid solutions. Limited pepsin digestion is usually used to solibulize a proportion of the total collagen in tissues [157,159]. This suggested a large proportion of the collagen in the arterial wall was type III collagen; however, type III collagen appears to be preferentially digested by pepsin [160]. A more accepted method of extraction to determine relative proportions of collagens is to fractionate and quantitate characteristic CNBr peptides of the α1(I) and α1(III) chains [159]. A third method used for quantitation of collagen in tissues is by immunofluorescence [152].

PROTEOGLYCANS. Most studies on proteoglycan (PG) structure have been done for cartilage; however, PG structures from other tissues are very similar [164]. The structure of PG is described as a "bottle brush" configuration that can reach MWs of 10^6, or more. Each PG may be made up of 50 or more glycosaminoglycan chains (GAG) attached to a protein core, forming a PG monomer. The GAG consists of an hexosamine, glucosamine or galactosamine, alternating with another sugar, glucuronic acid, iduronic acid or galactose. The GAG may vary in size and sulfate content within a single PG unit, producing a high degree of heterogeneity.

PG are only minor components of the vascular tissue but are important in maintaining structural integrity of the vessel wall and influencing collagen fibrillogenesis, blood coagulation, platelet aggregation, binding of lipoprotein lipase (LPL) and regulating fluid and electrolyte balance in the arterial wall. The PG of the arterial wall and their function have been extensively reviewed [165-

167,169,179]. A brief review of the characteristics of the arterial PG and their putative role in atherogenesis will be discussed here.

PG are found mostly in the subendothelial space and make up part of the IEL. PG are present in the gel-sieve matrix in the subendothelial region of the vessel wall, affecting the passage of macromolecules from the plasma. PG capacity for hydrophilic trapping, charge characteristics and interaction with collagen,elastin and fibronectin (FN) allows the molecules to form an effective gel-sieve environment in the subendothelium.

GAG have been isolated after extensive degradation of the tissue. There are known to be 7 types of GAG in mammalian tissues [164], but there are only two known to exist in the bovine and human aorta: a chondroitin sulfate (CS)/ dermatan sulfate (DS) hybrid (DS-CS) and heparan sulfate (HS) [165-167]. Endothelial cells are known to synthesize and secrete both HS and DS PG. They secrete at least 3 species of DS, including a high MW DSPG that can be covalently crosslinked to FN by a tissue TGase-catalyzed reaction [176]. SMC synthesize and secrete CSPG and DSPG along with some HSPG. The synthesis of CSPG by endothelial cells and SMC is known to be stimulated by macrophage-derived TGF-β [166] and incubation with LDL [180].

Generally, with the possible exception of hyaluronic acid (HA), connective tissue GAG occur in the native state covalently bound to a protein core to form the PG monomer. CS, HS and heparin are linked to protein by O-glycosidic bonds between the D-xylose and the hydroxyl group of serine. DS are linked to the protein backbone through xylose-lysyl linkages. The CS-DS PG are present in greater amounts and can be extracted from tissue with dissociative solvents, such as 4 M guanidine-HCl or $MgCl_2$ [170]. HS is not as easily extracted by dissociative solvents, with only 10% of the total amount extracted by this method; the remaining HS is firmly bound to elastic tissue and must be isolated

by elastase digestion. The only difficulty of elastase digestion is that the protease is nonspecific and can degrade the PG protein core. HS has been isolated by sequential extraction of bovine aorta by NaCl, followed by digestion with collagenase, then elastase, resulting in a PG with MW of 300 000 containing 19% protein and 18% uronate [168].

The molecular sieving capabilities of PG allows selective retention of macromolecules and cations, such as apo-B-containing lipoproteins, FGN lipid and calcium, all implicated in atherogenesis [165-167]. Recent studies have demonstrated that there are modifications of content, composition and molecular size of PG occuring in the lesions of atherosclerosis [171-175]. Some studies have shown that plaques in human arteries contain less uronic acid than normal areas, suggesting a total decrease in the amount of PG associated with lesion progression [172,175]. Cherchi et al [175] found that there is a relative increase in the GAG DS and, especially, HS in the lesioned areas of human arteries compared to normal regions. They also showed that there is an increased resistance to extraction, suggesting that increased interaction with other components of the arterial wall occur in the development of atherosclerosis.

PG modifications occurring in atherosclerotic regions increase interactions with LDL, emphasizing a role for LDL-PG interactions in atherosclerosis [176-178]. Papain digests of PG from atherosclerotic and normal regions of human arteries showed that components from atheroslcerotic samples react more strongly with LDL than those from normal areas of the same artery. GAG form both soluble and insoluble complexes selectively with plasma apo-B-containing lipoproteins [165-167,181]. LDL degradation by mouse peritoneal macrophages is increased several fold compared with controls when cells were incubated with LDL complexed with CS-DS PG aggregates from bovine aorta [181]. In humans, early lesions were found to contain lipoprotein-CS complexes

and peptides derived from collagen and elastin, while the complexes of later lesions were CS-HA [176-178]. As well, Falcone et al [182] showed that insoluble complexes of LDL, heparin, FGN and collagen were taken up more rapidly and to a greater extent by macrophages than normal LDL.

ELASTIN. Elastic fibres have been recognized as an histological entity for at least a century. Elastic fibres are composed of two morphologic components, a central amorphous material composed of elastin and microfibrils composed of a structural glycoprotein [188]. The primary material of elastic fibres is elastin. These elastic fibres are associated with collagenous material, in most tissues.

In the human body elastin is largely an insoluble protein, causing difficulties in its isolation and characterization. Early extraction techniques involved vigorous extraction and denaturation with hot alkali. The discovery of the more soluble precursor, tropoelastin, allowed the chemical nature of elastin to be studied [183]. Tropoelastin is a monomer,when secreted from the arterial SMC, with a MW of 70 000. It is soluble in alcohols and spontaneously precipitates when warmed, in aqueous solutions, to temperatures greater than 15°C. Elastin has an extremely high content of small hydrophobic amino acids, such as glycine, alanine and valine, with no methionine, tryptophan or histidine [184].

The tropoelastin molecule is rapidly crosslinked into insoluble polymers. The characteristic crosslink in the elastic fibres is called desmosine. The desmosines are adducts of four adjacent, but not contiguous, lysyl residues on two tropoelastin chains [188]. The synthesis of elastin can be quantitated by measuring the amount of incorporation of ^{3}H-lysine into the desmosines. During translation,certain lysyl and prolyl residues are hydroxylated by lysyl hydroxylase and prolyl hydroxylase, respectively. Crosslink formation is mediated by a

copper-dependent enzyme, lysyl oxidase, through the oxidative deamination of lysyl residues, since the crosslinking of elastin and collagens are inhibited by β-aminopropionitrile, a potent inhibitor of lysyl oxidase. Elastin then assembles into elastic fibres upon the binding of a structural glycoprotein (microfibrils) to the elastin polymer surface [185,186]. These microfibrillar structural glycoproteins serve as a nucleation site for condensation of tropoelastin molecules into mature elastic fibres.

Elastins have been shown to be integral in maintaining the integrity and function of blood vessels [187]. The fibres provide resilience and elasticity to many tissues, including the vessel wall.

Although elastin synthesis can be stimulated by injury in adult tissues, elastic tissues are not well equipped to undergo regenerative process [189,190]. In atherosclerotic lesions, there are lower amounts of elastin than in normal aortic tissue [191,192]. This decrease in elastin could be due to the increase in degradative enzymes, such as elastase, in the arterial wall as lesions progress [192,194]. There is an increased deposition of microfibrils with little or no elastin by SMC in atherosclerotic lesions. The elastin that is secreted is abnormal [191,193]. Kramsch and Hollander [193] found that atherosclerotic elastin had an increased amount of polar amino acids, as compared to normal. This is thought to be one of the factors contributing to the increased interaction of LDL and elastin extracted from atherosclerotic arteries [193,195] and the association of lipid droplets with elastin in electronmicrographs of lesions [196]. Also, large amounts of lipid-protein complexes containing apo-B can be released from atherosclerotic tissue after elastase treatment [197]. The role of these interactions in the progression of atherosclerosis is unsure, since LDL may not migrate far enough into the arterial wall to react with much elastin, due to the gel-sieve matrix of the IEL.

STRUCTURAL GLYCOPROTEINS. In the early sixties, it became clear that besides the preceding 3 constituents of the connective tissue matrix, there were locally synthesized constituents which did not fit in any of these categories of macromolecules. Since these glycoproteins appeared to play a structural role in the matrix, they were termed 'structural glycoproteins' (SG). The microfibrillar component of elastic tissues was one of the first SGs to be recognized. Most SG were thought to play a crucial role in cell-matrix interactions. The best understood example of an SG is fibronectin (FN). SG, specifically FN, have been extensively reviewed recently [198-201]; therefore the characteristics of FN will be only briefly discussed, along with its putative role in the progression of atherosclerosis.

FN is a high MW glycoprotein present in various body fluids and widely distributed in tissues. The structure of FN is known both from protein chemical studies and from gene sequencing [201,202]. This revealed the presence of several isoforms recognized by specific monoclonal antibodies.

FN was shown to interact with specific cell membrane receptors, called integrins, through a limited amino acid sequence: Arg-Gly-Asp, or more commonly known as R-G-D [201]. These adhesion receptors have been shown to be involved in several important recognition interactions, such as those between platelets and FGN, leukocytes or endothelial cells and thrombin, collagen and other agonists [203,204].

FN is known to specifically bind to matrix and cell surface molecules such as collagen, HS, HA and gangliosides [212-214]. It is also a substrate of plasma TGase (Factor XIIIa) [215] and tissue TGase [216].

FN interacts with several components of the hemostatic process. Plasma FN can be crosslinked by Factor XIIIa to the α-chain of fibrin during wound

healing [217]. The TGase activity can also crosslink FN to collagen, especially type III [223]. The crosslinked FN mediates cell adhesion to the fibrin [218]. FN is also thought to act as an opsonin for macrophages by aiding in the clearance of cellular and bacterial debris from the wound site [219]. FN can interact with the previously mentioned components and can, through its interaction with FN receptors on macrophages, clear debris through the mononuclear phagocytic system. FN is primarily associated with type III collagen in granulation tissue [220]. this is expected since, of all collagens, FN has the strongest interaction with type III collagen in vitro [221]. FN is secreted by and expressed on the surface of activated platelets, enhancing the spreading of platelets on collagen surfaces [222].

FN is synthesized and secreted by several types of cultured cells, most notably by fibroblasts, endothelial and SMC, as well as by macrophages [199-202]. FN is also especially abundant in the matrix of these cells as they occur in the arterial wall in vivo [205-208]. Radiolabelled FN has been detected in the early atheroma in vivo [209] and in the progressing lesions [208,210,211]. FN was localized in the arterial wall, by immunofluorescence, to be not in the matrix but immediately circling SMC [211]. Approximately equal amounts of soluble and FN covalently-bound to collagen are found in the normal intima and the developing plaques; however, concentration of collagen-bound FN in fibrous plaques is 2x less than that found in normal intima and developing lesions [210]. The presence of FN in the early lesions and in the cellular regions of fibrous plaques may indicate a proliferative stage in the plaque formation.

2. Fibrinogen (FGN)

FGN is one of the acute phase proteins, and produced mainly in the liver. High circulating and intimal concentrations of FGN are known to be risk factors in

the development of CHD [224,234,235]. The atherogenicity of FGN may be due to the lack of an effective method of clearing FGN/fibrin from the intima.

FGN is a glycoprotein with MW of 340 000 and is composed of two identical subunits [225]. Each subunit contains 3 polypeptide chains, Aα, Bβ and γ, connected by disulfide linkages, producing the final structure $[A\alpha B\beta\gamma]_2$. These two subunits form three domains: a 32 kD central domain, composed of the amino terminal ends of all 6 polypeptide chains and two globular terminal domains or lobes, at the carboxy terminus, connected to the central domain by two coiled coil structures. The structure and biosynthesis of FGN has been extensively reviewed [225] and will not be dealt with further.

The conversion of plasma FGN to the insoluble matrix of a fibrin blood clot is a multistep process involving limited proteolysis and aggregation [225]. The final steps of this process will be discussed. Upon the proteolytic activation of thrombin, a proteolytic enzyme, the process of fibrin formation begins. FGN has two proteolytic susceptible sites at the N-terminal ends of the α and β chains of both subunits. These two sites are cleavage points for two peptides, termed fibrinopeptide A (FPA) and B (FPB). Thrombin-catalyzed release of FPA is the first step in fibrin formation. The release of FPA exposes an essential N-terminal polymerization site on the α-chain. This site interacts with a complementary site on the γ lobe of the distal domain of another FGN molecule. This other molecule must also have at least one FPA cleaved, it interacts with the γ lobe of the first molecule. These interactions continue until the FGN molecules, minus FPA, are assembled into two-stranded fibrin protofibrils (fibrin I). The release of the 14 amino acid FPB exposes a second site. This site can interact with a complementary site on the β lobe of the distal domain of nearby molecules, to produce assembly into fibres (fibrin II). It is the interactions after FPB release that can produce lateral associations and branching.

During the formation of fibrin, Factor XIIIa catalyzes a crosslinking reaction between the repeating units of polymerized fibrin. These crosslinks occur between anti-parallel γ-chains in adjacent molecules to form γ–γ dimers. This crosslinking tends to stabilize the fibrin clot to provide the scaffolding necessary for tissue repair. FN is also preferentially crosslinked to γ-chain polymers by Factor XIIIa-catalyzed reactions [226].

Atherosclerotic lesions have long been known to contain large amounts of FGN/fibrin. Smith et al [227] have stated that it is a gross over simplification to think of atherosclerotic development as simply a case of lipid infiltration; lipids probably play a role in lesion progression, but FGN/fibrin may play a larger role in atherogenesis, since deposition of LDL was seen only in association with deposition of FGN [2].

Smith et al [227] developed a technique for quantitating FGN and fibrin or other insoluble fibrin-like antigens in arterial intima samples. They used electrophoresis directly from the tissue into antibody-containing gel for measurement of FGN and then exhaustive plasmin treatment, to degrade fibrin-related residual material, followed by immunoelectrophoresis of the fibrin-derived fragments. From these results, they reported that the normal intima contained about 2% fibrin and FGN:fibrin was 1:1.5. There was a small increase in fibrin in gelatinous lesions, but a large increase in FGN. The edges of larger plaques showed 3x normal FGN and 5x normal fibrin, with fibrin making up 10% of the dry tissue weight. About 80% of the soluble FGN-related antigen (FRA), was clottable with thrombin. The finding of large amounts of fibrin or some insoluble form of FGN bound to collagenous material in these plaques led to the hypothesis that FGN is bound up in the arterial wall by TGase [232].

Bini and colleagues [231] did further studies on FGN, fibrin I and II in normal and atherosclerotic arteries using monoclonal antibodies to various

regions of FGN. They found that FGN and fibrin I were in the intima and subintima around foam cells, in areas of connective tissue and around vascular cells (SMC and macrophages) in the early lesions. Fibrous plaques showed FGN and fibrin I within tissue or on the luminal surface; fibrin II was seen around cells or in bundles in the intima and media. In advanced lesions FGN and fibrin I were seen in large foci in connective tissue and around SMC and macrophages. FGN and fibrin I and II were all found around cholesterol crystals and calcium deposits. These results showed that with increasing severity of lesions there was an increase in fibrin II and also in plasmin degradation products of FGN/fibrin.

Smith [233] showed that at least half of the FRA in atherosclerotic lesions is intact FGN, the remainder were fragments of high MWs, with characteristic patterns in different lesions. Further study by Smith and co-workers [229] isolated FRA from intimal extracts by affinity chromatography on Sepharose-antiFGN columns, followed by elution with 8 M urea and separation by SDS-PAGE. They found that in gelatinous lesions, there is increased FGN entry into the arterial wall and reduced fibrinolysis. The fibrin in lesions is highly crosslinked. The FGN degradation products found in the arterial wall appeared to be formed by the simultaneous action of thrombin, TGase and plasmin on FGN, or that fibrin was formed prior to initiation of plasmin degradation.

FGN imbedded in the arterial wall exerts several effects on its environment. FGN fragments have been shown to be chemotactic for monocytes and macrophages [236] and cultured SMC [239]. Ishida and Tanaka [237] have shown that fibrin increases SMC proliferation, in culture, but FGN degradation products decrease SMC mitotic activity: Lorenzet et al [240] showed that plasmin digests of FGN caused the release of growth factors into the culture medium of

SMC but intact FGN had no effect. FGN and its low MW degradation products can damage endothelial cells in culture [238].

3. Plasma Lipoproteins

One of the most obvious characteristics of atherosclerotic lesions, of all types, is the accumulation of lipid, both intra- and extracellularly. Biochemical and ultrastructural evidence suggests that most of the lipid is derived from plasma LDL [244,245].

Essentially all plasma lipids are associated with proteins to form water-soluble macromolecular complexes, called lipoproteins. They have been isolated by density gradient centrifugation and have been named according to their densities: very low density (VLDL), low density LDL and high density (HDL). Each lipoprotein type has a characteristic composition of protein, phospholipids, unesterified and esterified cholesterol and triglycerides (Table 2). This discussion will focus on the atherogenic lipoproteins, specifically LDL.

High serum LDL-cholesterol levels constitute a known risk factor for CHD. The relationship between total cholesterol and CHD is correlated with elevated serum LDL [109]. Clinical trials in lipid research have suggested that lower LDL levels are beneficial since lower LDL levels are correlated with a reduction in the incidence of clinical manifestations of atherosclerosis [109].

The physiological role of circulating LDL is to provide cells with cholesterol [247]. This is accomplished by LDL binding to specific cell receptors. The cells take up the LDL particles, hydrolyse their cholesteryl esters in lysosomes and use the unesterified cholesterol for membrane synthesis. Intimal cells must acquire nutrients, including cholesterol, directly from the plasma; therefore LDL particles enter the intima to provide cholesterol to SMC. The LDL concentration in normal intima is twice that of the circulating plasma [241]. The

Table 2. Composition of human serum lipoproteins. Each value is percent dry weight per particle.

Lipoprotein particle	Protein	Phospholipid	Unesterified cholesterol	Esterified cholesterol	Triglycerides
Chylomicrons	2.0	7.0	2.0	5.0	84.0
VLDL	8.0	18.0	7.0	12.0	50.0
LDL	21.0	22.0	8.0	37.0	11.0
HDL	55.0	23.0	2.9	12.0	4.1

*[from reference 246]

major factors that appear to contribute to the hyperlipidemic environment of the arterial intima are the IEL barrier and the presence of a dense extracellular matrix [241]. In order to become atherogenic, native LDL must be chemically or physically modified, since cell receptors for native LDL are down-regulated by cellular cholesterol levels [122].

The major classes of lipid in arteries are free cholesterol, cholesteryl esters and phospholipids [248]. There is a continuous increase in all 3 forms of lipid in the artery during the first 15 years of life. Cholesteryl esters have not been shown to have an essential cellular function, instead they seem to be a storage form of cholesterol. There is a change, with age, in fatty acid content of cholesteryl ester from saturated fatty acids, such as palmitate and stearate , to unsaturated, such as linoleate and arachidonate. In atherosclerotic plaques,

there is a very large increase in cholesteryl ester content , as well as an increase in free cholesterol; suggesting , that most of the cholesterol ester in fatty streaks represents cholesterol taken up and reesterified by cells in the lesions.

It has been suggested by several studies [reviewed in 248] that native LDL must be modified to become atherogenic. Modified LDL isolated from atherogenic serum possesses the capacity to induce accumulation of intracellular cholesterol and to stimulate DNA and extracellular matrix synthesis in cell cultures [249-251]. Some of the possible modifications to LDL include, oxidation, acetylation, self aggregation and conjugation with connective tissue components and FGN degradation products [248]. In diabetic patients, lipoproteins have also been shown to be non-enzymatically glycated, altering their clearance from circulation [300]. There is considerably less amounts of saline-extractable apo-B in atherosclerotic lesions, than in normal arteries [244]. This decrease could be due to the uptake of modified LDL by cells in the lesions and the formation of bound LDL in the arterial intima. Of all protease digestions tried in one study, only plasmin digestion released maximum amounts of lipoprotein [253]. Most of these modifications appear to occur on the ε-amino groups of lysyl residues in the apo-B portion of the LDL particle [252].

4. Transglutaminases (TGases).

Transglutaminase (EC 2.3.2.13) structure and actions have been extensively reviewed; therefore the reactions catalyzed by the enzyme will only be briefly discussed along with the significance of its action in atherosclerosis.[254-256]. TGases are Ca^{2+}-dependent enzymes which catalyze the formation of an ε-(γ-glutamyl)lysine bond between the γ-carboxyl group of peptide-bound glutamine residues of one protein and the ε-amino group of a lysine residue in another protein or some amines (eg. putrescine,

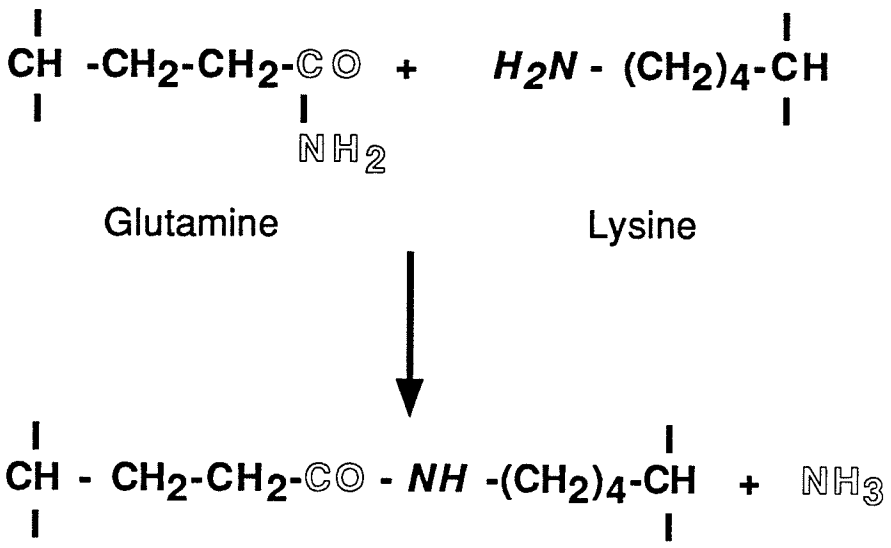


Figure 4. Transglutaminase-catalyzed crosslink. Transglutaminases catalyze the formation of an iso-peptide bond between the γ-glutamyl group of peptide-bound glutamine and the ε-amino of lysine residues or other amines.

spermidine) (see Figure 4) [254,255]. TGases are widely distributed in most organs, tissues and body fluids. These include liver or tissue TGase, which is found in many tissues, epidermal and hair follicle TGase, coagulation Factor XIII and prostate TGase. The different enzymes are distinguishable by their physical characteristics and the substrate sites they bind to.

Factor XIII is probably the best characterized and its role is best understood of the TGases. It is a plasma protein made of 4 subunits a_2b_2, with a MW of 320 000. The two a subunits are the catalytic subunits and are activated by proteolytic cleavage of activation peptides on these subunits by thrombin. Tissue TGase is found in many tissues, including liver and erythrocytes, has a different amino acid sequence than Factor XIII and it exists as a monomer with a MW of 75-80 000.

The reaction catalyzed by Factor XIIIa is well known to produce crosslinks between the γ-chains of two fibrin molecules to form γ–γ dimers stabilizing the fibrin plug. This discussion will focus on tissue TGase, termed TGase from this point.

The cellular functions and the endogenous substrates of the ubiquitous tissue TGase are not well understood. It has been suggested that TGase may play a role in the modification of membranes and membrane-associated proteins [257,258]. It has also been suggested that TGase is involved in receptor-mediated endocytosis [259]. Other possible functions of TGase are regulation of cell proliferation, ageing, stimulus-secretion coupling, cell differentiation and formation of the intracellular matrix [reviewed in 261]. Fesus and Thomazy [261] have suggested that TGase may also play a role in programmed cell death (apoptosis). One other role TGase may play is in the metabolism of connective tissue in wound healing [278,279].

The determination of function and substrates of the enzyme is further hampered by the possibility that there are two types of TGase. Initially, TGase was thought to occur solely as a cytosolic enzyme. Evidence now suggests that the enzyme may be membrane-bound on the membrane surface [260-267].

TGase activity has been known to be present in the arterial wall for a number of years and it has been implicated in the development of atherosclerosis [268,269]. The enzyme is present in many of the cells associated with lesion formation. Korner and colleagues [270] have characterized a TGase present in endothelial cells. TGase has also been localized in cultured SMC, endothelial cells and in supernatants of the homogenates of the human saphenous vein [271]. TGase has also been located and studied in macrophages [272-275].

As mentioned, the endogenous substrates of TGase are not known; however several proteins possess a specific substrate site for TGase and can be crosslinked to other proteins in vitro and in cell culture. FGN/fibrin have been known to be substrates for Factor XIIIa but they also possess a substrate site for TGase. This substrate site is close to the C-terminal end of the α-chain. Recently, Shainoff and co-workers [276] used a novel immunoelectrophoretic technique to characterize FGN and fibrin in plasma and atherosclerotic lesions. They discovered several α-linked FGN products were present in plasma and lesions. FGN and FN were shown to be crosslinked by TGase on the surface of hepatocytes in suspension [265,266]. FN has been shown to be a substrate for TGase and may be able to act as a carrier for the enzyme in the plasma after release of the enzyme through cell lysis [267]. Cocuzzi and colleagues have shown the apo-B protein of lipoprotein particles to be post-translationally modified by TGase [280]. PIIIP, another protein present in the arterial wall, is known to be a very good substrate for TGase [277]. This site has been characterized and shown to be centred around the glutaminyl residue at position 14 in the 15 kDa peptide by incorporation of ^{3}H-putrescine, an amine donor. PIIIP is a better substrate than casein, previously thought to be one of the best substrates for TGase. However, PIIIP is not a glutaminyl donor for Factor XIIIa.

II. HYPOTHESIS AND RATIONALE

The predominant view of the mechanism of the development of the atherosclerotic lesion is explained by Ross [1] as the 'response-to-inury hypothesis'. He states that repeated trauma to the endothelial layer of arteries, by various mechanisms, causes the mass infiltration of monocytes into the subendothelium and proliferation and migration of intimal and medial SMC in response to various mitogens and chemoattractants. These cells begin to accumulate lipid and secrete various substances. The SMC change their phenotype from a contractile to a more fibroblast-like synthetic phenotype, producing large amounts of connective tissue, especially collagens. For reasons unknown FGN and LDL remain bound in the arterial wall, usually to the ECM. This accumulation results in an impaired healing response by the endothelium and the rest of the arterial wall, causing the formation of complex lesions.

Skin wound healing studies showed increased TGase activity in rat wounds compared to normal tissue [279]. These studies also showed increases in the amount of PIIIP in early stages of healing in skin wounds as compared to normal tissue [288]. If the development of atherosclerotic lesions is a response to injury the arterial wall may behave as skin does during wound healing, with SMC playing the role of fibroblasts in ECM production and cellular repair.

The hypothesis to be examined in this thesis is that high concentrations of FGN and LDL in the arterial wall are crosslinked by SMC and/or macrophage TGase, present on the cell surface, to PIIIP and/or PCIII actively secreted in large amounts by SMC. This covalent linkage is thought to impair the clearance of FGN from the arterial wall and modify LDL so that it is recognized by the scavenger receptor on macrophages causing intracellular lipid accumulation and foam cell formation. In addition, feedback inhibition of collagen synthesis by PIIIP [153] may be abolished, producing fibrosis.

In Part A of this thesis the first approach to testing this hypothesis has been to incubate PIIIP and PCIII, excellent substrates for TGase, with TGase and FGN or fibrin in vitro to determine if the formation of insoluble complexes can occur. In solution these complexes can be detected by chromatography in SDS. Insoluble complexes can be isolated by centrifugation after boiling in SDS.

If TGase plays a role in atherogenesis, it may increase in the atherosclerotic artery compared to normal. Benko and Laki [294] showed that TGase activity increased in aortic homogenates of rabbits fed a high cholesterol diet as when compared with those aortas from rabbits fed a normal diet. In contrast, another study reported that buffer soluble extracts of advanced human lesions compared to adjacent normal-appearing tissue showed no difference or less TGase activity [289]. These studies did not separate the layers, nor did they try different extraction methods to more closely identify the source of the TGase activity. Moreover it was unsure whether this activity was tissue TGase or some other type. Part B determines the amount of TGase activity and tissueTGase antigen in extracts of three layers of the atherosclerotic artery. Since human tissue samples are difficult to obtain, it was decided to use diet induced atherosclerosis [292] in the rabbit model. In the early stages of development these lesions are cellularly and biochemically similar to human atherosclerosis [111]. The aorta can be carefully separated into 3 layers and activity assays performed on each layer. A monoclonal antibody to tissue TGase [291] was available to determine if activity is due to TGase and not Factor XIIIa.

Putrescine incorporation into protein has been used previously to identify the endogenous substrates of TGase [297,298]. In Part C this method has been tried with SMC. Incubation of SMC with ^{125}I-PIIIP plus FGN has been tried as a search procedure for specific covalent complex formation catalyzed by TGase.

III. EXPERIMENTAL

A. Transglutaminase-catalyzed Crosslinking of FGN and PIIIP.

1. Introduction

High concentrations of circulating FGN have been identified as a risk factor for ischemic heart disease. While there is evidence that it is found in a bound form in the arterial wall [2,224,234,235] it is not clear how it is bound and how it may influence the development of atherosclerosis. Recently, increased serum concentrations of PIIIP antigen have been reported in a number of patients with coronary artery disease [281]. In this section, TGase-catalysed interactions of FGN and PCIII and PIIIP are demonstrated in vitro.

2. Materials and Methods

^{3}H-Procollagen III and Fibrinogen. Fresh bovine aortae were transported on ice from the packing plant and small portions of the intima-media layer were minced and used as explants for culturing aortic SMC by standard procedures [282,283]. The cells were labelled with 20 μCi/ml of 2,3-^{3}H-proline (Amersham) in DMEM (Gibco) after preincubation with sodium ascorbate and β-aminoproprionitrile [282]. ^{3}H-PCIII was isolated by precipitation with ammonium sulphate and chromatography on DEAE-cellulose [282]. SDS-PAGE showed the presence of high MW material after pepsin digestion; this was converted to a single labelled band, after reduction, which corresponded in position with the α1(I) band of a collagen type I standard (Fig. 5). Together with the location of the labelled band before pepsin digestion, this identifies the labelled material as PCIII or PCIII lacking the C-terminal extension (PNIII). Since the reactions to be

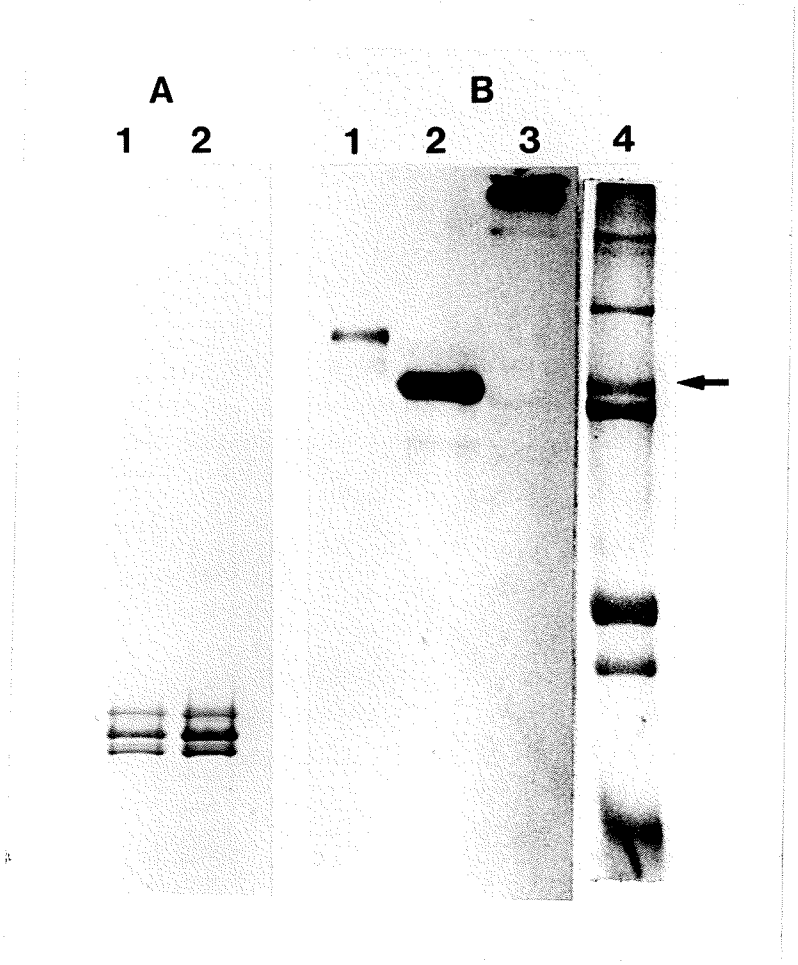


Figure 5. SDS-PAGE of FGN and ^{3}H-PCIII preparations. Gel A (3-10% gradient): lane 1, 20 µg FGN; lane 2, 40 µg FGN. Gel B (4%/6% discontinuous): lane 1, ^{3}H-PCIII reduced; lane 2, pepsin digest of ^{3}H-PCIII reduced; lane 3, same as lane 2 but unreduced; lane 4, α2(I) and α1(I) collagen chains (90 kDa), ovalbumin (45 kDa), chymotrypsinogen A (24 kDa) and cytochrome C (12.2 kDa). A1 and 2 and B4 were visualized by Coomassie blue stain and B1-3 by fluorography.

studied here involve only the N-terminal extension, these two procollagen forms were not separated or distinguished. Some unlabeled protein was also present in the preparation. The content of PCIII was determined by densitometry of a gel stained with Coomassie blue.

Unlabeled PCIII was prepared from fetal calf skin by the method of Timpl and co-workers [145].

Bovine FGN (Sigma) was dissolved and then dialysed against 0.1M Tris/HCl, pH 8.3 (TGase buffer) with 1mM EDTA added, then stored at -20°C in small aliquots. The purity of the FGN preparation is shown by the presence of only 3 stained bands representing the α, β and γ chains (Fig. 5A). Some of this material was refractionated by glycine precipitation but this was not found to increase the purity or alter the results obtained. In Table 3, lines 1-4 were obtained with refractionated material and lines 5 and 6 with unfractionated.

Tissue TGase was prepared from rabbit liver by an established procedure [284]. The preparation was concentrated to give a protein concentration of 3 mg/ml and then stored at -20°C in 50 µl aliquots.

Chromatography. A 1.6 x 45 cm column of Sepharose CL-2B (Pharmacia) was equilibrated with the running buffer, 0.15M NaCl and 0.1% SDS in 50mM Tris/HCl, pH 8.0. Materials to be tested for crosslinking (Fig. 6) were incubated in microcentrifuge tubes in a total volume of 0.3 ml TGase buffer, containing 1mM.DTT, 5mM $CaCl_2$ and 5 µl of 4x diluted liver TGase, for 1 h at 37°C. The samples were then diluted to 1 ml containing 10mM EDTA, 1% SDS and 50mM DTT and boiled in a water bath for 2 min. After cooling, bromophenol blue was added as a marker and the sample applied to the column. Elution was with the running buffer applied to the column in an upward direction by a peristaltic pump at 12 ml/h. Fractions of 1 ml were collected and assayed by scintillation counting by adding Beckman Ready Protein+ scintillant to the samples.

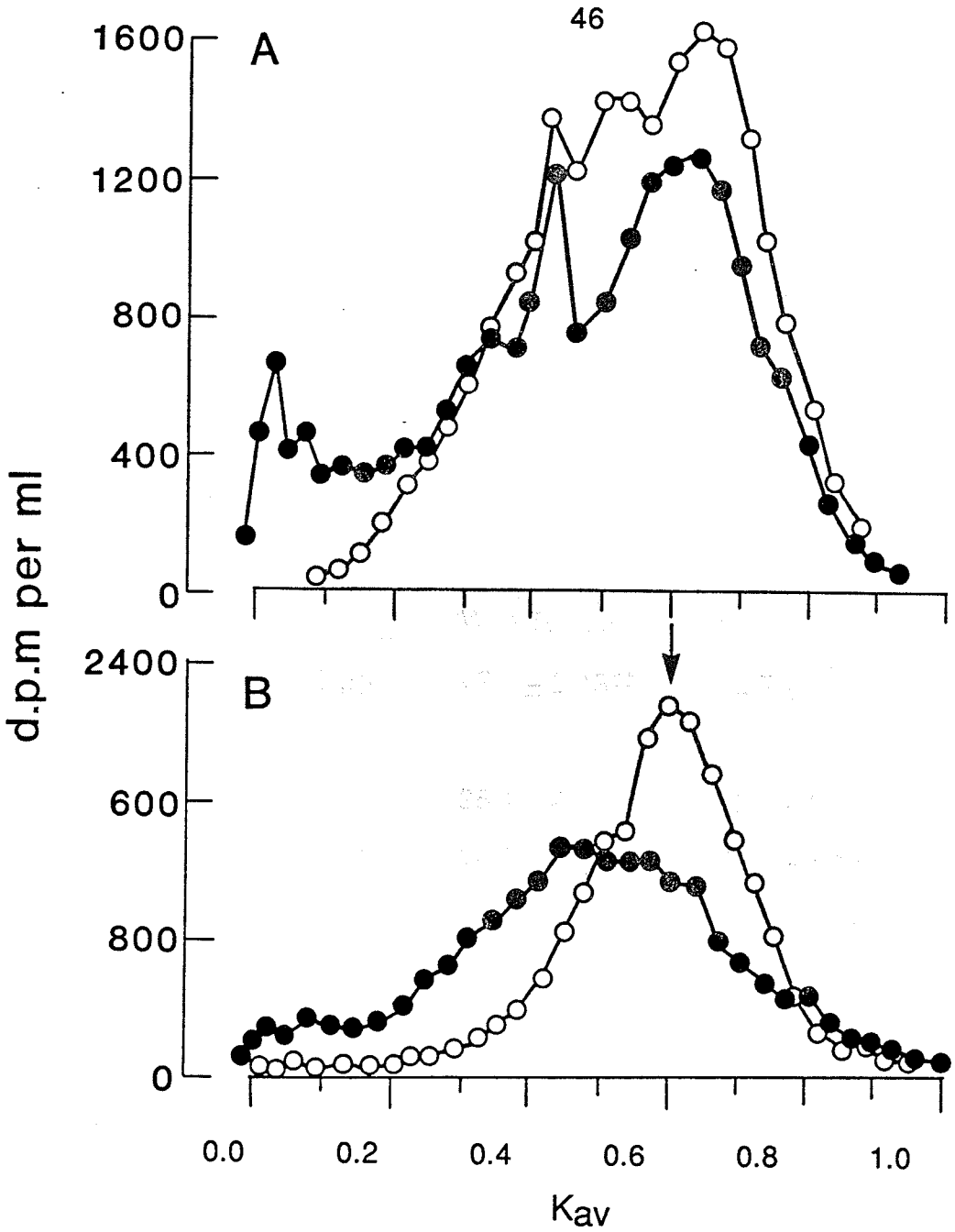


Figure 6. TGase-catalysed crosslinking shown by chromatography on Sepharose CL-2B in 0.1% SDS.

A. ^{3}H-PCIII (40 000 dpm, 0.1 µg) incubated with TGase and 40 µg FGN (●—●) or 50 µg plasma proteins (o—o).

B. ^{3}H-PCIII (as in A) incubated with TGase alone (o—o) or plus 5 µg FGN (●—●). Arrow marks elution point of reduced ^{3}H-PCIII alone.

$K_{av} = (V_e-V_o) / (V_t-V_o)$, where V_o is the void volume of the column, V_e is the effluent volume collected up to a specific point and V_t is the total volume of the column.

Table 3. Precipitation of SDS-insoluble material after incubation with TGase. Incubation was with materials listed as described in 'Methods'.

Incubation Mixture	% dpm in pellet (mean ± SD)		n
^{3}H-PCIII:	With TGase	Without TGase	
1. (0.04 µg)+FGN (40 µg) + Thrombin	47.7 ± 2.43[ab]	2.7 ± 1.09	6
2. (0.04 µg)+Fibrin	28.4 ± 4.06[ab]	3.1 ± 0.28	6
3. (0.04 µg)+FGN (40 µg)	14.0 ± 3.43[ab]	3.8 ± 1.13	5
4. (0.04 µg) alone	5.9 ± 1.45	4.4 ± 0.69	6
5. (3.5 µg)+FGN (10 µg) + Thrombin	17.0 ± 1.38[ab]	7.8 ± 0.82	5
6. (3.5 µg) alone	6.0 ± 0.97	6.3 ± 0.85	5

[a] $P<0.01$ in pair t-test against incubations without TGase.

[b] $P<0.01$ in pair t-test against incubations without FGN.

Precipitation Assays. The materials listed in Table 3 were incubated for 2 h at 37°C in a total volume of 0.3 ml TGase buffer containing 1mM DTT and 5mM $CaCl_2$. 16 000 dpm of ^{3}H-PCIII was used in each incubation with unlabeled PCIII added when amounts greater than 0.04 µg were used. 5 µl of 4x diluted TGase and 2 NIH units of thrombin were used where listed. Fibrin, where listed, was prepared by preincubation of 40 µg of FGN with 2 NIH units of thrombin. All incubations were terminated by adding 30 µl of 0.1M EDTA. For controls (without TGase), 5 µl of diluted TGase was added at this stage. The mixtures were then centrifuged at 8000 x g for 10 min. The supernatants were removed and pellets washed with 1% SDS in TGase buffer. After centrifuging, the washes were pooled with the first supernatants and the precipitates were dissolved in 1 ml TGase buffer with 1% SDS and 50 mM DTT, by boiling for 2 min. The pooled supernatants and the dissolved precipitates were counted after the addition of scintillant.

3. Results

Reduced ^{3}H-PCIII gives a single symmetrical peak (arrow in Fig 6B) when chromatographed in SDS on Sepharose CL-2B. After incubation with TGase, some higher MW material is produced, as shown by the shoulders in the open circle profile of Fig. 6B. However, incubation with FGN plus TGase (Fig. 6A) produces very high MW material (close to V_o) which is not produced when ^{3}H-PCIII is incubated with BSA or a plasma protein preparation (Pentex) (Fig. 6A). The plasma proteins produce a peak at 0.42-0.45 K_{av} which is also found with FGN (Fig. 6A, 40 µg and Fig. 6B, 5 µg), but this peak may be due to the presence of FGN in the plasma protein sample.

A small amount of the ^{3}H-PCIII preparation sediments in SDS after centrifugation, with or without TGase incubation (lines 4 and 6 in Table 3). A larger fraction is sedimented when 1000-fold excess of FGN is used with TGase

plus thrombin (line 1). Preformed fibrin or FGN alone produce less sedimentation. Presumably much of the high MW material seen in Fig. 6 is not sedimented under these conditions.

4. Discussion

As mentioned, the aminopropeptide of type III collagen contains a specific glutamine which forms part of an excellent substrate site for tissue TGase; FGN and fibrin are poor substrates for this enzyme [254,277]. Fig. 6 shows that there is a fairly specific TGase-catalysed interaction between PCIII or PIIIP and excess FGN; presumably PIIIP is the glutaminyl donor. The absence of a peak at Vo, when PCIII is incubated with BSA or other plasma proteins, indicates that only FGN produces very high MW material which is not split up by boiling in SDS and DTT and therefore is presumably stabilized by covalent crosslinks. Table 3 shows that a non-reduced high MW complex can be sedimented by centrifugation even in the presence of SDS. Increased resistance to solibulization by denaturing media, such as SDS, is typically associated with crosslinking by TGase [285]. Preformed fibrin produces more labelled precipitate than FGN with ^{3}H-PCIII, but even more is formed when FGN and thrombin are present in the incubation medium with TGase. This indicates that PCIII is more readily crosslinked to fibrin monomers or oligomers than to FGN or fibrin polymers. A 2.85-fold excess of fibrin monomer was the minimum for which significant differences were found between incubations with and without TGase (Table 3,line 5). A 1000-fold excess produces more labelled precipitate (Table 3, line 1), but in no case is all the ^{3}H-PCIII sedimented. This may be due to the presence of soluble non-sedimentable complexes, such as those seen in Fig. 6, as well as to the presence of unbound ^{3}H-PCIII.

Further experimentation showed that TGase catalyses the formation of high MW crosslinks between ^{3}H-PCIII or ^{125}I-PIIIP and IDL or LDL, but not HDL [286,287]. These high MW crosslinks of PIIIP were found in the floating fraction of sera of infarct patients after density gradient ultracentrifugation. These interactions, along with those with FGN could provide a link between FGN and LDL which is consistent with the response to injury hypothesis of atherosclerosis [1].

Skin wound healing studies [279,288] suggest that PCIII may be temporarily crosslinked to fibrin or other extracellular structures in the early stages of fibre repair. Type III collagen is known to occur with type I in the intimal and medial layers of arteries, as well as in atherosclerotic plaques [133,134,157-162], but the concentration of PCIII in the intima is unknown. Up to 0.1 mg/g has been obtained from fetal calf skin [145] but the concentration is known to be lower in adult skin and it is unlikely to be more than this in the intima. The concentrations of LDL, FGN and fibrin in the arterial intima are known to be increased in atherosclerosis [33] and may be up to 50x the estimate of 0.1mg/g for PCIII. Under these conditions, it appears that crosslinking by TGase is possible. The increase in serum concentration of PIIIP antigen in some patients with CHD [281] is consistent with an increase in biosynthesis of PCIII in atherosclerosis. It has not been established whether this increase in serum antigen is due to the presence of free PIIIP or to a form which is joined to some other molecule.

B. Increased TGase in the Aortas of Cholesterol-fed Rabbits.

1. Introduction

It has been reported that TGase activity in homogenates of aortas from cholesterol-fed rabbits is higher than in aortas from rabbits on a normal diet [294]. However in human advanced atherosclerotic plaques from the thoracic aorta, the soluble TGase activity was 25% lower than in adjacent normal tissue [289].

It has been shown in Section III:A [286] that TGase in vitro can form high MW compounds involving FGN or low density lipoprotein and PIIIP. It is hypothesized that this process occurs locally in the arterial wall, promoting the development of atherosclerotic lesions, if the concentrations of both the enzyme and the blood-borne risk factors are abnormally high. Since elevated concentrations of FGN and low density lipoprotein are known to be associated with the atherosclerotic process [2] it seemed important to obtain further information about TGase levels.

In the present work, three layers of normal and cholesterol-fed rabbit aortas have been examined by two different assay procedures: TGase activity by ^{3}H-putrescine incorporation into casein [277] and tissue TGase antigen by an e.l.i.s.a. method using a monoclonal antibody [290].

2. Materials and Methods

1,4-^{3}H-Putrescine dihydrochloride (17 Ci/mmol)was purchased from Amersham Canada, Ltd. Casein (Hammarsten) was from BDH Chemicals Ltd. Dithiothreitol (DTT) was purchased from Boehringer-Mannheim Canada Ltd. . DMEM and FBS were from Flow Laboratories . All other chemicals and supplies were from Sigma or Canlab , except where mentioned in the text. Rabbit liver TGase was prepared by standard procedures [284] . Monoclonal antibody to

guinea pig liver TGase [291] was a generous gift from Dr. P. J. Birckbichler of the Samuel Roberts Noble Foundation, Oklahoma, U. S. A.

Animals and Tissue Preparation. Male New Zealand white rabbits, 3.0-4.0 kg, were fed either a normal diet of Puratone rabbit pellets (Winkler Feed Service Ltd.) or the normal diet supplemented with one percent cholesterol and six percent peanut oil for ten to twelve weeks [292]. After this period of time, the rabbits were anaesthetized with sodium pentobarbital by intravenous injection and killed by heart puncture and removal of blood. The aortas were removed, dissected free of fat and fascia, and washed with isotonic saline at 37°C to remove all blood. They were then cut open longitudinally and pinned down, luminal side up. The inner layer was peeled off with forceps and placed into a polycarbonate tube on ice. The remaining middle layer was scraped away from the outer layer with a scalpel blade and placed into a separate tube, while the outer layer was cut up and placed into another tube. The inner layer contained all of the endothelium and the intima, but the peeling procedure also left some of the media in this layer. The middle layer was more fragile and less coherent than the outer layer and therefore the former presumably corresponds with the smooth muscle and elastic tissue adventitia.

The weight of each tissue layer was determined and 0.1 M Tris-HCl, pH 8.3, containing 1 mM EDTA was added to give a tissue concentration of 40 mg/ml buffer. The tissues were then homogenized three times for 30 s each using a Polytron homogenizer (Brinkmann Instruments) at speed setting seven in a 4°C cold room. The homogenates were allowed to extract at 4°C for one hour, then centrifuged for 20 min and 12 000 x g and the supernatant removed (buffer extract). The pellets were re-extracted in the same way except that the buffer contained 0.05% (v/v) Triton X-100. The supernatant was removed and called the Triton extract. The remaining pellet, buffer and Triton X-100 insoluble, was

resuspended in buffer and called the residue fraction. The extracts and fractions were stored at -20°C when not in use.

TGase Activity Assay. The buffer extract, Triton extract and residues were assayed for TGase activity by incorporation of ^{3}H-putrescine into casein, using previously described procedures [277,279], with some modifications. The assay mixture, 0.3 ml in a 1.5 ml microcentrifuge tube, contained 100 µg casein, 1 µCi ^{3}H-putrescine, 0.1 mM putrescine, 2 mM DTT, 10 mM $CaCl_2$, 50 µM MGBG and 0.1 ml of sample extract, all in 0.1 M Tris-HCl, pH 8.3. Controls contained 30 mM EDTA in addition to the rest of the assay mixture. Standards of 5 µl of 2 x diluted rabbit liver TGase (approximately 1.2 µg protein) were run in place of the sample with each set of assays to check reproducibility.

The assay mixtures were incubated at 37°C for 90 min before addition of 100 µL of 50% (w/v) trichloroacetic acid. The mixtures were allowed to precipitate overnight at 4°C. The supernatant was discarded and the pellet washed with 1 ml of 10% (w/v) TCA, 1 ml ethanol:ether (1:1;v/v) and finally 1 ml ether. After removal of the ether, the pellets were allowed to dry, resuspended in 0.5 M NaOH and boiled 5 min before being transferred to mini-scintillation vials (Beckmann Instruments), mixed with 4 ml Beckman Ready Protein scintillation fluid and 100 µL glacial acetic acid and counted in a Beckman LS 3801 scintillation counter. Control values were subtracted from test values to yield the results shown in Figure 7.

TGase Antigen Assay. The procedure was a modification of a previously described inhibition e.l.i.s.a. [290] , using a monoclonal antibody to guinea pig liver TGase [291].

The hybridoma liquid (ascites fluid) containing the monoclonal antibody was diluted five times with DMEM containing 10% (v/v) FBS and 10 µL of the diluted antibody was placed into sterile tubes (Falcon 205) with 90 µL of the

sample and 150 µL of DMEM with 10% (v/v) FBS. The rabbit liver enzyme (0.5 mg protein/ml) was diluted 2000 times in 0.1 M Tris-HCl, pH 8.3 and standards were set up containing 0, 5, 10, 20, 25, 30 and 40 µL of the diluted enzyme and Tris-HCl buffer to give volume of 90 µL. The tubes were then shaken for 2 h at 20°C before standing overnight at 4°C.

The following day, the enzyme preparation (0.5 mg/ml protein) was diluted 3000 times with PBS (0.15 M NaCl, 10 mM sodium phosphate, pH 7.4) and 50 µL of the diluted enzyme was placed in each well of a polyvinyl microtitre plate (Falcon 3913). The plate was shaken at 20°C for one hour, the liquid discarded and replaced with 200 µL of 5% (w/v) BSA in PBS in each well, then incubated for one hour an 20°C. The liquid was discarded and 100 µL of the antibody-sample mixture was added to duplicate wells and incubated, with shaking, at 20°C for 3 h. The liquid was then discarded, the wells washed 3 x with PBS and 100 µl of a 200 x dilution of the β-D-galactopyranosidase-linked second antibody (from a hybridoma screening kit, Bethesda Research Laboratories Life Technologies, Inc.) in PBS was added to each well. The plates were incubated at 25°C for 2 h. The liquid was then discarded, the wells washed 3 x with PBS and 100 µl 1.0 mg/ml p-nitrophenyl β-D-galactopyranoside was added to each well. The plates were incubated at room temperature for 1 h, then 50 µl of 1% sodium carbonate was added to each well and colour development was read at 410 nm on an MR700 plate reader (Dynatech Laboratories, Inc. in the laboratory of Dr. R. Yatscoff). The tests were compared to the standard curve and expressed as ng antigen/mg wet tissue weight (Figure 8).

3. Results

Animals. A total of 23 rabbits were used for the study; 8 rabbits fed a normal diet and the remaining 15 fed the high cholesterol diet. Two of the 15 rabbits became jaundiced and were not assayed.

The aortas of the cholesterol-fed rabbits appeared more fatty than those of the normal rabbits and all had raised lesions on the luminal surface. In accordance with previous findings [292] , the rabbits developed varying degrees of atherosclerosis and two groups could be distinguished on the basis of visual inspection of the aorta without staining: group I, having less than 25% of the luminal surface lesioned and group II having more then 25% of the luminal surface lesioned (up to 80%). The distribution of the lesions observed without staining corresponded very closely to that described by Duff & McMillan [293] in atherosclerotic rabbit aortas stained with Sudan IV. Thus, group I aortas correspond with their grades 1 and 2 and group II aortas correspond with their grades 3 and 4. Because of the small size of many of the lesions it was not possible to dissect them to obtain a comparison of the activity in lesions with that in non-lesioned areas. Seven rabbits fell into group I and six into group II.

The weights (means $\pm$ SE) of the three layers (inner, middle and outer, in that order) were 227.1 $\pm$ 9.2; 119.3 $\pm$ 16.2; 89.2 $\pm$ 11.2 mg for the normal aortas, 243.3 $\pm$ 14.9; 127.5 $\pm$ 24.0; 58.1 $\pm$ 5.1 for the group I atherosclerotic aortas and 363.1 $\pm$ 18.5; 157.7 $\pm$ 17.7; 87.9 $\pm$ 13.5 for the group II aortas. Plasma cholesterol concentrations in normal rabbits ranged from 0.24 - 0.66 mg/ml, while cholesterol-fed rabbits had a range of 13.20 - 34.98 mg/ml as measured by the method of Webster [299]. No significant difference in concentration was found between groups I and II and there was no significant correlation between cholesterol and TGase activity.

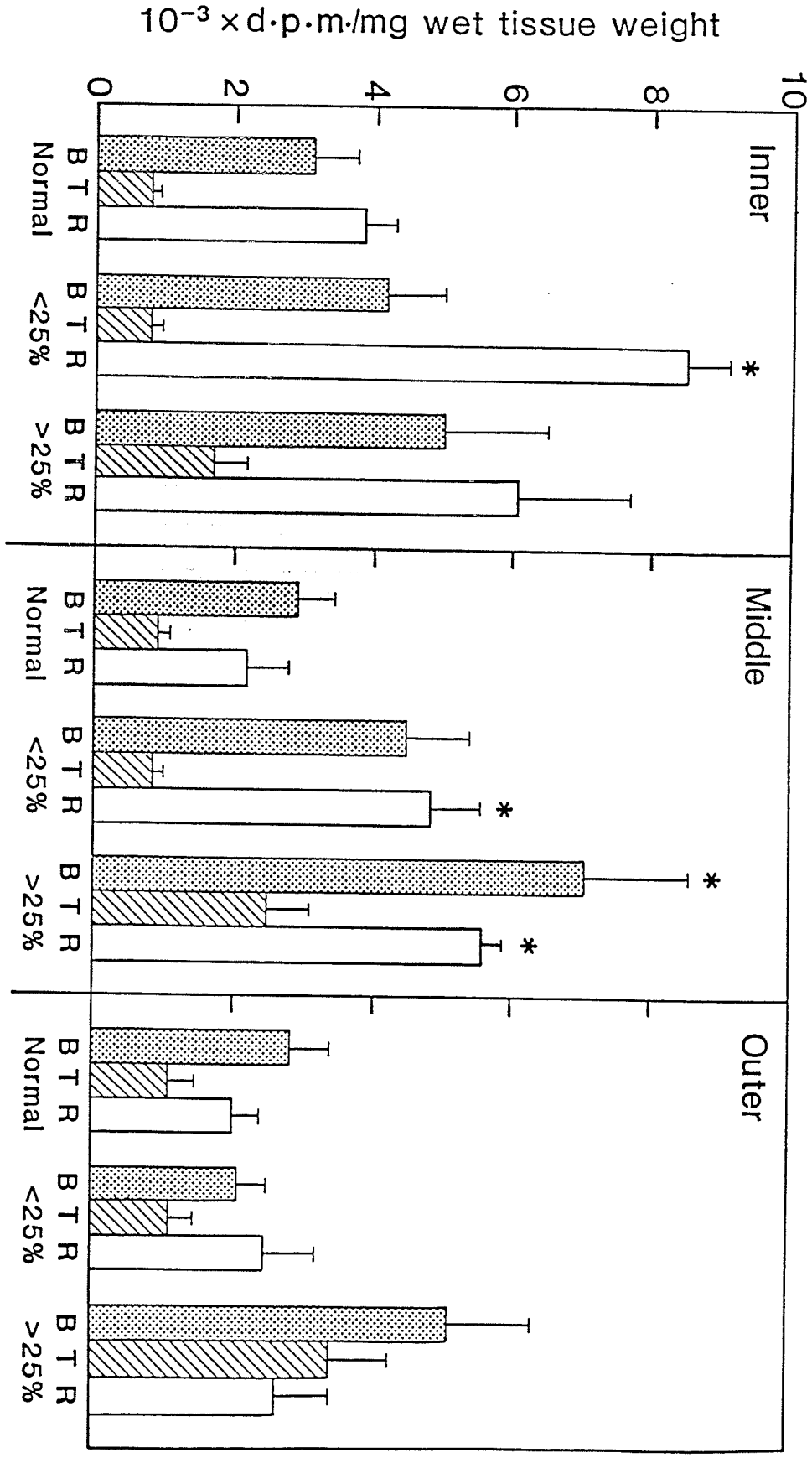
Activity and Antigen Assays. The activities shown in Figure 7 are entirely calcium-dependent, since the relatively low incorporations of ^{3}H-putrescine in the presence of EDTA (in all cases less than 500 dpm/mg wet tissue weight) were subtracted from each test result. The incorporation of ^{3}H-putrescine was not due to the presence of amine oxidase activity, since the inhibitor MGBG was used in all assay mixtures. Separate experiments (results not shown) demonstrated that the ^{3}H-putrescine incorporation was inhibited at least 80% by 0.2 mM dansyl cadaverine, a potent inhibitor of TGase. These findings indicate that the observed activity is similar to that for other transglutaminases [254,255].

Figure 7. TGase activity in three layers of normal and atherosclerotic rabbit aortas. B, buffer extract; T, Triton X-100 extract; R, residue. <25% and >25%, aortas with lesions on less or more than 25% of their luminal surface. The bar graphs show means and the lines above them the SE. *P<0.02 in pair t-test against normals.

Figure 8. Tissue TGase antigen in three layers of normal and atherosclerotic rabbit aortas. Abbreviations are as in Fig. 7. Triton X-100 extracts were made in each case, but the assay results were too low to be reliable.

*P<0.02 in pair t-test against normals.

Figure 7.


10^{-3} x d·p·m./mg wet tissue weight
0
2
4
6
8
10
Inner
Middle
Outer
Normal
<25%
>25%
B
T
R
*

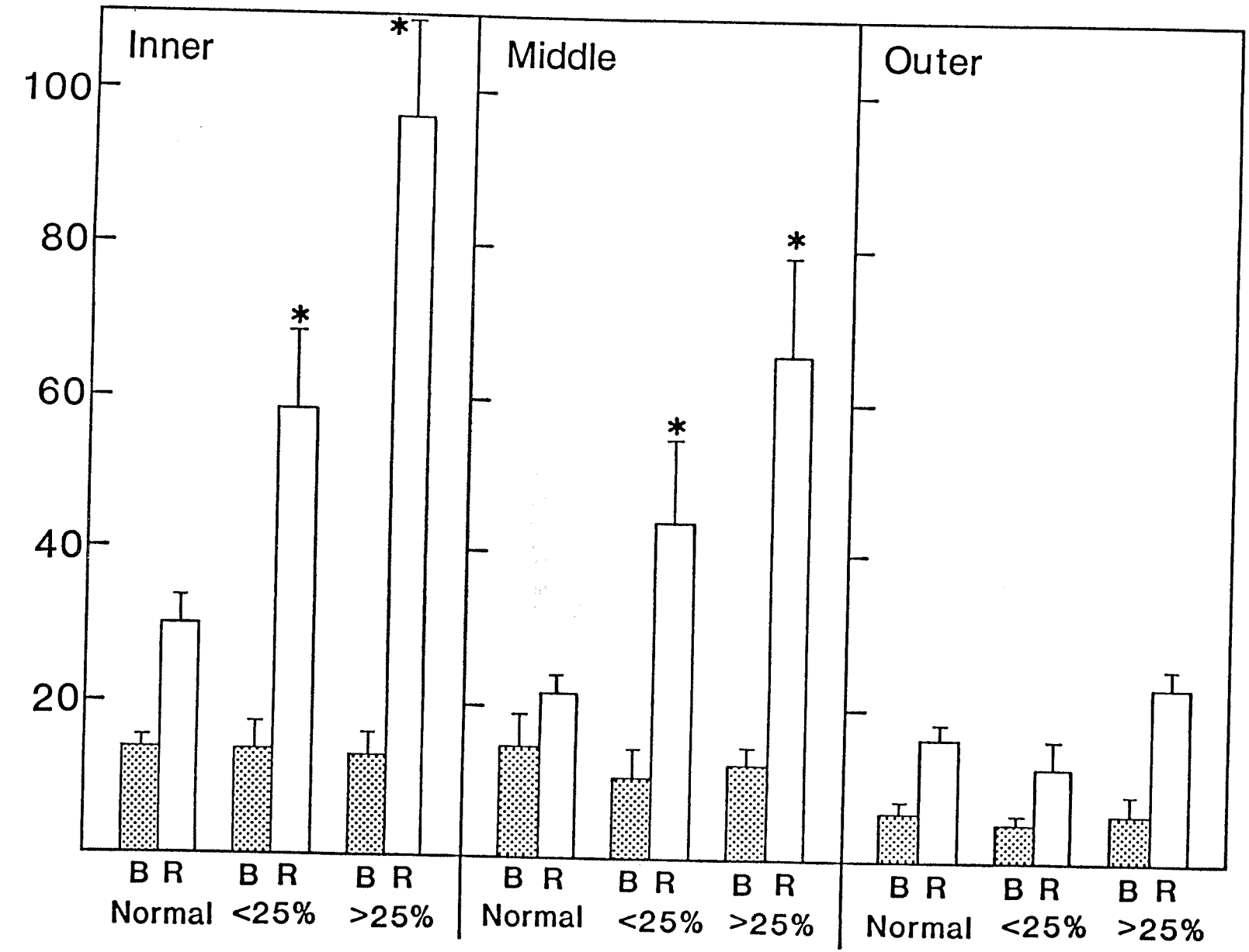

Inner
Middle
Outer
ng antigen/mg wet tissue weight
100
80
60
40
20
*
B R
Normal
<25%
>25%

Figure 8.

The data for ng antigen in Figure 8 were obtained by comparison with a standard curve for a purified rabbit liver TGase preparation which gave maximum antigenic activity per ng of protein and reproducible readings. The specific activities shown in Table 4 are derived from the activity data of Figure 7 and the ng antigen data of Figure 8.

Due to the difficulty in obtaining complete dispersion in the assay mixtures, the activity and antigen assays on the insoluble residues probably represent minimum values.

Table 4. Specific activities of TGase in three layers of normal and atherosclerotic rabbit aortas.

TISSUE	INNER		MIDDLE		OUTER	
	Buffer	Residue	Buffer	Residue	Buffer	Residue
Normal	201 ± 46	138 ± 13	209 ±56	89 ± 12	387 ± 123	118 ± 7
< 25%	357 ± 125	176 ± 41	584 ± 242	151 ± 25*	379 ± 126	232 ± 111
> 25%	357 ± 117	74 ± 26*	591 ±220	106 ± 16	585 ± 145+	122 ± 26

The results shown are means ± SE expressed as dpm/ng TGase antigen. The number of tissue samples was 8 for the normal aortas, 7 for the aortas with <25% of the surface area covered with lesions and 6 for the >25% group.
*P<0.05 and +P<0.01 in pair t-test against the normal groups.

4. Discussion

The results of the present work confirm the finding of Benko and Laki [294] that TGase activity in the aortas of cholesterol-fed rabbits is higher than in normal aortas. In addition, they show that the increase is not localized to the inner layer, which contains the intima and the atherosclerotic lesions, but is also found in the middle layers (Figure 7) which contain most of the media. A general effect of this type is presumably due to stimulation by a factor or factors spreading from the hyperlipidemic plasma of the atherosclerotic lesions into the medial portion of the artery. It is possible that the lesions themselves do not have raised TGase levels, as has been reported for advanced human lesions by York and Benjamin [289]. It seems more likely, however, that increased TGase activities do occur in the lesions, but are associated only with the insoluble residues, as is suggested by Figure 1 in the present work. Only supernatant extracts of the human lesions were assayed by York and Benjamin [289], whereas Benko and Laki [294]used tissue homogenates for their assays.

In all three layers of both normal and atherosclerotic aortas a large fraction of both the TGase activity and the tissue TGase antigen was not extractable with buffer or Triton X-100. In every case the insoluble residues contain the largest fraction of the antigen (Figure 8) and in one instance (the inner layer with <25% luminal area lesioned) the largest fraction of the activity.

The effects of cholesterol feeding on the buffer-soluble TGase fraction differ from those on the buffer-insoluble fraction. The results of the e.l.i.s.a. for tissue TGase show that this antigen is higher in the buffer-insoluble residues, from the inner and middle layers of cholesterol-fed rabbits, than in the same layers from normal animals. In the buffer extracts of all three layers there is a consistent trend to an increase in activity with increased severity of atherosclerosis (Figure 7). This effect is statistically significant in the case of the

middle layer. In contrast, Figure 8 shows that there is no significant difference in the antigen content of the buffer extracts from any layer of the normal and atherosclerotic aortas. Consequently, the activities per ng of antigen in the buffer extracts are higher in the atherosclerotic aortas (Table 4). It is possible that Factor XIIIa, a TGase which does not react with the antibody to tissue TGase , might infiltrate the atherosclerotic lesions and contribute to this increase. However, the increase is statistically significant only in the middle layer, which would be expected to receive less infiltrate from the blood than the inner layer, which is adjacent to the lumen of the aorta. In addition, for the insoluble residues, there is a significant decrease in the activity per ng of antigen in intimas with >25% lesions. In all layers of the aortas, activities per unit of antigen were lower for the insoluble residues than for the buffer extracts.

TGase activity and tissue TGase antigen have previously been reported in endothelial cells [270] and in cultured smooth muscle cells, endothelial cells and in supernatants of homogenates of human saphenous vein [271]. Only 4-22% of the activity was found in the particulate fraction from endothelial cells. This is considerably lower than the percentage found in the buffer-insoluble fraction of the homogenates in the present work. The nature of the binding of TGase in the buffer-insoluble residues is not yet established. The possibility that it is a plasma membrane component, as suggested by the work of Tyrell et al. [262,295] on hepatocytes, does not explain the divergence between the present results and those for endothelial cells. Juprelle-Soret et al [296] found that a cytosolic enzyme from liver bound to plasma membranes mixed with collagen during the in vitro homogenization procedure. It is possible that this process may contribute to the buffer-insoluble activity observed in the present work.

The relationship between the observed changes in TGase and the process of atherosclerosis remain to be determined. Fesus et al [261] have

shown that increased levels of TGase are associated with programmed cell death (apoptosis) in hepatocytes. As yet, there have been no studies of the possible occurrence of apoptosis in arterial cells. It has been shown that tissue TGase in vitro will crosslink PIIIP (which is a normal product of collagen biosynthesis in the artery) to FGN or fibrin [Section III.A]. Crosslinked aminopropeptide compounds were also shown to bind to lipoproteins [287]. The existence of these crosslinked complexes in the arterial wall, and their effects, remain to be demonstrated.

C. Binding of PIIIP or Putrescine in SMC Cultures.

1. Introduction

The endogenous substrates of tissue TGase are unknown. There are several proteins which exhibit the characteristic binding site for TGase. One of these proteins is PIIIP, which is an excellent substrate for TGase in vitro [277]. Recent evidence suggests that TGase may not only be a cytosolic enzyme but may be either membrane-bound intracellularly [262,263,295,296] or bound extracellularly to the ECM or plasma membrane [267,296].

Cultured SMC contain both TGase activity and antigen [271]. It is not clear where this enzyme is located. The previous study [Section III.B] showed the presence of a large amount of TGase activity and antigen in the particulate fraction of aorta homogenates. It is possible that this may be either membrane-bound or extracellular.

Incubation of cell cultures and tissues with radiolabeled putrescine and other amines followed by homogenization and SDS-PAGE have been used in previous studies to attempt to examine the endogenous substrate(s) of TGase [297,298]. The work described in this section uses a similar method to look for substrates of TGase in cultured bovine aortic SMC. It also studies whether ^{125}I-PIIIP can be crosslinked by SMC cultures under various conditions and examines possible locations for the enzyme activity.

2. Methods and Materials

Cell Culture. Fresh bovine aortae were transported, on ice, from the packing plant and small portions of the intima-media were minced and used as explants to grow SMC by established procedures [1] Cells were grown in Waymouth's MB 752/1 medium (Gibco) to confluency, incubated in trypsin/EDTA (Boehringer

Mannheim) and passaged 1-100 mm plastic culture dishes to 3- or 4-100 mm dishes (Falcon). All incubations were on cell cultures between the second and eighth passages.

^{3}H-Putrescine Labeling and Sample Preparation. (Fig. 9) Confluent and non-confluent SMC cultures in 100 mm plastic culture dishes were incubated with 40 µCi each of ^{3}H-putrescine (Amersham) for 48 h in DMEM, with or without 10% calf serum. Some cultures were preincubated 24 h with 1 mM butyrate and/or 50 µg/ml ascorbate. After incubation, cells were washed 3x with PBS then scraped into tubes with 0.5 ml TGase buffer containing 0.2 mM PMSF and 5 mM EDTA. The medium and washes were pooled and dialysed against TGase buffer containing 5 mM EDTA and then scintillation counted.

The cells were freeze/thawed twice and sonicated for 15 s at 40 watts (Brinkman Instuments). Small aliquots of the cell homogenate were counted in a scintillation counter (Beckman) after the addition of Ready Protein+ scintillant (Beckman). The remainder of each homogenate was precipitated with the addition of ice cold TCA to give a concentration of 25% (w/v). The precipitate was centrifuged at 12 000 x g for 10 min, the supernatant discarded and the pellet washed 3x with 10% TCA (w/v), once with ethanol:ether (1:1), and 2x with ether and dried. The remaining protein was resolubilized by boiling in a small volume of TGase buffer containing 1% SDS and 50 mM DTT before, running on a 3%-10% Laemmli gradient SDS-PAGE. The gel was then soaked in Autofluor (National Diagnostics) for 90 min and exposed to X-ray film at -80°C.

^{125}I-PIIIP Labeling. (Fig. 10-12) Confluent cultures of SMC in 100 mm plastic culture dishes, between passages 2 and 8, were incubated with 150-200 000 cpm of ^{125}I-PIIIP (Behring) for 90 min either alone in serum-free DMEM (Gibco) or with one of the following in DMEM: 50 µg/ml FGN, 50 µg/ml BSA, 200 µg/ml casein, 0.2 mM dansyl cadaverine (Sigma). Some cultures were preincubated

with 1 mM n-butyric acid (Sigma) for 90 min before the incubations. Total incubation volume in each case was 2 ml. After incubation, medium was removed with a pasteur pipet and cells were washed twice with 2 ml PBS and pooled with the medium. EDTA was added (to 10 mM) to the medium pools to terminate the incubation.

Some incubations were done with conditioned medium in the absence of SMC. For these samples, a total of 2 ml of serum-free DMEM was added to cells alone or with 0.2 mM dansyl cadaverine or 200 µg/ml casein. The medium was removed after a 90 min incubation and centrifuged at 400 x g for 10 min to remove cellular material. ^{125}I-PIIIP was added to the supernatants alone or with 50 µg/ml FGN and incubated again for 90 min in sterile plastic culture tubes (Falcon) in a 37°C water bath. Control incubations were done with unconditioned medium or with PBS and $CaCl_2$ (Fig. 12, lanes 5 and 6). All incubations were terminated with EDTA as above.

Protease inhibitors (pepstatin A, 10 µg/ml; PMSF, to 0.2 mM; NEM, to 10 mM) were then added to the cell media from the incubations and centrifuged at 400 x g for 10 min to remove cellular material. The supernatants were dialyzed against water and lyophilized.

The lyophilized supernatants were resolubilized in TGase buffer with 1% SDS and 50 mM DTT and boiled for 5 min prior to running on a Laemmli 3%-12% gradient SDS-PAGE. The gels were dried and exposed to X-ray film (Kodak).

3. Results

The proteins labeled with ^{3}H-putrescine in confluent and non-confluent SMC cultures were similar but the high MW proteins with incorporated label were increased in the confluent cultures (Fig. 9). It was also found that incorporation of ^{3}H-putrescine into the cell layer was increased in the presence

of ascorbate and butyrate (Table 5). In all cases, the labeling of the medium after dialysis was less than 8% of total label incorporation into (cells + medium) protein.

Figure 9 shows the labeling of several low MW proteins in the region of 14,000 Da. There were also very high MW complexes labeled; some did not enter the 3% acrylamide stacking gel and others did not enter the 10% running gel.

The incubation of confluent SMC cultures with ^{125}I-PIIIP and either FGN or BSA produced a number of high MW bands in the medium (Fig. 10). In all incubations with ^{125}I-PIIIP, incorporation of label into the cell layer was less than 10% of total label added. In Fig. 10 it appears that the presence of FGN (lane 2) produced more of the high MW bands than BSA (lane 1), with a decreased

Table 5. Total counts in TCA precipitates of confluent SMC layers labeled with ^{3}H-putrescine.

DMEM containing:	dpm/mg dried TCA precipitate ± SE
no additions	68 316 ± 5190
ascorbate	115 694 ± 3307
butyrate	178 772 ± 7166

n=3 for all samples.
All samples were prepared as in 'methods'.

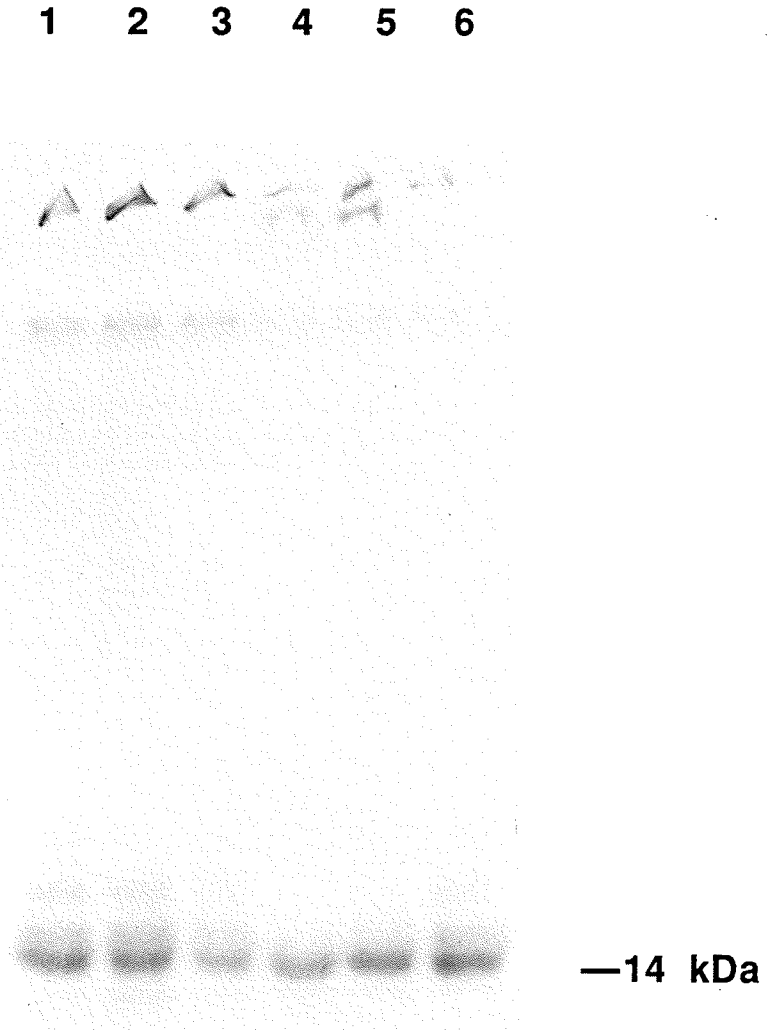


Figure 9. Fluorograph of SDS-PAGE of cell extracts after labeling with ^{3}H-putrescine. Confluent (lanes 1-3) and non-confluent (lanes 4-6) SMC cultures were incubated with ^{3}H-putrescine and samples prepared as described earlier. Cultures were incubated with ^{3}H-putrescine in DMEM plus: lane 1, ascorbate with 10% serum; lane 2, 10% serum alone; lane 3, ascorbate without serum; lane 4, no additions; lane 5, ascorbate; lane 6, ascorbate and butyrate.

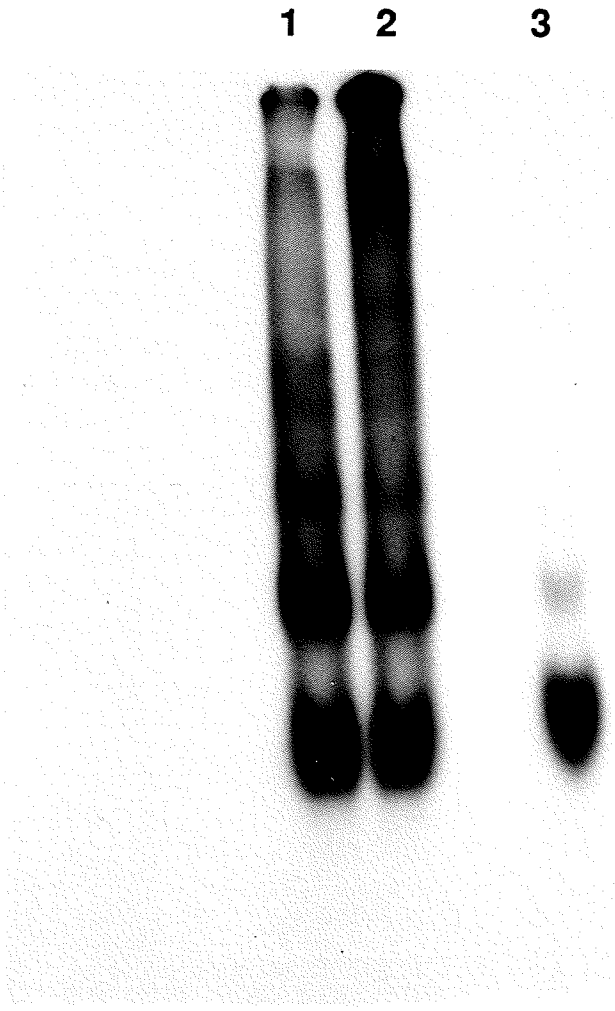


Figure 10. Gradient SDS-PAGE of lyophilized medium after incubation of ^{125}I-PIIIP with SMC. Lanes 1 and 2 show the products after incubation of SMC with ^{125}I-PIIIP in serum-free DMEM, with the addition of 50 µg/ml BSA (lane 1) and 50 µg/ml FGN (lane 2). Lane 3 contains only a standard of ^{125}I-PIIIP without incubation.

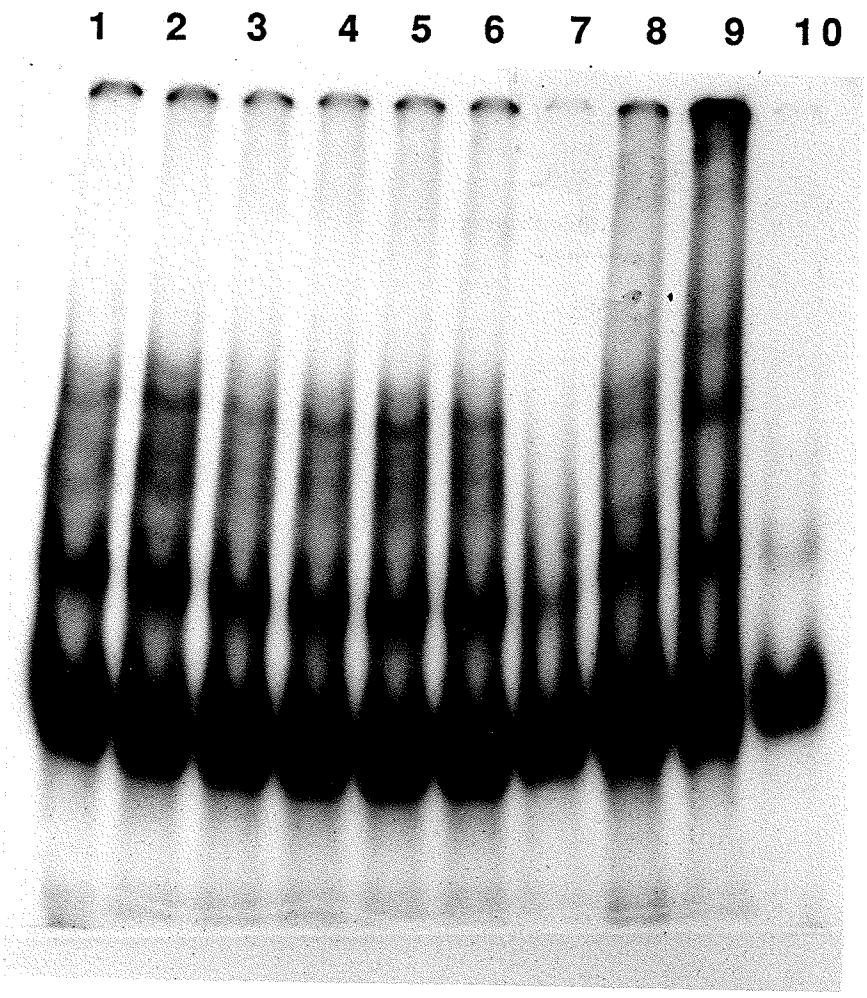


Figure 11. Gradient SDS-PAGE of lyophilized medium after incubation of ^{125}I-PIIIP with SMC or medium. Incubations for lanes 1-6 and 9 were with confluent SMC cultures in DMEM containing: lane 1, ^{125}I-PIIIP and 50 µg/ml FGN; lane 2, ^{125}I-PIIIP and 50 µg/ml BSA; .lane 3, ^{125}I-PIIIP and 50 µg/ml FGN (SMC were preincubated for 24 h with butyrate for lanes 3 and 4); lane 4, ^{125}I-PIIIP and 50 µg/ml FGN and 15 ng cold PIIIP; lane 5, ^{125}I-PIIIP alone; lane 6, ^{125}I-PIIIP and 50 µg/ml casein. Lane 7, ^{125}I-PIIIP and 200 µg/ml casein were incubated in conditioned medium without SMC. Lane 8, ^{125}I-PIIIP was incubated wih conditioned medium obtained by incubating SMC with 0.2 mM dansyl cadaverine. Lane 9, ^{125}I-PIIIP was incubated with SMC in DMEM containing 0.2 mM dansyl cadaverine. Lane 10 shows standard ^{125}I-PIIIP without any incubation.

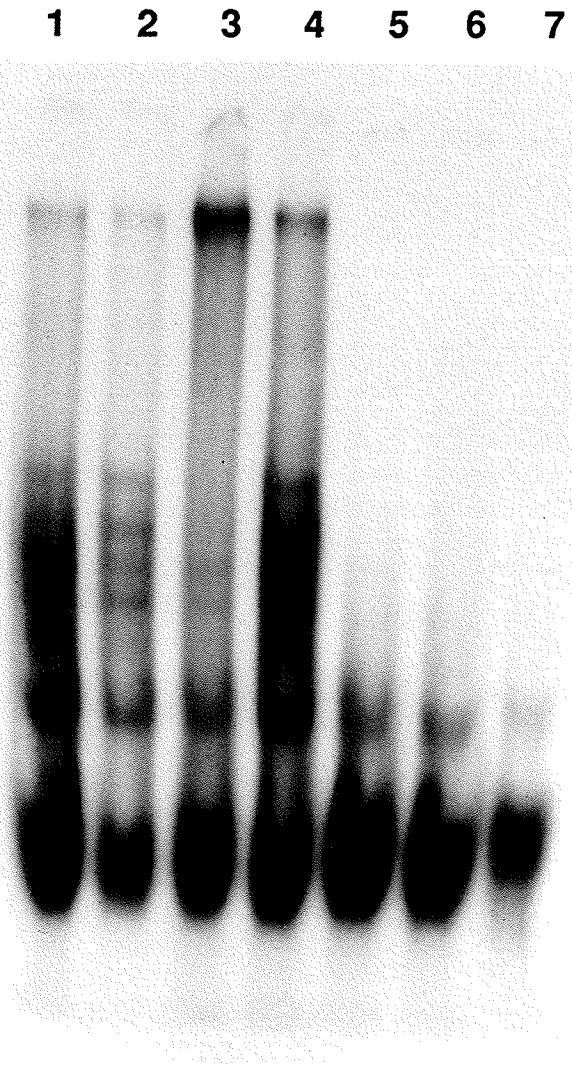


Figure 12. Gradient SDS-PAGE of lyophilized medium after incubation of ^{125}I-PIIIP with SMC or medium. SMC were incubated with ^{125}I-PIIIP, as described on p. 64 and 65, with the addition of 50 µg/ml FGN (lane 1), 50 µg/ml BSA (lane 2), 50 µg/ml casein (lane 3) and 50 µg/ml FGN with a butyrate preincubation (lane 4). Lane 5 shows the product after the incubation of ^{125}I-PIIIP plus 50 µg/ml FGN in conditioned media removed from cells after 90 min incubation. Lane 6 shows the 90 min incubation of ^{125}I-PIIIP with 50 µg/ml FGN in PBS plus 5 mM $CaCl_2$ in the absence of SMC. Lane 7 shows standard ^{125}I-PIIIP.

amount of the lower MW. However, when this incubation was repeated (Fig. 11, lanes 1 and 2) this difference is not apparent. When SMC were preincubated with butyrate there was an increased incorporation of the ^{125}I-PIIIP into large complexes (Fig. 12, lane 4) compared to just FGN. Once again, this difference was not evident in a repeated incubation (Fig. 11; lane 1-4). Addition of casein (Fig. 11; lane 6) and cold PIIIP (Fig. 11; lane 5) appeared to produce the same banding patterns as FGN in figures 11 and 12. When medium was removed from the cultures and incubated with ^{125}I- PIIIP very little of the high MW complexes were formed (Fig. 11, lane 7; Fig. 12, lane 5). Incubation of ^{125}I-PIIIP and FGN in PBS also showed no high MW band formation (Fig. 12, lane 6).

In an effort to determine if these high MW complexes were TGase-catalyzed the SMC were incubated with the inhibitor dansyl cadaverine. The gels from these experiments (Fig. 11, lanes 8 and 9) showed the formation of a large amount of high MW complexes in the dialysed media. The cells did not respond well to these incubations. The cells were quite unstable and did not adhere well to the culture dish. These suggested that the experimental procedure should be revised, as discussed below.

4. Discussion

The endogenous substrates of TGases are unknown and several investigators have attempted to determine these substrates by crosslinking putrescine, or other amines, to various proteins and cellular systems [277,297,298]. Hand et al [297] labelled normal hepatocytes and hepatocyte carcinomas with ^{14}C-methylamine and, after SDS-PAGE, found 14 kD bands along with very high MW complexes that did not enter the gels. Beninati et al [298] labeled CHO cells in culture with ^{3}H-putrescine and found two different pathways of protein labeling, one via hypusine synthesis producing low MW (18

kDa) bands on SDS-PAGE and another through the covalent binding of the putrescine label to protein through γ-glutamyl bonding producing very high molecular complexes. These γ-glutamyl bonds were suggested to be produced by TGase. The results shown in this study are consistent with those of Hand et al [297] and Beninati et al [298]. The low MW bands in Figure 9 may be due to hypusine synthesis and the high MW complexes due to TGase activity.

Birckbichler et al [242] showed that TGase activity was increased in lung fibroblast cultures when incubated with sodium butyrate. The present work shows that there is greater incorporation of ^{3}H-putrescine when the SMC are preincubated with butyrate or ascorbate (Table 5). These incubations were done in an effort to enhance the incorporation of label by increasing TGase activity and determine if an increase in collagen synthesis would produce a noticeable incorporation into collagen.

The putrescine labeling studies with SMC show that the banding patterns on the gels were similar to those of other investigators with different cell types [297,298]; therefore it seems likely that similar proteins are labelled in all the cell types examined so far. The observation that ^{3}H-putrescine labelling of high MW bands is a greater fraction of the total label in confluent cultures than in non-confluent cultures is also of interest (Fig. 9, lanes 3 and 5). This suggests that TGase activity is associated with cell contact. Further work is required to follow up this observation.

The results of the ^{125}I-PIIIP experiments show that SMC must be present for high MW complex formation to occur (Fig. 10-12). The high MW complexes are not formed in medium alone with ^{125}I-PIIIP, nor is there an enzyme catalyzing this reaction secreted into the medium during the incubation (Fig. 11, lane 7; Fig. 12, lanes 5 and 6); therefore the complexes must be formed by the

SMC. The results suggest that crosslinked complex formation during 90 minute incubation may be catalyzed on the surface of the cell layer.

Incubation times were kept at a minimum to reduce incorporation of the labeled PIIIP into the cell layer. It is possible that the PIIIP may have been endocytosed into the cells as has been shown for liver endothelial cells [243]. Smedsrod [243] recently showed that PIIIP is removed from circulation through receptor-mediated endocytosis by liver endothelial cells. Once internalized, the peptide is degraded. The incubations were limited to 90 minutes to keep endocytosis and degradation of the PIIIP to a minimum. It is not known if SMC possess such a receptor, nor is it known whether any high MW complexes are produced during degradation in liver endothelial cells. However, degradation would produce low MW bands on the gels and these are not seen in Figs. 9-11. Therefore, it is possible that covalent complex formation is catalyzed by an enzyme on the surface of the SMC.

The addition of FGN, BSA or casein to cell incubations containing ^{125}I-PIIIP gave varied results and the experiments are therefore inconclusive though 2 out of 3 experiments showed more higher MW material formed with FGN than BSA. The addition of the potent TGase inhibitor dansyl cadaverine seems to disrupt the cells, perhaps releasing large amounts of cytosolic enzyme into the medium (Fig. 11, lanes 8 and 9). The enzyme in the medium may catalyze covalent bond formation during dialysis, when no inhibitor was present. It is clear that in future work the experimental procedure should be revised by adding an inhibitor of TGase which does not harm the cells for incubations and adding a further inhibitor to all the dialysis fluids.

The covalent incorporation of putrescine into high MW substrates and the formation of high MW PIIIP complexes indicate that SMC in culture possess an active TGase enzyme. This activity may be located on the surface of the SMC as

is suggested for several other cell types [262,263,265,266,275,295,296]. This may allow for the covalent incorporation of FGN and LDL into the arterial wall, through linkage to PCIII and PIIIP in the ECM This process may contribute to the development of atherosclerotic lesions.

IV. SUMMARY

Section III.A showed by chromatography that bovine ^{3}H-labeled procollagen type III (PCIII) forms very high molecular weight compounds with excess bovine fibrinogen after incubation with purified transglutaminase. Equivalent complexes were formed by transglutaminase with 3H-PCIII plus plasma protein samples or BSA. Larger complexes formed with fibrinogen or fibrin monomers can be separated by centrifugation and are insoluble even after washing with 1% SDS.

Other work from this laboratory [286] showed by ultracentrifugation that ^{3}H-PCIII forms low density complexes after incubation with transglutaminase and LDL, but not with HDL. SDS-PAGE also showed that ^{125}I-aminopropeptide of type III procollagen (^{125}I-PIIIP) also forms high MW complexes after incubation with transglutaminase and excess LDL but not with HDL. Bowness and Tarr [287] later showed that human plasma samples contain similar high molecular weight, low density fractions of PIIIP antigen. These fractions were large in 2 of 7 myocardial infarct patients.

In section III.B transglutaminase activity and antigen were shown to be higher in the aortas of cholesterol-fed rabbits than in rabbits fed a normal diet. Three layers from aortas of rabbits fed a high cholesterol diet or a normal diet were separated and assayed for transglutaminase activity and tissue transglutaminase antigen. The inner and middle layers of the aortas with atherosclerotic lesions, from cholesterol-fed rabbits, showed increased transglutaminase activity and antigen compared to aortas from normal rabbits. All three layers showed a large amount of transglutaminase activity and antigen that was not extractable by buffer or Triton X-100. The presence of a particulate enzyme as described by Tyrell et al [262,295] or an association with collagen

during homogenization as described by Juprelle-Soret et al [296] may explain the large amount of transglutaminase activity and antigen in the residue fractions.

In section III.C it was shown that the protein bands labeled by 3H-putrescine in smooth muscle cells were similar to those found for other cells by other investigators. Incubation of ^{125}I-PIIIP with smooth muscle cells or culture medium showed that the formation of high molecular weight material labeled with ^{125}I-PIIIP did not occur in the absence of smooth muscle cell culture. Other workers have shown that the transglutaminase present in macrophages can catalyze crosslinking of fibrinogen from the medium and at the cell surface [275]. These results suggest that the same may be true for smooth muscle cells. In any case, it is known that atherosclerotic lesions contain macrophages as well as smooth muscle cells [1,16-24].

All these results obtained are consistent with the hypothesis shown in Fig. 1 of section I.A.1, though they do not prove it. Elevated concentrations of fibrinogen and LDL in the arterial wall are known to be associated with the atherosclerotic process [2]. It is hypothesized that with the normal low concentrations of fibrinogen and LDL in the arterial wall transglutaminase does not form significant amounts of crosslinks. However, under the conditions where fibrinogen and LDL are greatly increased, transglutaminase may catalyze crosslinks between fibrinogen or LDL and PCIII or PIIIP. It is suggested that the binding of the PIIIP to LDL and fibrinogen would modify them, increasing their accumulation by macrophages and smooth muscle cells and result in foam cell formation. The sequestering of PIIIP in these complexes could also interfere with the feedback inhibition of smooth muscle cell collagen production by PIIIP, thus causing the accumulation of collagen found in atherosclerotic lesions.

These results provide no answers to the cure of cardiovascular disease, but the results obtained here do advance our knowledge of the processes that are active in the development of atherosclerosis. Further studies may include the determination of the amount of transglutaminase-catalyzed crosslink present in each layer of human atherosclerotic plaques. This would conclusively show the amount of activity of transglutaminases in the arterial wall that is associated with the development of atherosclerosis. Future studies may then involve the use of specific inhibitors of tissue transglutamiunase to promote the arrest of the development of atherosclerotic lesions or eventually their regression.

The work in this thesis contributed to presentations at the following meetings:

Can. Fed. Biol. Soc. (1988)
Can. Fed. Biol. Soc. (1989)
West. Connec. Tiss. Soc. (1990)

and publishing of the following papers:

Bowness, J.M., Tarr, A.H. and Wiebe, R.I. (1989) Transglutaminase-catalyzed crosslinking: a potential mechanism for the interaction of fibrinogen, low density lipoprotein and arterial type III procollagen. *Thromb. Res.* **54**, 357-367.

Wiebe, R.I., Tarr, A.H. and Bowness, J.M. (1991) Increased transglutaminase in the aortas of cholesterol-fed rabbits; occurence of buffer soluble and insoluble forms and an inhibitor. *Biochem. Cell Biol.* (accepted for publishing).

V. REFERENCES

1. Ross, R. (1986) *N. Eng. J. Med.* **314**, 488.
2. Smith, E.B. (1991) *Biochem. Soc. Trans.* **19**, 235.
3. Duckert, F. and Nyman, D. (1978) *Thromb. Haemostas.* **63**, 391.
4. Bowness, J.M., et al (1988) *Biochim. Biophys. Acta* **967**, 234.
5. Velican, C and Velican, D (1989) **Natural History of Coronary Atherosclerosis**: CRC Press Inc., Boca Raton, Fla.
6. McGill, H.C.J. (1988) *Clin. Chem.* **34**, B33.
7. Schwartz, C.J., et al (1989) *Am. J. Cardiol.* **64**, 23G.
8. Munro, M.J. and Cotran, R.S. (1988) *Lab. Invest.* **58**, 249.
9. Campbell, J.H. and Campbell, G.R. (1989) *Clin. Exper. Hyper.* **A11**, 901.
10. Haust, M.B. (1972) *Monographs in Atherosclerosis*
11. Stary, H.C. (1987) *Atherosclerosis* **64**, 91.
12. Velican, C. and Velican, D. (1979) *Atherosclerosis* **33**, 301.
13. Velican, C. and Velican, D. (1976) *Atherosclerosis* **23**, 345.
14. Gerrity, R.G., et al (1979) *Am. J. Pathol.* **95**, 775.
15. McGill, H.C.Jr. (1974) *Atherosclerosis III* , 27.
16. Rosenfeld, M.E., et al (1987) *Arteriosclerosis* **7**, 9.
17. Mazzone, T., et al (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 5094.
18. Gerrity, R.G., et al (1985) *Arteriosclerosis* **5**, 55.
19. Rosenfeld, M.E., et al (1987) *Arteriosclerosis* **7**, 24.
20. Faggioto, A., et al (1984) *Arteriosclerosis* **4**, 323.
21. Faggioto, A. and Ross, R. (1984) *Arteriosclerosis* **4**, 341.
22. Gerrity, R.G. (1981) *Am. J. Pathol.* **103**, 181.
23. Gerrity, R.G. (1981) *Am. J. Pathol.* **103**, 191.
24. Masuda, J. and Ross, R. (1990) *Arteriosclerosis* **10**, 164.
25. Quinn, M.T., et al (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 2995.
26. Parthasarathy, S., et al (1989) *Arteriosclerosis* **9**, 398.
27. McGill, H.C.Jr. (1984) *Arteriosclerosis* **4**, 443.
28. Robertson, W.B., et al (1963) *Exp. Mol. Pathol.* **S1**, 28.
29. Greer, J.C., et al (1968) *Lab. Invest.* **18**, 565.
30. Katz, S.S. (1981) *J. Biol. Chem.* **256**,12275.
31. Smith, E.B. (1975) *Adv. Exp. Med. Biol.* **57**, 254.

32. Smith, E.B. and Smith, R.M. (1976) *Atherosclerosis Rev.* **1**, 119.
33. Smith, E.B. and Staples, E.M. (1981) *Lancet* **i**, 1171.
34. Haust, M.B. (1971) *Am. J. Pathol.* **86**, 665.
35. Smith, E.B. (1977) *Am. J. Pathol.* **39**, 521.
36. Woolf, (1961) *Am. J. Pathol.* **39**, 521.
37. Ross, R. and Wight, T.N. (1984) *Am. J. Pathol.* **114**, 79.
38. Jonasson, L., et al (1986) *Arteriosclerosis* **6**, 131.
39. Jaffe, E.A. (1987) *Human Pathol.* **18**, 234.
40. Wallis, W.J., et al (1986) *Pathol. Immunopathol. Res.* **5**, 73.
41. Stern, D.M., et al (1988) *Haemostasis* **4-6**, 202.
42. Moncado, S., et al (1977) *Thromb. Res.* **11**,323.
43. Furchgott, R.F. (1983) *Circ. Res.* **53**, 557.
44. Gajdusek, C.M., et al (1980) *J. Cell Biol.* **85,** 467.
45. DiCorleto, P.E., et al (1983) *J. Cell Physiol.* **114**, 339.
46. DiCorleto, P.E. and Bowen-Pope, D.F. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 1919.
47. Castellot, J.J.Jr., et al (1981) *J. Cell Biol.* **90**, 372.
48. Castellot, J.J.Jr., et al (1982) *J. Biol. Chem.* **257**, 11256.
49. Fox, P.L. and DiCorleto, P.E. (1984) *J. Cell Physiol.* **121**,298.
50. Barrett, T.B., et al (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 6772.
51. Schwartz, S.M. and Benditt, E.P. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**,651.
52. Schwartz, S.M. and Benditt, E.P. (1977) *Circ. Res.* **41**, 248.
53. Gimbrone, M.A.Jr. (1976) *Prog. Hemost. Thromb.* **3**, 1.
54. Gajdusek, C.M., et al (1986) *J. Cell Biol.* **103**, 419.
55. Esman, C.T. and Owen, W.G. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 2249.
56. Owen, W.G. and Esman, C.T. (1981) *J. Biol. Chem.* **256**, 5532.
57. Walker, F.J., et al (1979) *Biochim. Biophys. Acta* **571**, 333.
58. Walker, F.J. (1981) *J. Biol. Chem.* **256**, 11128.
59. Savion, N., et al (9181) *J. Biol. Chem.* **256**, 4514.
60. Collen, D.(1980) *Thromb. Haemost.* **43**, 77.
61. Willis, A.L., et al (1986) *Lancet* **ii**, 682.
62. Moncada, S. (1982) *Arteriosclerosis* **2**,193.
63. Hopkins, N.K. and Gorman, R.R. (1981) *J. Clin. Invest.* **67,** 540.
64. Tremoli, E., et al (1985) *Arteriosclerosis* **5**, 178.
65. Burke, J.M. and Ross, R. (1979) *Int. Rev. Connect. Tiss. Res.* **8**, 119.

66. Muratak, K., et al (1986) *Atherosclerosis* **60**, 251.
67. Yla-Herttuala, S., et al (1986) *Lab. Invest.* **54**, 402.
68. Chait, A., et al (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 4084.
69. Witte, L.D. and Cornicelli, J.A. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 5962.
70. Rubin, K., et al (1988) *Lancet* **i**, 1353.
71. Madri, J.A., et al (1990) *Ann. N.Y. Acad. Sci.* **593**, 243.
72. Clowes, A.W. and Schwartz, S.M. (1985) *Circ. Res.* **56**, 139.
73. Webster, W.S., et al (1974) *Am. J. Pathol.* **76**, 245.
74. Schwartz, S.M., et al (1985) *Ann. N. Y. Acad. Sci.* **454**, 292.
75. Clowes, A.W., et al (1983) *Lab. Invest.* **49**, 208.
76. Gabbiani, G., et al (1982) *Lab. Invest.* **47**, 265.
77. Chanley-Campbell, G.R., et al (1981) *J. Cell Biol.* **89**, 379.
78. Stadler, E., et al (1989) *J. Cradiovasc. Pharm.* **14**, S1.
79. Campbell, G.R. and Campbell, J.H. (1990) *Ann. N. Y. Acad. Sci.* **598**, 143.
80. Campbell, G.R. and Campbell, J.H. (1985) *Exp. Mol. Pathol.* **42**, 139.
81. Bjokerud, S. and Ekroth, R. (1980) *Artery* **8**, 329.
82. Mosse, P.R.L., et al (1985) *Lab. Invest.* **53**, 556.
83. Mosse, P.R.L., et al (1986) *Arteriosclerosis* **6**, 664.
84. Skalli, O. and Gabbiani, G. (1985) *Pathol. Res. Pract.* **180**, 338.
85. Ang, A.H., et al (1990) *Biochem. J.* **265**, 461.
86. Campbell, J.H., et al (1983) *Atherosclerosis* **47**, 279.
87. Campbell, G.R., et al (1988) *Arch. Pathol. Lab. Med.* **112**, 977.
88. Rennick, R.E., et al (1988) *Atherosclerosis* **71**, 35.
89. Wissler, R.W. (1968) *J. Atherosc. Res.* **8**, 201.
90. Ross, R., et al (1990) *Science* **248**, 1009.
91. Goodman, L.V. and Majach, R. (1989) *J. Biol. Chem.* **264**, 5241.
92. Walker, L.N., et al (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 7311.
93. Fager, G., et al (1988) *Exp. Cell Res.* **176**, 319.
94. Fager, R., et al (1989) *In Vitro* **25**, 511.
95. Hosang, M. and Rouge, M. (1989) *J. Cardiovasc. Pharmacol.* **14**, S22.
96. Koyama, N., et al (1990) *Biochem. Biophys. Res. Comm.* **169**, 725.
97. Luscher, T.F. (1990) *Clin. Exper. Hypertens.* **A12**, 897.
98. Antio, I., et al (1989) *Artery* **16**, 72.
99. Gotendorst, G.R., et al (1982) *J. Cell Physiol.* **113**, 261.
100. Barath, P., et al (1990) *Am. J. Cardiol.* **65**, 297.
101. Assoian, R.K. and Sporn, M.B. (1986) *J. Cell Biol.* **102**, 1217.

102. Rappolee, D.A., et al (1988) *Science* **241**, 708.
103. Antonelli-Orlidge, A., et al (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 4544.
104. Merrilees, M.J. and Scott, L. (1990) *Atherosclerosis* **81**, 245.
105. Merrilees, M.J. and Scott, L. (1990) *Atherosclerosis* **81**, 255.
106. Hirata, Y., et al (1989) *Atherosclerosis* **78**, 225.
107. Nakaki, T., et al (1989) *Biochem. Biophys. Res. Comm.* **158**, 880.
108. Leibovich, S.J., et al (1987) *Nature* **329**, 630.
109. Lipid Research Clinics Program (1984) *JAMA* **251**, 365.
110. DiCorleto, E. and de la Motte, C.A. (1985) *J. Clin. Invest.* **75**, 1153.
111. Vesselinovitch, D., et al (1988) *Arch. Pathol. Lab. Med.* **112**, 1011.
112. Hansson, G.K., et al (1989) *Arteriosclerosis* **9**, 567.
113. Vranue, E.R. (1976) *Am. J. Pathol.* **83**, 396.
114. Leibovich, S.J. and Ross, R. (1975) *Am. J. Pathol.* **78,** 71.
115. Nathan, C.F. (1987) *J. Clin. Invest.* **79**, 319.
116. Valente, A.J., et al (1988) *Biochemistry* **27**, 4162.
117. Steinberg, D., et al (1988) *N. Eng. J. Med.* **320**, 915.
118. Gerrity, R.G. (1981) *Am. J. Pathol.* **103**, 191.
119. Gerrity, R.G., et al (1985) *Arteriosclerosis* **5**, 55.
120. Faggioto, A., et al (1984) *Arteriosclerosis* **4,** 323.
121. Ball, R.Y., et al (1989) *J. Cell Sci.* **93**, 163.
122. Brown, M.S. and Golstein, (1983) *Annu. Rev. Biochem.* **52**, 223.
123. Kodama, T., et al (1990) *Nature* **343**, 531.
124. Roher, L., et al (1990) *Nature* **343**, 570.
125. Hoff, H.F., et al (1990) *Eur. Heart J.* **11E**, 105.
126. Sparrow, C.P., et al (1989) *J. Biol. Chem.* **264**, 2599.
127. Arai, H., et al (1989) *Biochem. Biophys. Res. Comm.* **159**, 1375.
128. Rostlethwait, A.E. and Kang, A.H. (1976) *J. Exp. Med.* **143**, 1299.
129. Yonemasa, K., et al (1983) *Microbiol. Immunol.* **27,** 283.
130. Senior, R.M., et al (1980) *J. Clin. Invest.* **66**, 859.
131. Fleishmajer, R., et al (eds.) "Structure and Molecular Biology and Pathology of Collagen" (1990) *Ann. N. Y. Acad. Sci.* 580.
132. Burgeson, R.E. and Morris, N.P. in "Connective Tissue Disease" (1987) Uitto, J. and Perejda, ed.: Marcell-Dekker, Inc., N.Y.
133. Nimini, M.E. (1988) "Collagen" **v. 1,2,3**: CRC Press, Boca Raton, Fla.
134. Barnes, M.J. (1985) *Coll. Rel. Res.* **5**, 65.
135. Mayne, R. (1986) *Arteriosclerosis* **6**, 585.

136. Church, R.L. and Tanzer, M.L. (1975) *FEBS Lett.* **53**, 105.
137. Fessler, J.H., et al (1985) *Ann. N. Y. Acad. Sci.* **460**, 181.
138. Blobel, G. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 1496.
139. Kivirikko, K.I. and Myllyla, R. (1985) *Ann. N. Y. Acad. Sci.* **460**, 187.
140. Adams, E. and Frank, L. (1980) *Annu. Rev. Biochem.* **49**, 1005.
141. Kivirikko, K.I. and Myllyla, R. (1979) *Int. Rev. Conn. Tiss. Res.* **8**, 23.
142. Shinkai, H. and Lapiere, C.M. (1983) *Biochim. Biophys. Acta* **758**, 30.
143. Kuhn, K., et al (1982) *Conn. Tiss. Res.* **10**, 43.
144. Kuhn, K. (1984) *Coll. Rel. Res.* **4**, 309.
145. Timpl, R., et al (1975) *Z. Physiol. Chem.* **356**, 1783.
146. Cheung, P.T., et al (1983) *J. Biol. Chem.* **258**, 7774.
147. Fessler, I., et al (1981) *J. Biol. Chem.* **256**, 2531.
148. Ricard-Blum, S. and Ville, G. (1989) *Int. J. Biochem.* **11**, 1185.
149. Nusgens, B.U., et al (1980) *Biochem. J.* **191**, 699.
150. Fleishmajer, R., et al (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 3354.
151. Nimini, M.E. (1983) *Sem. Arthr. Rheum.* **13**, 1.
152. Fleischmajer, R., et al (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 7360.
153. Wiestner, M., et al (1979) *J. Biol. Chem.* **254**, 7016.
154. Fleischmajer, R., et al (1985) *Ann. N. Y. Acad. Sci.* **460**, 246.
155. Fleischmajer, R., et al (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 7360.
156. Miller, E.J. (1985) *Ann. N. Y. Acad. Sci.* **460**, 1.
157. McCullaugh, K.G. and Ballan, G. (1975) *Nature* **258**, 73.
158. Rauterberg, J., et al (1977) *Z. Physiol. Chem.* **358**, 401.
159. Morton, L.F. and Barnes, M.J. (1982) *Atherosclerosis* **42,** 41.
160. Szyamanowicz, A., et al (1982) *Artery* **10**, 250.
161. Ooshima, A. (1981) *Science* **213**, 666.
162. Labat-Robert, J., et al (1985) *Pathol. Biol.* **33**, 261.
163. DeCrombruggle, B., et al (1990) *Ann. N. Y. Acad. Sci.* **580**, 88.
164. Silbert, J.E. in "Connective Tissue Disease": Uitto, J. and Perejda, A.J., eds. (1987) Marcel-Dekker, Inc., New York.
165. Berenson, G.S., et al (1985) *Ann. N. Y. Acad. Sci.* **454**, 69.
166. Wight, T.N. (1989) *Arteriosclerosis* **9**, 1.
167. Radhakrishnamurthy, B. (1990) *Eur. Heart J.* **11E**, 148.
168. Vijayagopal, P. (1983) *Biochim. Biophys. Acta* **758**, 70.
169. Oegema, T.R. (1979) *J. Biol. Chem.* **254**, 1312.
170. McMurtrey, J. (1979) *J. Biol. Chem.* **254**, 1612.

171. Wagner, W.D., et al (1986) *Arteriosclerosis* **6**, 407.
172. Dalfrese, E.R. (1987) *Exp. Mol. Pathol.* **47**, 363.
173. Berenson, G.S., et al (1988) *Arch. Pathol. Lab. Med.* **112**, 1002.
174. Murata, K. and Yokoyama, Y. (1989) *Atherosclerosis* **78**, 69.
175. Cherchi, G.M., et al (1990) *Matrix* **10**, 362.
176. Kinesella, M.G. and Wight, T.N. (1990) *J. Biol. Chem.* **265**, 17891.
177. Wagner, W.D., et al (1989) *Atherosclerosis* **75**, 49.
178. Srinivasan, S.R., et al (1989) *Biochim. Biophys. Acta* **106**, 159.
179. Vijayagopal, P., et al (1980) *Lab. Invest.* **42**, 190.
180. Vijayagopal, P., et al (1988) *Biochem. J.* **255**, 639.
181. Vijayagopal, P., et al (1985) *Biochim. Biophys. Acta* **837**, 251.
182. Falcone, D.J., et al (1984) *J. Cell Biol.* **99**, 1266.
183. Smith, D.W., et al (1968) *Biochem. Biophys. Res. Comm.* **31**, 309.
184. Foster, J.A., et al (1980) *Biocemistry* **19**, 857.
185. Siegel, R.C. (1979) *Int. Rev. Connect. Tiss. Res.* **8**, 73.
186. Ross, R. and Bornstein, P. (1969) *J. Cell Biol.* **40**, 366.
187. Page, R.C. and Benditt, E.P. (1972) *Lab. Invest.* **26**, 22.
188. Jacques, A. and Serafin-Fracassini, L. (1986) *J. Ultrastruct. Mol. Struct. Res.* **95**, 218.
189. Giro, M.G., et al (1985) *J. Clin. Invest.* **75**, 672.
190. Moczar, M. (1989) *Matrix* **9**, 28.
191. Yea-Herttuba, S., et al (1986) *Lab. Invest.* **54**, 402.
192. Katsuda, S., et al (19900 *Exp. Mol. Pathol.* **52**,13.
193. Kramsch, D.M. and Hollander, W. (1973) *J. Clin. Invest.* **52**, 236.
194. Robert, L., et al (1984) *Mech. Ageing Dev.* **28**, 155.
195. Podet, E.J., et al (1991) *Arteriosclerosis* **11**, 116.
196. Bocan, T.M.A. (1986) *Am. J. Pathol.* **123**, 413.
197. Srinivasan, S.R. (1981) *Atherosclerosis* **38**, 137.
198. Labat-Robert, J., et al (1986) in "Fronteirs of Matrix Biology", v11, Skarger, Basel.
199. Yamada, K.M. and Olden, K. (1978) *Nature* **275**, 179.
200. Hynes, R.O. and Yamada, K.M. (1982) *J. Cell Biol.* **95**, 369.
201. Ruoslahti, E. (1988) *Annu. Rev. Biochem.* **57**, 375.
202. Hynes, R.O. (1985) *Annu. Rev. Cell Biol.* **1**, 67.
203. Ruoslahti, E. and Pierschabacher, D. (1987) *Science* **238**, 491.
204. Ginsberg, M.H., et al (1988) *Thromb. Haemost.* **59**, 1.

205. Couchman, J.R., et al (1979) *Arch. Dermatol. Res.* **266**, 295.
206. Stenman, S. and Valheri, A. (1978) *J. Exp. Med.* **147**, 1054.
207. Jensen, B.A., et al (1983) *Histochemistry* **77**, 395.
208. Shekhonin, B.V., et al (1987) *Atherosclerosis* **67**, 9.
209. Viehara, A., et al (1988) *J. Nucl. Med.* **7**, 1264.
210. Smith, E.B. and Ashal, K. (1986) *Biochim. Biophys. Acta* **880**, 10.
211. Shekhonin, B.V., et al (1990) *Atherosclerosis* **82**, 213.
212. Hahn, L.H.E. and Yamada, K.M. (1979) *Cell* **18**, 1043.
213. Furie, M.B., et al (1980) *J. Biol. Chem.* **255**, 4391.
214. Ruoslahti, E. (1979) *J. Biol. Chem.* **254**, 6054.
215. Mosher, D.F., et al (1980) *J. Biol. Chem.* **255**, 1181.
216. Birckbichler, P.J. and Patterson, M.K.Jr. (1978) *Ann. N. Y. Acad. Sci.* **312**, 354.
217. Mosher, D.F. (1975) *J. Biol. Chem.* **250**, 6614.
218. Grinnell, F., et al (1980) *Cell* **19**, 517.
219. Saba, T.M. and Jaffe, E. (1980) *Am. J. Med.* **68**, 577.
220. Grinnell, F., et al (1981) *J. Invest. Derm.* **76**, 181.
221. Engvall, E., et al (1978) *J. Exp. Med.* **147**, 1584.
222. Plow, E.F. and Ginsberg, M.H. (1981) *J. Biol. Chem.* **256**, 9477.
223. Mosher, D.F. (1984) *Mol. Cell. Biochem.* **58**, 63.
224. Ernst, E. (1990) *J. Int. Med.* **227**, 365.
225. Doolittle, R.F. (1984) *Annu. Rev. Biochem.* **53**, 195.
226. Okada, M., et al (1985) *J. Biol. Chem.* **260**, 1181.
227. Smith, E.B., et al (1976) *Atherosclerosis* **23**, 19.
228. Smith, E.B. and Staples, E.M. (1981) *Lancet* **i**, 1171.
229. Smith, E.B., et al (1990) *Arteriosclerosis* **10**, 263.
230. Shekhonin, B.V., et al (1990) *Atherosclerosis* **82**, 213.
231. Bini, A., et al (1989) *Arteriosclerosis* **9**, 104.
232. Laki, K., et al (1972) *Ann. N. Y. Acad. Sci.* **202**, 235.
233. Smith, E.B. (1986) *Clin. Heamatol.* **15**, 355.
234. Wolhelmsen, L., et al (1984) *N. Eng. J. Med.* **311**, 501.
235. Kannel, W.B., et al (1987) *JAMA* **258**, 1183.
236. Richardson, D.L., et al (1976) *Br. J. Haematol.* **32**, 507.
237. Ishida, T. and Tanaka, K. (1982) *Atherosclerosis* **44**, 161.
238. Watanabe, K. and Tanaka, K. (1983) *Atherosclerosis* **48**, 57.
239. Naito, M., et al (1989) *FEBS Lett.* **247**, 358.

240. Lorenzet, R., et al (1987) *J. Cell Biol.* **105**, 224.
241. Smith, E.B. (1990) *Eur. Heart J.* **11**, E72.
242. Birckbichler, P.J., et al (1983) *Biochim. Biophys. Acta* **723**, 27.
243. Smedsrod, B. (1988) *Coll. Rel. Res.* **8**, 375.
244. Smith, E.B. (1974) *Adv. Lipid Res.* **12**, 1.
245. Guyton, J.R., et al (1985) *Arteriosclerosis* **5**, 644.
246. Shen, B., et al (1976) *J. Lipid Res.* **17**, 125.
247. Kovanen, P.T. (1990) *Eur. Heart J.* **11**, E238.
248. Yla-Herttulla, S. (1991) *Ann. N. Y. Acad. Sci.* **623**, 40.
249. Orekhov, A.N., et al (1988) *Circ. Res.* **62**, 421.
250. Tertov, V.V. (1989) *Exp. Mol. Pathol.* **50**, 337.
251. Orekhov, A.N., et al (1990) *Circ. Res.* **66**, 311.
252. Weigensberg, B.I. (1985) *Atherosclerosis* **57**, 87.
253. Smith, E.B., et al (1990) *Atherosclerosis* **84**, 165.
254. Folk, J.E. (1980) *Annu. Rev. Biochem.* **49**, 517.
255. Lorand, L. and Conrad, S.M. (1984) *Mol. Cell. Biochem.* **58**, 9.
256. Ichinose, A., et al (1990) *J. Biol. Chem.* **265**, 13411.
257. Lorand, L., et al (1987) *Biochemistry* **26**, 308.
258. Maccioni, R.B. and Arechaga, J. (1986) *Exp. Cell Res.* **167**, 266.
259. Davies, P.A. and Murtaugh, M.P. (1984) *Mol. Cell. Biochem.* **58**, 69.
260. Chang, S.K. and Chung, S.I. (1986) *J. Biol. Chem.* **261**, 8112.
261. Fesus, L. and Thomazy, V. (1988) *Adv. Exp. Med. Biol.* **231**, 119.
262. Tyrell, D.J., et al (1988) *J. Biol. Chem.* **263**, 1946.
263. Hormann, H., et al (1987) *Thromb. Res.* **46**, 39.
264. Wiestner, M., et al (1979) *J. Biol. Chem.* **254**, 7016.
265. Barsigian, C., et al (1988) *J. Biol. Chem.* **263**, 14015.
266. Fellin, F.M., et al (1988) *J. Biol. Chem.*, **263**, 1791.
267. Lorand, L., et al (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 1057.
268. Lorand, L., et al (1966) *Biochem. Biophys. Res. Comm.* **23**, 828.
269. Laki, K., et al (1972) *Ann. N. Y. Acad. Sci.* **202**, 235.
270. Korner, G., et al (1989) *Biochem. J.* **262**, 633.
271. Greenberg, C.S., et al (1987) *Blood* **70**, 702.
272. Roth, W.J., et al (1988) *Adv. Exp. Med. Biol.* **231**, 161.
273. Murtaugh, M.P., et al (1983) *J. Biol. Chem.* **258**, 11074.
274. Roth, W.J., et al (1983) *J. Leuk. Biol.* **42**, 9.
275. Conkling, P.R., et al (1989) *Thromb. Res.* **55**, 57.

276. Shainoff, J.R., et al (1991) *J. Biol. Chem.* **266**, 6429.
277. Bowness, J.M., et al (1987) *J. Biol. Chem.* **262**, 1022.
278. Lee, S.Y. and Chung, S.I. (1979) *Thromb. Diath. Haemorrh.* **42**, 379.
279. Bowness, J.M., et al (1988) *Biochim. Biophys. Acta* **967**, 234.
280. Cocuzzi, E., et al (1990) *Biochem. J.* **265**, 707.
281. Bonnet, J., et al (1988) *Europ. J. Clin. Invest.* **18**, 18.
282. Sage, H. and Bornstein, P. (1982) *Meth. Enzymol.* **82**, 96.
283. Ross, R. (1971) *J. Cell Biol.* **50**, 172.
284. Abe, T., et al (1977) *Biochemistry* **16**, 5495.
285. Folk, J.E. and Finlayson, J.S. (1977) *Adv. Prot. Chem.* **31**,2.
286. Bowness, J.M., et al (1989) *Thromb. Res.* **54**, 357.
287. Bowness, J.M. and Tarr, A.H. (1990) *Biochem. Biophys. Res. Comm.* **170**, 519.
288. Bowness, J.M., et al (1987) *Connect. Tiss. Res.* **16**, 57.
289. York, J.L. and Benjamin, W.S. (1981) *Thromb. Res.* **21**, 475.
290. Lee, K.N., et al (1987) *Biochim. Biophys. Acta* **928**, 22.
291. Birckbichler, P.J., et al (1985) *Hybridoma* **4**, 179.
292. Opsahl, W.P., et al (1987) *Arteriosclerosis* **7**, 470.
293. Duff, G.L. and McMillan, G.C. (1949) *J. Exp. Med.* **89**, 611.
294. Benko, A. and Laki, K. (1968) *Biochem. Biophys. Res. Comm.* **31**, 231.
295. Tyrell, D.J., et al (1986) *J. Biol. Chem.* **261**, 14833.
296. Juprelle-Soret, M., et al (1988) *Biochem. J.* **250**, 421.
297. Hand, D., et al (1990) *Biochim. Biophys. Acta* **1033**, 57.
298. Beninati, S., et al (1988) *Biochim. Biophys. Acta* **952**, 325.
299. Webster, D. (1962) *Clin. Chem. Acta* **7**,277.
300. Sasaki, J., et al (1982) *Biochim. Biophys. Acta* **713**, 199.