NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

THE UNIVERSITY OF ALBERTA

INITIAL RESPONSES TO ENDOTHELIAL INJURY: OBSERVATIONS ON PLATELET ADHESION AND LIPID METABOLISM

BY

THUR WANG

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF PHYSIOLOGY

EDMONTON, ALBERTA

SPRING 1989

ISBN 0-315-52999-7

THE UNIVERSITY OF ALBERTA

RELEASE FORM

NAME OF AUTHOR: THUR WANG

TITLE OF THESIS: INITIAL RESPONSES TO ENDOTHELIAL INJURY: OBSERVATIONS ON PLATELET ADHESION AND LIPID METABOLISM

DEGREE: MASTER OF SCIENCE

YEAR THIS DEGREE GRANTED: 1989

Permission is hereby granted to THE UNIVERSITY OF ALBERTA LIBRARY to reproduce single copies of this thesis and to lend or sell such copies for private, scholarly or scientific research purposes only.

The author reserves other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without the author's written permission.

Wang Thur
(Student's signature)

448E Michener Park
Edmonton Alberta
(Student's permanent address)

Date: April 20, 1989

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled INITIAL RESPONSES TO ENDOTHELIAL INJURY: OBSERVATIONS ON PLATELET ADHESION AND LIPID METABOLISM submitted by Thur Wang in partial fulfilment of the requirements for the degree of Master of Science.

.....
(Supervisor)

.....

.....

.....

.....
(External Examiner)

Date: 31st March 1989

DEDICATION

To my parents and my brother in China.

I've missed being with you these past three years.

ABSTRACT

In this present study the initial responses to endothelial cell (EC) injury were investigated in terms of interactions of platelets with injured EC *in vivo* and incorporation of radio-labelled precursors into the arterial wall *in vitro*.

In vivo EC injury was produced by exposure to either low concentration (0.01-0.7%) triton-X100 (rat aorta) or dry air (rabbit carotid arteries). EC injury was classified, based on morphological changes, into several categories, such as apparently normal endothelial cells, mildly injured EC, lysed EC and de-endothelialization. Platelet density was quantified by SEM. The relationships between platelet adhesion and the grade of EC injury were analyzed both quantitatively and morphologically. Acute EC injury by triton showed progression during a 60 minute period. *In vivo* platelet adhesion and activation on the arterial wall was EC injury degree-dependent. These relationships were further demonstrated and confirmed in triton foam- and air dry-injured arteries where a full spectrum of graded EC injury was produced in the same specimen. It would appear that any adherent part of injured EC has a structural barrier role, preventing circulating platelets from adhering to the artery. Similarly, EC perturbation or dysfunction does not readily initiate *in vivo* platelet adhesion without exposure of sub-endothelium.

In the metabolic study, an *in vitro* perfusion technique

was used. This technique preserved the integrity of EC lining and metabolic activity of the arterial wall. Such an approach represents a physiologic attempt to examine a complex cell system *in vitro*. In the metabolic study, there was no significant effect of *in vitro* mechanical de-endothelialization on the incorporation of ^{3}H-acetate into polar lipid and cholesterol in arterial tissue perfused with a serum containing medium. The *in vitro* air-dry injury under the conditions used in this study reduced the metabolic activity of the arterial wall. The potential of surrounding tissue contamination in the study of metabolism of the arterial wall was also noted.

ACKNOWLEDGEMENTS

I would like to thank Dr. Ken Hutchison for his supervision, encouragement and, in particular, patience throughout this work.

I am also indebted to Dr. Mark Poznansky for his collaboration and comments in part of this project.

I am very grateful to Dr. Lawrence Wang for making his computer facilities available to me, to Wendy Person for her assistance with the animal surgery, to Edith Schwaldt for her assistance with the electron microscopy and to Jeff Westly for his excellent computer graphics.

My deepest gratitude also goes to my loving wife, Dejuan Li and daughter, Ying-ying Wang for their sharing joyful and sometimes difficult times with me abroad.

Finally, many thanks to all members of the Physiology Department for their friendship, especially David Campbell.

TABLE OF CONTENTS

Chapter	Page
1. INTRODUCTION	1
1.1. Structure and Composition of the Normal Arterial Wall	1
1.1.1. Basic Organization	1
1.1.2. Basic Tissue Components	2
1.1.2.1. Endothelium	2
1.1.2.2. Vascular Smooth Muscle	3
1.1.2.3. Vascular Connective Tissue . .	3
1.2. Atherosclerosis: Nature and Mechanisms . . .	4
1.3. Platelet-Vessel Wall Interactions	7
1.3.1. Introduction	7
1.3.2. The Nature of the Arterial Surface . .	8
1.3.3. The Rheologic Factor	11
1.3.4. Platelets Spreading on the Sub-endothelium Prevent Further Platelet Deposition	13
1.3.5. Time Factor	14
1.3.6. Fibrin Deposition and Heparin	15
1.4. Function and Dysfunction of EC with Emphasis on Thrombogenicity	16
1.4.1. Endothelium as " Nature's Blood Compatible Container"	16

1.4.2. EC Dysfunction and Thrombogenic Potential 17
1.4.2.1. Introduction 17
1.4.2.2. Importance of EC Dysfunction . 18
1.4.2.3. Consequence of EC Dysfunction . 19
1.4.2.4. Detection of EC Dysfunction . . 21
1.5. Responses of Arterial Wall to EC Injury . . . 22
1.6. Objectives 24

2. MATERIALS and METHODS 26
2.1. Materials 26
2.2. Surgical Procedures 27
2.2.1. Surgery in Rats 27
2.2.2. Surgery in Rabbits 28
2.3. Endothelial Injury *In Vivo* 29
2.3.1. Chemical Injury of Endothelial Cells by Triton in Rats 29
2.3.2. Physical Injury of Endothelial Cells by Air-Drying in Rabbits 30
2.4. *In Situ* Perfusion-Fixation and Preparation for SEM 31
2.5. Evaluation of EC Injury and Quantification of Platelet Adhesion by SEM 33
2.5.1. Method of Sampling Injured Surfaces . 33
2.5.2. Categorization of Endothelial Cell Injury 33

2.5.3. Categorization of Adherent Platelets . 38
2.5.4. Methods of Quantification 40
2.5.5. Evaluation of EC Injury 40
2.5.6. Quantification of Platelet Adhesion . 41
2.6. Perfusion Apparatus for Metabolic Study . . . 42
2.7. Preparation of Perfusate and PBS 42
2.8. Transfer of the Arteries to Perfusion Apparatus 43
2.9. Tissue Analysis 43
2.10. Experimental Design 45
2.10.1. Morphological Study 45
2.10.1.1. Quantification of EC Injury and Platelet Adhesion 45
2.10.1.2. Morphological Demonstration in Triton Foam Injury and Air-drying Injury 45
2.10.1.3. Period of Blood-Vessel Wall Interaction 46
2.10.2. Metabolic Study 46
2.10.2.1. Effect of EC injury in Incubated Arteries 46
2.10.2.2. Effect of EC Injury in Perfused Arteries 47
2.10.2.3. Evaluation of Tissue Viability During Perfusion 48

2.10.2.4. Evaluation of Possible Contamination of Surrounding Membranous Tissue 48
2.10.2.5. Tissue Perfusion in a Glass Chamber 50
2.10.2.6. Metabolic Study in New Zealand White Rabbit Carotid 50
2.10.2.7. Evaluation of Endothelial Cell Integrity of Perfused Artery by SEM 50
2.11. Statistical Analysis 51

3. RESULTS 52
3.1. General Condition of the Rats 52
3.2. Morphological Study of the Relationship between Platelet Adhesion and EC Injury 52
3.2.1. Low Power Results with Triton Injury . 52
3.2.2. Medium Power Results with Triton Injury 55
3.2.3. Platelet Adhesion 58
3.2.3.1. Platelet Distribution 58
3.2.3.3. Observation of Interactions of Platelets with the Injured Surface at High Magnification 72
3.2.3.4. Platelet Adhesion at Branch Orifices 72

3.3. Labelled Precursor Incorporation 76
3.3.1. Tissue Incubation 76
3.3.2. Perfusion of Arterial Segments 76
3.3.3. Tissue Viability 80
3.3.4. Incorporation into Surrounding Tissue 85
3.3.5. Comparison of Chamber Types 85
3.3.6. Incorporation Study in New Zealand White Rabbit 85
3.3.7. SEM of In Vitro Perfused Artery . . . 90

4. DISCUSSION 93
4.1. Endothelial Injury 93
4.2. Interactions of Platelets with the Injured Arterial Wall 96
4.3. Perfusion Study of Arterial Wall Metabolism . 101

5. CONCLUSION 105

6. REFERENCES 107

LIST OF TABLES

Table	Description	Page
I	Density of platelets in each category of EC injury independent of dose and time.	66
II	Incorporation of ^{3}H-acetate into lipid and protein, and ^{35}S-methionine into protein in intact artery or *in vivo* air dry-injured artery.	81
III	Incorporation of ^{3}H-acetate into lipid and protein in intact or *in vitro* air dry-injured artery of New Zealand white rabbit.	89

LIST OF FIGURES

Figure	Description	Page
1	General condition of the rats examined at 5 and 60 minutes after EC injury.	53
2	Percentage of De-EC area of the injured rat aortas examined at 5 minutes and 60 minutes after graded triton injury.	56
3	Incidence of different types of EC injury examined at 5 (a) and 60 minutes (b).	59
4	Density of platelets adherent on the surface of the graded triton-injured rat aortas examined after 5 and 60 minutes of restoration of blood flow.	60
5	Density of total platelets in different types of EC injury area.	62
6	Density of activated platelets in different types of EC injury area.	63
7	Density of unactivated platelets in different types of EC injury area.	65
8	Incorporation of ^{3}H-acetate into polar lipid (a) and cholesterol (b) in incubated normal arteries or *in vivo* air dry-injured arteries.	77
9	Incorporation of ^{3}H-acetate into polar lipid (a) and cholesterol (b) in perfused normal arteries or *in vitro* mechanically injured arteries.	78
10	Incorporation of ^{3}H-acetate into polar lipid (a) and ^{35}S-methionine into protein (b) in perfused normal arteries or *in vitro* air dry-injured arteries.	79
11	Incorporation of ^{3}H-acetate into polar lipids (a) and cholesterol (b) in intact arteries perfused or incubated with 10% LPDS in MEM.	82
12	Accumulative incorporation of ^{3}H-acetate into polar lipid and cholesterol in perfused normal arteries after 4 to 16 hours of pulse.	83

13 Accumulative incorporation of ^{3}H-acetate and ^{35}S-methionine into protein in normal arteries as described in the Figure 12. 84

14 Incorporation of ^{3}H-acetate into polar lipids (a) and cholesterol (b) in perfused normal arteries and their surrounding tissue. 86

15 Incorporation of ^{3}H-acetate into polar lipids (a) and cholesterol (b) in surrounding tissue of perfused normal arteries or injured arteries. 87

16 Incorporation of ^{3}H-acetate into polar lipids in arteries perfused in glass or plastic chambers. 88

LIST OF PLATES

Plate		Page
1	SEM view of the surface of a normal rat aorta perfusion-fixed *in situ*.	35
2	SEM view of 0.05% triton treated rat aorta fixed after 5-minute restoration of blood flow.	36
3	SEM view of 0.05% triton treated rat aorta fixed after 5-minute restoration of blood flow.	37
4	The surface of 0.7% triton injured rat aorta fixed after 60-minute restoration of blood flow.	39
5	Low magnification view of 0.05% triton treated rat aorta.	54
6	SEM view of 0.01% triton treated rat aorta fixed after 60-minute restoration of blood flow.	57
7	SEM view of triton (0.1%) foam-injured surface of a rat aorta fixed after 60-minute restoration of blood flow.	67
8	Detailed view of the same specimen of Plate 7.	69
9	SEM view of an air-dry injured rabbit carotid artery.	70
10	SEM view of an air-dry injured surface of a rabbit carotid.	71
11	High magnification view of a triton-injured EC.	73
12	Triton injured surface in the region around a branching orifice in a rat aorta.	74
13	High magnification micrograph of injured rat aorta by 0.05% triton and fixed after a 5-minute restoration of blood flow.	75
14	SEM of perfused rabbit carotid artery with intact EC.	91
15	SEM of perfused carotid artery previously subjected to *in vivo* air-dry injury followed by a 3-hour restoration of blood flow.	92

LIST OF ABBREVIATIONS

BM	basement membrane
CPM	counts per minute
De-EC	de-endothelialized area
De-EC(B)	sub-endothelium without platelet adhesion
De-EC(P)	sub-endothelium with platelet adhesion
EC(s)	(normal) endothelial cell(s)
FB	fibrinogen
FN	fibronectin
GP	glycoprotein
HRP	horseradish peroxidase
IEC	injured endothelial cell(s)
IEL	internal elastic laminar
LDL	low density lipoprotein
LEC	destroyed endothelial cell(s)
LM	light microscopy
LPDS	lipoprotein-deficient serum
MEM	minimum essential medium
PC	phosphatidylcholine
PDGF	platelet-derived growth factor
PES-EC	partially exposed sub-endothelium
PGI_2	prostacyclin
RBC	Red blood cells
Sil G	silica gel G

SEM	scanning electron microscopy
SMC	smooth muscle cell(s)
TCA	trichloroacetic acid
TEM	transmission electron microscopy
TLC	thin layer chromatography
TSP	thrombospondin
VWF	von-Willebrand factor

1. INTRODUCTION

1.1. Structure and Composition of the Normal Arterial Wall

1.1.1. Basic Organization

All larger blood vessels exhibit a common plan of organization, and each specific type of vessel shows adaption for a particular purpose (Leeson and Leeson, 1985). The arterial tissue components are, by convention, arranged and classified as three concentric layers (tunics) (Bunce, 1974; Rhodin, 1974; Simionescu and Simionescu, 1977). These are the intima, the innermost layer, which is a three-layered structure consisting of endothelial cells (EC), sub-endothelial connective tissue and internal elastic lamina (IEL); the media which is composed of alternating layers of elastin lamellae and circumferentially arranged smooth muscle cells (SMC); and the adventitia, the outermost layer of the arterial wall, which consists of fibrous connective tissue, small blood vessels (vasa vasorum), lymphatic vessels and nerves. The adventitia merges with the connective tissue surrounding the artery.

1.1.2. Basic Tissue Components

Three basic structural constituents can be recognized in the arterial wall: the EC, the SMC and the connective tissue with large elastin and collagenous components.

1.1.2.1. Endothelium

The endothelium is the interfacing cell lining between the vessel wall and the blood. The unique position of EC's demands that they modulate thrombosis and vascular permeability (Stemerman, 1982). When an EC is viewed from the lumen, it is an approximately uniform repeating unit, polygonal in shape. Its prominent elongated nucleus is oriented in the long axis of the vessel and presumably arranged in the longitudinal vector fields generated by shearing effects of the blood flow (Flaherty et al., 1972b; Langille and Adamson, 1981; Nerem et al.,1981). The characteristic feature is the presence of numerous, uniform infoldings of cell membrane called micropinocytotic vesicles. These are open either on the blood front or on the tissue front or free in the cytoplasm (Rhodin, 1962). In addition, the EC contains a unique organelle called a Weibel-Palade body (Weibel and Palade, 1964) which is a reliable tag for the identification of isolated EC (Simionescu and Simionescu, 1977). At the abluminal surface of EC, an

amorphous matrix, called basement membrane, separates the EC from the deeper layer of sub-endothelium. Arterial EC's have more elaborate intercellular junctions (tight junctions and gap junctions) than EC's in other vessels. The EC may extend processes which penetrate the IEL and occasionally make close contact with the adjoining SMC (myoendothelial junction) (Simionescu and Simionescu, 1977).

1.1.2.2. Vascular Smooth Muscle

Smooth muscle cells (SMC) represent the only cellular element in the media of mammalian elastic arteries and are occasionally found in intima and adventitia especially in arteries subjected to repeated bending, such as carotid, axillary and iliac arteries (Pease and Molinari, 1960; Simionescu and Simionescu, 1977). Under pathological stimuli, SMC are capable of producing most connective tissue components (elastic fibres, collagen and glycosaminoglycans). They may migrate into the intima and proliferate resulting in intimal thickening.

1.1.2.3. Vascular Connective Tissue

Elastin fibres are organized in scattered bundles or in regular sheets alternating with SMC in the media. Collagen fibres are found throughout the wall. The collagen fibre has

a characteristic 64-nm periodicity, and are easily identified by transmission electron microscopy (TEM) (Stemerman, 1982). Collagen fibres are known to be highly reactive both to platelets and the coagulation system (Coller, 1987). It is believed that the sub-endothelium is synthesized by the EC on top of it and the SMC beneath or within it (Sixma and DeGroot, 1986). Therefore, the sub-endothelium is a highly varied structure both morphologically and chemically. It morphologically consists of BM, elastin and microfibrils. The BM is an amorphous structure of moderate electron density. Collagen fibres are seen in the sub-endothelial region but are not common (Stemerman, 1974). BM is composed of a unique type of collagen (type IV) which reacts less with platelets (Barnes and MacIntyre, 1979; Stemerman, 1982; Packham and Mustard, 1984).

Vascular connective tissue also contains a complex population of cells including fibroblasts, mast cells, macrophages and plasma cells.

1.2. Atherosclerosis: Nature and Mechanisms

Atherosclerosis represents the major cause of death from cardiovascular disease in the western hemisphere. That it is a disease of multiple risk factors has been amply substantiated and emphasized by Steinberg (1979) and more recently by Ross (1986).

The hallmark of this disease is atherosclerotic plaque formation which is characterized by a complex of processes including accumulation of extra- and intra-cellular lipid, proliferation of intimo-medial smooth muscle cells and formation of mural thrombi. According to Roessner's description of the morphogenesis of the plaque (Roessner, et al., 1987), atherosclerotic lesions are classified into several stages. The early changes are manifested by macroscopic yellow fatty patches or streaks, histologically identified as intimal aggregations of macrophages which have been transformed into foam cells by increased lipid storage. A progression of these early changes is considered to be the fibrous plaque. This contains in addition to foam cells, proliferating SMC embedded in a matrix of collagen, elastic lamellae and glycoproteins. Disruption of the endothelium over a fibrous plaque induces thrombotic aggregations and haemorrhages and produces a complicated atherosclerotic lesion.

The mechanism of the initiation and formation of the atherosclerotic plaque remains unclear. A number of theories, based on morphological investigations, have been developed to explain the pathogenesis of the disease such as the filtration theory, the thrombogenic theory and the response-to-injury theory (Reossner et al., 1987). Currently the most popular is the response-to-injury theory. It was proposed by Ross and Glomset (1976) and suggests that the loss of arterial EC is the primary lesion. This results in platelet adhesion to the

exposed sub-endothelium and consequent release of platelet derived growth factor (PDGF). PDGF causes proliferation of smooth muscle cells (SMC), a prominent feature of the atherosclerotic plaque (Ross and Glomset, 1976; Ross et al., 1978; Ross, 1986). In support of this hypothesis the following have been shown: (1) atherosclerotic lesions both in human and in experimental animal models tend to develop preferentially in certain regions of the arterial tree, and are not uniformly or randomly distributed (Flaherty et al., 1972a). Many of the early lesions are topographically related to arterial orifices and branching points where blood flow is disturbed and consequently the EC may be altered (Schwartz et al., 1983); (2) de-endothelialization caused by experimental trauma initiates rapid platelet adhesion, vascular SMC migration and proliferation and lipid accumulation in the intima - an atherogenic-like response (Stemerman and Ross, 1972; Bjorkerud and Bondjers, 1973; Fishman et al., 1975; Schwartz et al., 1975; Davies et al., 1976; Minick et al., 1977; Clowes et al., 1978; Moore, 1979); (3) animals with von Willebrand disease (VWD) show a marked resistance to the formation of atherosclerotic plaque as their platelet interaction with the sub-endothelium is defective (Weiss et al., 1978).

The response-to-injury hypothesis has been revised recently (Ross, 1986) as apparent EC denudation was not observed prior to plaque development (Faggiotto and Ross, 1984; Faggiotto et al., 1984). EC dysfunction without cell

loss, which may have no morphological manifestations (Kruth, 1985), is now considered the possible first event in atherogenesis (Stemerman et al., 1984). Recently, Roessner et al. (1987) proposed that the monocyte/endothelial interactions, possibly induced by functional alterations of the endothelium, may be the primary alteration. The monocytes which have reached the intima are transformed to macrophages. These are of special importance to lipoprotein metabolism, and either act as "scavenger"-cells or are transformed to foam cells.

1.3. Platelet-Vessel Wall Interactions

1.3.1. Introduction

Platelets normally circulate in the form of flattened discs at the periphery of the circulating cell mass. Platelets in the resting state have smooth surface contours. Typically, they have a large number of secretory granules (White, 1987).

The main function of the platelets is to arrest blood loss by adhering to sites of vascular injury (adhesion) and to each other (aggregation), producing a haemostatic platelet plug. This process involves several distinct steps known as platelet responses: adhesion, shape change, aggregation, secretion and arachidonate liberation (Zucker and Nachmias, 1985). However, to produce complete haemostasis, the

coagulation system must be activated on the surface of the platelets in the plug. This forms a mesh work of fibrin (Steen and Holmsen, 1987). The number of responses elicited depends on the number of stimulating agents and their potency. These platelet responses have been studied extensively in vitro. Since platelets are multi-responding cells, the responses of platelets in vivo must be complex processes and many biochemical and cellular factors determine the outcome of the responses (see reviews by Coller, 1987; Dejana, 1987; Mustard et al., 1987; Weiss et al., 1987; Gerrard, 1988). These factors have been organized into several broad categories: (1) the nature of the surface; (2) the integrity of the platelets; (3) the plasma protein cofactors; and (4) the activators and inhibitors. In addition, other factors also control the kinetics of the platelet-vessel wall interactions such as rheology and time. The following is an overview of the factors which are most important in vivo.

1.3.2. The Nature of the Arterial Surface

It is generally accepted that the EC of normal blood vessels is characteristically a non-thrombogenic, blood-compatible surface (Mustard et al., 1987). The reasons for this are complex and not yet fully understood (see further discussion in section 1.4.1.).

Damage to a blood vessel in vivo results in loss of

integrity of the EC lining and thus the connective tissue matrix becomes exposed to circulating platelets. It is proposed that the type of injury or the depth of the sub-endothelial layer exposed determines the amount of platelet adhesion to the surface (Dejana, 1987). This is probably because the sub-endothelial reactivity to platelet deposition depends on the amount of reactive components exposed. The composition of the sub-endothelial matrix is variable and still poorly defined. The best characterized of these reactive components is collagen, which has long been recognized as an important substrate for platelet adhesion (Sixma and DeGroot, 1986; Coller, 1987; Mustard et al.,1987). Considerable evidence indicates that type I and III collagen found in medial connective tissue (fibrillar form) are very reactive to platelets (Barnes and MacIntyre, 1979; Packham and Mustard, 1984). In vitro studies showed that platelet binding to purified collagen I and III is VWF and fibronectin dependent (Houdijk et al., 1985), whereas other evidence has indicated that collagen is not the binding site for plasma VWF in the sub-endothelium (Sixma et al., 1984). VWF deposited in the vessel wall by the EC was responsible for 40% of platelet adhesion and plasma VWF was required for optimal adhesion (Stel et al., 1985; Turitto et al., 1985). It is not known to which sub-endothelial matrix components plasma VWF binds (Sixma and DeGroot, 1986).

Compared with collagen type I and III, basement membrane

(BM), mostly composed of collagen type IV, is less reactive (Barnes and MacIntyre, 1979; Packham and Mustard, 1984). BM amorphous material and microfibrils are poor substrates for platelet adhesion. Therefore, collagen, if present, may be masked by other basement membrane materials (Baumgartner and Handenschild, 1972). Recently, it was proposed that the low reactivity of basement membrane may be due to the presence of the glycoprotein laminin which conceivably prevents "access" of platelets to collagen (Barnes, 1986). Another study (Buchanan et al., 1987) showed that the EC basement membrane when exposed by air-dry injury to the EC was initially (within one hour after injury) non-thrombogenic. This thrombo-resistance was gradually lost. It was proposed that this is due to an EC-derived chemorepellant called 13-hydroxyoctadecalinoic acid (13-HODE) which is supposed to decrease with time after EC injury. This mechanism was also suggested as an explanation for the difference in reactivity of sub-endothelium prepared by other techniques. For instance, the marked reactivity of sub-endothelium exposed by balloon-catheters is likely due to the more extensive injury produced by that technique. This leads to the mechanical removal of the more superficial portions of the sub-endothelium into which 13-HODE is released by the EC (Buchanan et al., 1987).

1.3.3. The Rheologic Factor

The high incidence of venous thrombosis associated with immobilization and tissue trauma (e.g. post-operatively) may be largely attributable to the combination of stimulation of coagulation and stasis from severely restricted blood flow (Mustard et al., 1987). It is also recognized that in atherosclerotic arteries, extensive thrombus formation occurs only in regions where the flow is disturbed and eddies form, such as in stenotic regions. This disturbed flow may activate platelets and cause their accumulation at the vessel surface (Mustard et al., 1987).

Initial studies on adhesion utilized the sticking of platelets to artificial surfaces in a static system. Since the development of the Baumgartner perfusion system (Baumgartner and Muggli, 1976), many in vitro studies on the platelet-biological surface interactions have been carried out. Elaborate cell culture techniques or vessel wall explants under well defined conditions of blood flow and blood composition have been used (Bourgain et al., 1986; Mustard et al., 1987).

Such studies have demonstrated totally different phenomena from those observed in static conditions. Exposing native blood to de-endothelialized aorta in vitro has shown, paradoxically, that increasing the shear rate increases

platelet deposition and decreases fibrin deposition (Weiss et al., 1986). The wall shear rate or flow dependency of platelet adhesion to sub-endothelium (Wekster, 1987) has been analyzed using classic mass-transport theory (Weiss et al., 1987). This analysis indicated that at low shear rates platelet attachment to the sub-endothelium was determined predominantly by physical factors controlling the rate of platelet transport to the sub-endothelium. In contrast, the influence of platelet surface reactivity becomes more evident at higher shear rates (Turitto and Baumgartner, 1979). This can be used to explain the phenomena observed in von-Willebrand disease and Bernard-Souler disease, where platelet interaction with sub-endothelial tissue (adhesion) is defective at high shear rates (microcirculation) but not at arterial shear rates (below 100/s) (Weiss et al., 1978).

The rheologic effect on the rate of removal of agonists for platelet activation and activated coagulation factors may also have a profound effect on the final result of platelet and fibrin deposition on the surface (Leonard, 1982; Drouet and Caen, 1986; Coller, 1987). In addition to the soluble substances, the red blood cells (RBC), which represent close to 50% of the circulating blood volume, may affect platelet-vessel wall interactions. There is a good correlation between the number of RBC and the incidence of thrombotic complications in the arterial system. Hemodilution is efficient in preventing arterial thrombosis (Drouet and Caen, 1986)

indicating platelet deposition may be highly dependent on the concentration of RBC. This effect was attributed in part to the fact that RBC increase the radial movement of platelets by shear-induced collisions (Weiss et al., 1987). This effect is also the explanation for the observed non-uniform distribution of platelets in the blood stream and the reason why an increasing shear rate increases platelet adhesion.

1.3.4. Platelets Spreading on the Sub-endothelium Prevent Further Platelet Deposition

When the sub-endothelium becomes covered with platelets, it also becomes rapidly (in 10 minutes) non reactive to further platelet deposition (Groves et al., 1979; Kinlough-Rathbone et al., 1983) (i.e. the platelets present a non-thrombogenic surface to the blood). Although the explanation is not clear (Turitto and Baumgartner, 1982), it may be that the receptors on the surface of adherent platelets are no longer capable of interacting with circulating platelets. Also the circulating platelets themselves may not be altered sufficiently to be able to adhere to the adherent platelets on the sub-endothelium (Mustard et al., 1987). Another explanation is that laminar blood flow rapidly removes the ADP and thromboxane A_2 (aggregating agents) that are released from the adherent platelets. The converse of these two explanations can be used to account for why additional platelets associate with

platelets on the wall and with each other forming an aggregate or platelet thrombus in regions where flow is disturbed and vortices and eddies can form (Packham and Mustard, 1984 and White, 1987). The initial adhesion may be followed by further platelet activation if the sticky surface exposes collagen fibrils or if the platelets are stimulated in other ways. Platelets that have merely contacted the surface and have not yet changed their shape to an appreciable extent, and those platelets in the initial stages of adhering, may be removed by the force of blood flow. However, platelets that have spread on the surface do not appear to detach readily (White, 1987).

1.3.5. Time Factor

In experimental models apparent thrombogenicity of exposed sub-endothelial surfaces lasts for only a few hours. At 10 minutes after de-endothelialization, the maximum formation of platelet thrombi has been reached. After 2 hours only a monolayer of platelets persists. In the following hours this layer becomes less and less populated, until after several days no further platelets adhere to the exposed sub-endothelial surface. This occurs long before EC cover the surface again (Groves et al., 1979; Drouet et al., 1983). The mechanisms are not established, but increased PGI_2 production by regenerating SMC after injury has been ruled out (Dejana et

al., 1980; Eldor et al., 1981).

1.3.6. Fibrin Deposition and Heparin

It is well known that lipids on the platelet surface can accelerate blood coagulation and that collagen can activate the coagulation mechanism (Turitto and Baumgartner, 1982). However most investigations of platelet function have been conducted in anticoagulated blood. Therefore, it is important to know how blood coagulation can affect platelet behaviour at surfaces. Studies have shown that the first platelet deposition on the sub-endothelium is not inhibited by heparin (Groves et al., 1979 and 1981; Dejana, 1987). This is also suggested by the fact that thrombin and fibrin do not contribute to this process (Groves et al., 1979; Packham and Mustard, 1984 and Dejana, 1987). Similarly, absence of fibrin formation, as in blood from fibrigenemic or haemophilic patients, does not alter platelet adhesion (Baumgartner et al., 1980). In contrast, heparin (at concentrations that inhibit coagulation) prevents thrombus formation and reduces platelet accumulation on the sub-endothelium (aggregation) (Groves et al., 1979 and 1982). The development of a fibrin meshwork is certainly necessary for the stabilization of the haemostatic plug (Turitto and Baumgartner, 1982). The blood coagulation-dependent platelet accumulation may be a platelet aggregate trapped in fibrin (Groves et al., 1979). This can also explain

the effect of blood flow and severity of injury on both platelet deposition and fibrin deposition. It was observed that platelet-fibrin thrombi develop where flow is not laminar or where there has been severe damage to a vessel. This can be due to activation of both the extrinsic and intrinsic pathways of coagulation (Mustard et al., 1987).

1.4. Function and Dysfunction of EC with Emphasis on Thrombogenicity

1.4.1. Endothelium as " Nature's Blood Compatible Container"

Under normal conditions the vascular EC lining is a non-thrombogenic surface (Dejana, 1987), "Nature's blood-compatible container" (Gimbrone, 1987). Unstimulated platelets do not adhere to the intact ECs in vivo (Stemerman, 1974; Dejana et al., 1980) and only minimally to intact EC monolayers in vitro. The latter apparently adherent platelets actually adhere to exposed sub-endothelium rather than to the cells themselves (Booyse et al., 1975; Curwen et al., 1980). Until recently this non-thrombogenic property was considered to be more passive than active (Dejana, 1987; Gimbrone, 1987) (i.e. the EC surface failed to activate the plasma coagulation system or to induce platelet adhesion). Thus the EC lining was viewed as a sort of insulation, preventing blood from interacting with more reactive sub-endothelial tissues. With the discovery that

EC can synthesize PGI_2 (the most potent naturally occurring inhibitor of platelet aggregation), a more active anti-thrombotic role for EC was suggested (Gimbrone, 1987). With the discovery of more and more biological activities of the EC several anti-thrombotic mechanisms have been described. These are the heparin-anti-thrombin mechanism, the protein C-thrombomodulin mechanism and the tissue plasminogen activator mechanism. Since most of these activities of EC have been characterized very recently, it remains to be clarified whether any of these activities are important in maintaining the non-thrombogenicity of EC (Groves et al., 1979; Dejana, 1987).

1.4.2. EC Dysfunction and Thrombogenic Potential

1.4.2.1. Introduction

Despite previous opinions to the contrary, endothelial cells when stimulated, appear to display thrombogenic activities (i.e. they might promote coagulation or platelet aggregation) (Dejana, 1987). This seems especially true since EC are capable of inducible functions. Thus they can respond to stimuli in ways that alter their haemostatic and immunologic potential and that alter their shape and behaviour (Ryan, 1987). These responses have been referred to as EC perturbation (Stemerman et al., 1984), dysfunction (Gimbrone, 1986), injury (Ross, 1986) or activation (Ryan, 1987). The

descriptions of such phenomena, as imperfect as they may be, are basically common. Any reversible or irreversible insult that perturbates or disrupts the normal working or adaptive functional state of the EC with or without morphologic manifestations will serve as a basis for this definition (Stemerman et al., 1984; Ross, 1986; Gimbrone, 1987).

1.4.2.2. Importance of EC Dysfunction

As previously noted, EC dysfunction is a new and important concept applied to vascular pathology and thrombosis because loss of EC has never been observed except in animals subjected to experimental trauma (Stemerman et al., 1984). Even in animal and human atherosclerosis, EC denudation is extremely rare. Therefore whether EC denudation plays any role in the initiation of atherosclerosis is questionable (Hansson and Bondjers, 1987). Many "risk factors" can disturb EC behaviour and potentially cause initiation of thrombosis and probably atherosclerosis. These factors include virus infection (Curwen et al., 1980; Gimbrone and Buchanan, 1982), fluid shear stress (Dewey et al., 1981; Davies et al., 1986), cigarette smoking (Pittilo et al., 1982), endotoxin (Colucci et al., 1983; Brox et al., 1984), hypercholesterolemia (Hansson and Bondjers, 1980; Morrel et al., 1987) and thrombin (Brox et al., 1984). Evidence indicates that the ECs may become altered and reactive to blood elements without being lost from the vascular surface

(Dejana, 1987).

1.4.2.3. Consequence of EC Dysfunction

In addition to increased migration (Ando et al., 1987), turnover (Davies et al., 1986) and pinocytosis (Dewey et al., 1981; Sprague et al., 1987) of stressed EC, EC dysfunction might entail the loss of the normal non-thrombogenic surface property. This would be manifest by increased platelet adherence and/or activation of the intrinsic or extrinsic coagulation pathway (Stern et al., 1985). By using a perfusion chamber, Sixma and DeGroot (1986) investigated the reactivity of platelets to the extracellular matrix synthesized by thrombin-perturbed EC. They showed an initial increase in reactivity (in hours), then a decreased reactivity towards platelets (in days). Thrombin, which has been shown to increase PGI_2 production, causes release of ADP and VWF from EC and also increases the release of tissue plasminogen activator (Dejana, 1987; Jaffe, 1987; Sixma et al., 1987). These changes in reactivity coincided well with the change of VWF and fibronectin content in the matrix (Sixma and DeGroot, 1986; DeGroot et al., 1987). It is suggested EC respond to long-term perturbation by increasing their PGI_2 synthesis and decreasing the amount of VWF and FB in their matrix, making it more difficult for platelets to adhere to the injured surface of the vessel wall (Sixma and DeGroot, 1986).

Intact EC possess little or no tissue factor activity. When EC are exposed to thrombin or endotoxin, or in the presence of either lymphocytes, platelets, or macrophages, their tissue factor activity increases without any apparent cellular damage or cell detachment (Colucci et al., 1983; Brox et al., 1984; Jaffe, 1987). Angiotensin II, vasopressin or ionophore A23187 release platelet activating factor (PAF) specifically from EC (Dejana, 1987). EC binds coagulation factor V, IX, and X and promotes their activation in the absence of platelets or exogenous phospholipid, thus leading to localized activation of the coagulation cascade and thrombin formation (Jaffe, 1987). EC can also selectively interact with fibrinogen (FB) and fibrin (Dejana et al., 1985). When activated, platelets expose their receptors for FB and then the platelet-FB interaction promotes platelet-platelet interaction (aggregation). Since the FB binding sites for activated platelets and EC are different, EC-FB interaction may lead to increased platelet adhesivity to the endothelial surface in the presence of aggregating agents. This mechanism could play an important role in the localization and growth of thrombi (Dejana, 1987). Also EC dysfunction could have abnormal biosynthesis of heparin-like molecules and thus could result in thrombosis. Lastly, EC have been shown to activate factor XII via a membrane-bound enzyme (Jaffe, 1987).

It should be noted that all the evidence indicating the thrombogenic potential of injured or dysfunctional EC was

derived from *in vitro* studies. Little is known about how circulating platelets interact with injured EC *in vivo*.

1.4.2.4. Detection of EC Dysfunction

In addition to loss of surface non-thrombogenic property as mentioned above, loss of barrier function represents another form of EC dysfunction. It is now possible to detect EC dysfunction *in vivo* by using tracers. Areas of increased Evans Blue permeability are correlated with elevated uptake of albumin, fibrinogen and cholesterol (Morrel *et al.*, 1987). Utilizing horseradish peroxidase (HRP) as a macroscopic marker of enhanced permeability, Stemerman (1981) noted numerous spots of enhanced permeability to HRP scattered over the surface of the rabbit aorta and most prevalent in the arch and upper abdominal segments. Uptake of LDL within these foci of high HRP permeability was much greater when compared with other zones (Stemerman *et al.*, 1986). Similarly distributed foci exhibit an increased cell replication, implying that there is an increased cell turnover in areas with increased trans-endothelial permeability (Hansson and Bondjers, 1987). Using IgG (which can bind to the cytoskeletal structure) as a viability tracer, Hansson and Bondjers (1987) found that IgG accumulation occurs only in irreversible forms of cell injury. This occurs when there are significant permeability defects in the cell membrane and IgG can penetrate into the cell or

because of the formation of new antigens during the cell injury process.

1.5. Responses of Arterial Wall to EC Injury

Injury to EC has been caused experimentally by a variety of trauma in animals (Moore, 1973; Fishman et al., 1975; Minick et al., 1979; Reidy and Silver, 1985) but different techniques for removing EC have elicited differing responses from the arterial wall.

Moore (1973) has attempted to produce atherosclerotic lesions following continued injury to EC by using an implanted catheter in a rabbit aorta. Moore noted an intimal thickening containing lipid-filled cells in non-endothelialized and thrombus-covered lesions within at least 2 weeks. A single de-endothelialization model developed by Fishman et al (1975), using an air-drying technique, showed complete re-endothelialization by 7 days, but massive intimal thickening in the central region of the denuded segment by 14 days. This central region was the last area to be covered with regenerating EC. Minick et al. (1979), using a balloon catheter method, observed that the intimal thickening and lipid accumulation were greater in areas covered with regenerated EC compared to areas without EC cover. In a more recent study, Reidy and Silver (1985) selectively removed a small defined area of EC with a small nylon catheter. The

denuded areas were re-endothelialized by 3 - 8 days depending on size, but no intimal thickening or SMC replication activity was observed. Their previous study showed a detectable SMC proliferation within 4 days after balloon catheter injury. They pointed out that not only the area but also the depth of injury inflicted on an artery can cause differing vascular responses.

Biochemical studies (Day et al., 1974; Reidy and Silver, 1985; Groves et al., 1986) showed changes in content of cholesterol and its ester consistent with changes in the morphological study. These lipids preferentially accumulate in re-endothelialized regions. Other approaches include studies in lipid metabolism with labelled precursor incorporation (Day et al., 1974) and analysis of cholesterol ester metabolizing enzymes (Hajjar et al., 1981). These indicate that arterial lipid accumulation is not simply a result of passive filtration but results from alterations in arterial wall metabolism induced by injury and may be modified by EC regeneration (Falcone et al., 1980; Hajjar et al., 1981).

Recently, Simatos (1987) has shown a serum-dependent increase in ^{3}H-acetate incorporation into polar lipid in de-endothelialized rabbit carotid artery perfused in vitro. This incorporation was principally into phosphatidylcholine (PC). The content of PC phosphorus was unchanged. He suggests a greater turnover and not increased net PC synthesis. He also presented an enhanced turnover in the sn-2 fatty acyl chains

of PC and implied a platelet derived growth factor mediated response.

1.6. Objectives

Although considerable insight has developed by using in vitro and ex vivo models on the interaction of platelets with sub-endothelium, much less is known about how platelets interact with the more superficial layer of sub-endothelium. Even less is known about how platelets interact with mildly injured or dysfunctional ECs in vivo which now appears to be more important in vascular pathophysiology than desquamation of EC.

The original objective of this study was to produce an in vivo model of mildly injured EC in the artery and to use this model to study both interaction of platelets with the injured arterial wall in vivo and the effect of EC injury on arterial tissue metabolism.

The conditions for the in vivo model of EC injury were as follows: (1) large arteries should be used; (2) during the entire in vivo experimental period the animal must be maintained in a physiological condition; (3) the injury itself should be restricted to the EC and should be graded ranging from relatively normal EC to de-endothelialization. Two techniques were examined in an attempt to meet these aims. The first technique involved the perfusion of low

concentrations of the chemical triton X-100. This was a modification of the technique described by Langille and O'Donnell (1986) where high concentration triton (2.0%) was used to completely remove the arterial EC. The second technique involved physical injury of EC by means of perfusion of dry-air. This was a modification of the method described by Fishman et al., (1975). Morphological characterization of EC injury was by SEM and quantification of platelet adhesion. Lipid metabolism of the arterial wall was studied using an in vitro perfusion technique developed by Simatos (1987) which assessed the incorporation of a radio-labelled precursor. The responses were assessed to (1) in vitro mechanical injury as used by Simatos (1987); (2) in vitro air-dry injury; (3) in vivo air dry injury followed by re-establishment of normal blood flow for 4 hours.

2. MATERIALS and METHODS

2.1. Materials

In this study, white rats (Sprague-Dawley, male, 400-500 g) were used in the EC triton-injury experiments. Lop-eared rabbits (3-3.5 kg, both sexes) were used for air-drying EC injury experiments and the metabolic study. Animals were maintained on standard chow diet. Triton X-100 was purchased from Sigma, and diluted with physiological saline into appropriate concentrations (v/v). 2.5% glutaraldehyde was prepared in Millonig's buffer. Other agents used for arterial tissue preparation for SEM were prepared by standard methods. ^{3}H-acetate, sodium salt (100 mCi/mmol), ^{35}S-methionine (1127 Ci/mmol) were obtained from New England Nuclear. Aqueous counting scintillant was purchased from Amersham Canada. Chloroform, methanol, hexane and ether were purchased from Fisher Scientific Co. Chloroform and methanol were redistilled prior to use. The tissue perfusate medium was minimum essential medium (MEM) from Gibco Canada. Human lipoprotein deficient serum (LPDS, density>1.215g/ml) was prepared by differential ultracentrifugation of plasma from healthy volunteers in Dr. Poznansky's laboratory. Silica Gel G (Sil G) plastic backed thin layer chromatography (TLC) plates were purchased from Brinkman Canada. Lipid standards were obtained

from Sigma. An eight circuit roller minipulse pump was purchased from Gilson.

2.2. Surgical Procedures

2.2.1. Surgery in Rats

Rats were anaesthetized with sodium pentobarbital (50 mg/kg, I.P.). The anaesthetized rat was placed on its back on a warm plate at a temperature of $38^{o}C$-$39^{o}C$. After tracheotomy and intubation, the rat was ventilated mechanically (5 ml of tidal volume, rate 40/min.). The jugular vein was cannulated for administration of heparin. A ventral midline incision was made along the whole length of the abdomen. All bleeding points were cauterized or tied to minimize blood loss. The abdominal aorta was exposed and the segment (approx. 2.0 cm) between the lower renal artery (usually left renal artery) and the bifurcation was gently isolated from surrounding tissue. The lower renal artery and one of the common iliac arteries were exposed and prepared for cannulation. All other branches from this segment were ligated to avoid spill over of the perfusate into the general circulation. After heparinization (heparin 2500 u.s.p., i.v.), the lower renal artery was cannulated (0.75 mm O.D., 10 cm long). The cannula tip was placed in the root of the renal artery. The drainage cannula (1 mm O.D., 4 cm long) was introduced into the prepared

common iliac artery with its tip situated just below the aortic bifurcation. The cannulation procedures were carried out with the help of a dissecting microscope. The renal and iliac arteries were ligated distal to each cannula. Blood pressure was measured before EC injury through the drainage cannula in the iliac artery.

2.2.2. Surgery in Rabbits

Following pre-anaesthetic medication with atropine, anaesthesia was produced and maintained by bolus intravenous administration of a mixture of ketamin and xylazine (2:1, v:v) combined with bolus sodium pentobarbital (1/3 anaesthetic dosage, IV). The anaesthetized rabbit was placed on its back on a surgery bench and kept warm. A midline incision was made along the whole length of the neck. An airway was introduced through a tracheotomy to prevent tracheal obstruction. Both common carotid arteries were gently exposed. In order to perfuse the segments, all the lateral branches were tied off to prevent leakage and movable ligatures were placed 4.0 cm apart on the exposed artery. For the metabolic experiments, exposure and isolation of carotid arteries was done in a similar way as described above except that all branches leading from the segments were tied and cauterized several millimetres away from the origins of the branches. Thus the carotid artery was isolated with its adjacent surrounding tissue attached.

This was to ensure that all branches were tied and cauterized without interfering with the arterial wall, and also to prevent damage to the arterial wall from surface drying and handling.

2.3. Endothelial Injury *In Vivo*

2.3.1. Chemical Injury of Endothelial Cells by Triton in Rats

The perfusion apparatus consisted of a dual infusion/withdraw pump (Harvard) and two 30 cc glass syringes which were mounted on the pump and connected to the infusion cannula through a 3-way stopcock. The arrangement of the perfusion apparatus provided an easy shift from one perfusate to another without cessation of flow and loss of pressure. The perfusion started with a 1-minute pre-wash with Millonig's buffer at 10 ml/min. Once the buffer was flowing, the aorta was occluded with a movable surgical silk loop at the level just above the cannulated renal artery. The non-cannulated iliac artery was occluded in the same way. After pre-wash, the diluted triton solution was perfused at 10 ml/min. for 1 minute. The arterial lumen was then post-washed in a manner similar to the pre-wash. During the entire perfusion period, a luminal pressure was maintained at 80 mm Hg by connecting the out-flow cannula to a pressurized reservoir. After post-washing, both occlusions were released and blood flow was restored for either 5 or 60 minutes. These periods of time

permitted blood-vessel wall interaction. During the entire experiment, the animal was kept anaesthetized and ventilated, and the animal's condition was observed and recorded (e.g. bleeding, leakage of perfusate, the body temperature and tongue color). Bleeding was estimated according to the number of sponges used (5.0 cm X 5.0 cm, Curity, Kendall Canada), based on the estimation that each sponge contains 4 ml of blood when saturated. At the end of the blood-vessel wall reaction period, the blood pressure was measured through the drainage cannula in the iliac artery.

2.3.2. Physical Injury of Endothelial Cells by Air-Drying in Rabbits

Following heparinization, a butterfly needle (25G) was inserted at the distal end of the selected carotid artery segment toward the proximal end. Saline solution was infused at a rate of 10 ml/min for prewashing the blood. As soon as the perfusion started, blood flow was stopped by using a self-closing artery forcep on the distal side of the infusion needle. An exit for perfusate was made at the proximal end of the segment by punching a small hole with a 26G needle. The segment was then isolated from the circulation by clamping proximal to the exit hole. After prewashing (about 1 min.), a stream of dry air was perfused through the segment at an inlet pressure of 100 mmHg for 3-5 minutes. The air entered

through the distal needle and exited through the proximal small hole. After air-drying, the segment was post-washed with saline to remove any air remaining in the lumen. The two artery forceps and the needle were then removed and blood flow was restored for 0.5-3 hrs. Any bleeding was stopped by direct pressure.

2.4. In Situ Perfusion-Fixation and Preparation for SEM

It was noted that when the internal hydraulic pressure drops, collapse and circular constriction of the arterial wall are unavoidable. The morphology of collapsed arteries, however, can vary remarkably between different vessels and between different regions of the same vessel. The cellular elements are contracted, resulting in many surface artifacts such as wrinkling of longitudinal folds, artifactual projections (ridges) and undulations of the EC (Bunce, 1965 and 1974). Therefore, to obtain the detail of morphological information faithfully and exactly, an in situ perfusion-fixation technique was used in preparation for SEM examination because it prevents the following:

(1) Artifacts caused by specimen handling prior to fixation.

(2) Blood component deposition on the arterial surface.

(3) Cell structure alteration due to changes occurring prior to arrest of blood flow.

(4) Distortion in cell shape and relationships between cells

which could occur when the artery collapses. This is because perfusion pressure was maintained at the same level as the mean BP measured right before fixation at all times.

Before perfusion fixation, the injured segments of rat aorta or rabbit carotid artery were prewashed and ligated at both ends as before. 2.5% glutaraldehyde was perfused at 10 ml/minute. The perfusion pressure was kept at the same level as the blood pressure measured just before perfusion fixation. Once the fixation was started, the animal was killed by intravenous infusion of KCl while still under anaesthesia. After a perfusion period of 20 minutes, the fixed segment was gently removed with its orientation marked and then immersed in 2.5% glutaraldehyde overnight at $4^{o}C$. The fixed artery was gently dissected free of extra surrounding tissue and cut open along the midline, flattened and pinned on a cork plate. The tissue was then post-fixed in 0.5% osmium tetroxide and dehydrated through graded acetone. The tissue was cut into small pieces (0.8 cm long) with the orientation marked again. The tissue was critical-point dried with CO_2, which was then mounted onto a SEM stub with glue, coated with gold, and examined in a Philips Model 505 SEM.

2.5. Evaluation of EC Injury and Quantification of Platelet Adhesion by SEM

2.5.1. Method of Sampling Injured Surfaces

The prepared artery specimen (about 0.8 cm long) was examined by SEM (one specimen from each animal). A series of electromicrographs were taken for each specimen as follows:

(1) One low magnification photograph (1.95-7.8 X 10) was taken to give an overall view of the surface of the whole specimen.

(2) To obtain an unbiased sample, 3 moderate magnification photographs (6.25 X 10^2) were taken at random in 3 locations of the surface for quantifying the platelet adhesion. This was accomplished by positioning the SEM over the specimen at low magnification, then zooming in and without further alteration taking the picture at higher magnification.

(3) In some specimens, higher power photographs (2.50 X 10^3)were taken to give a more detailed view of the morphology of the surface.

2.5.2. Categorization of Endothelial Cell Injury

The categorization of endothelial cell injury was as follows:

(1) Apparently Normal EC(EC): In SEM the normal arterial EC was elongate in shape with a raised central area (likely representing the nucleus) and a smooth surface (Plate 1). This category was designated as EC and marked number 1 in photographs.

(2) Injured EC (IEC): In this category, the EC was apparently lightly injured with abnormal surface appearances including roughness and brightness. This, presumably, indicates a structural change in the cell membrane but may also be due to the deposition of unformed blood material. In addition, the cell contour was poorly defined (Plate 2). This category was designated as IEC and marked number 2 in photographs.

(3) Lysed EC (LEC): The cells were more seriously injured and lysed. The cell bodies were distorted and the cell nuclei were naked. Therefore, the underlying sub-endothelium was exposed through the crevices in the residue of the lysed cell (Plate 2). This category was designated as LEC and marked with a number 3 in photographs.

In this area some injured EC were partially detached leaving the sub-endothelium partially exposed. These discrete sub-endothelial areas were sometimes covered with much fewer platelets (Plate 3), and designated as PES-EC (partially exposed sub-endothelium) and marked 3a in photographs.

Plate 1. SEM view of the surface of a normal rat aorta perfusion-fixed <u>in situ</u>. The normal EC is elongated and aligned with the direction of blood flow (flow is from the top to the bottom as indicated by arrow). Cells contact each other and the boundary of the cell can easily be seen. The cell surface has a smooth appearance with a raised area in the center. There are no formed blood components associated with the surface.

1=EC.

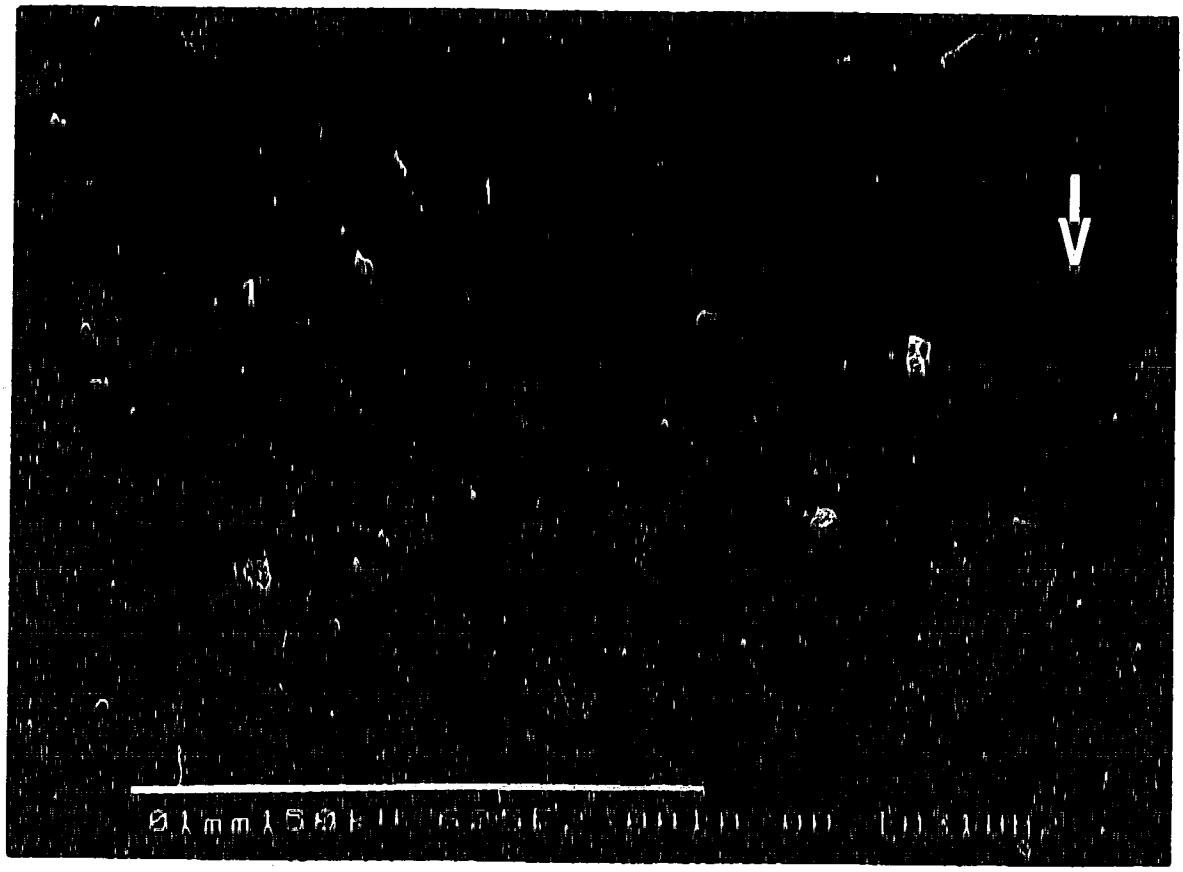


Plate 2. SEM view of 0.05% triton treated rat aorta. The blood flow was restored for 5 minutes after the treatment. This plate shows an unevenly injured EC with uneven distribution of adherent platelets. In the IEC area the EC were injured with the cell residue covering the sub-endothelium and most of the adherent platelets were unactivated. In the more seriously injured and distorted area, the EC were lysed (LEC) where the sub-endothelium was exposed and most of the platelets were activated.

2=IEC; 3=LEC.

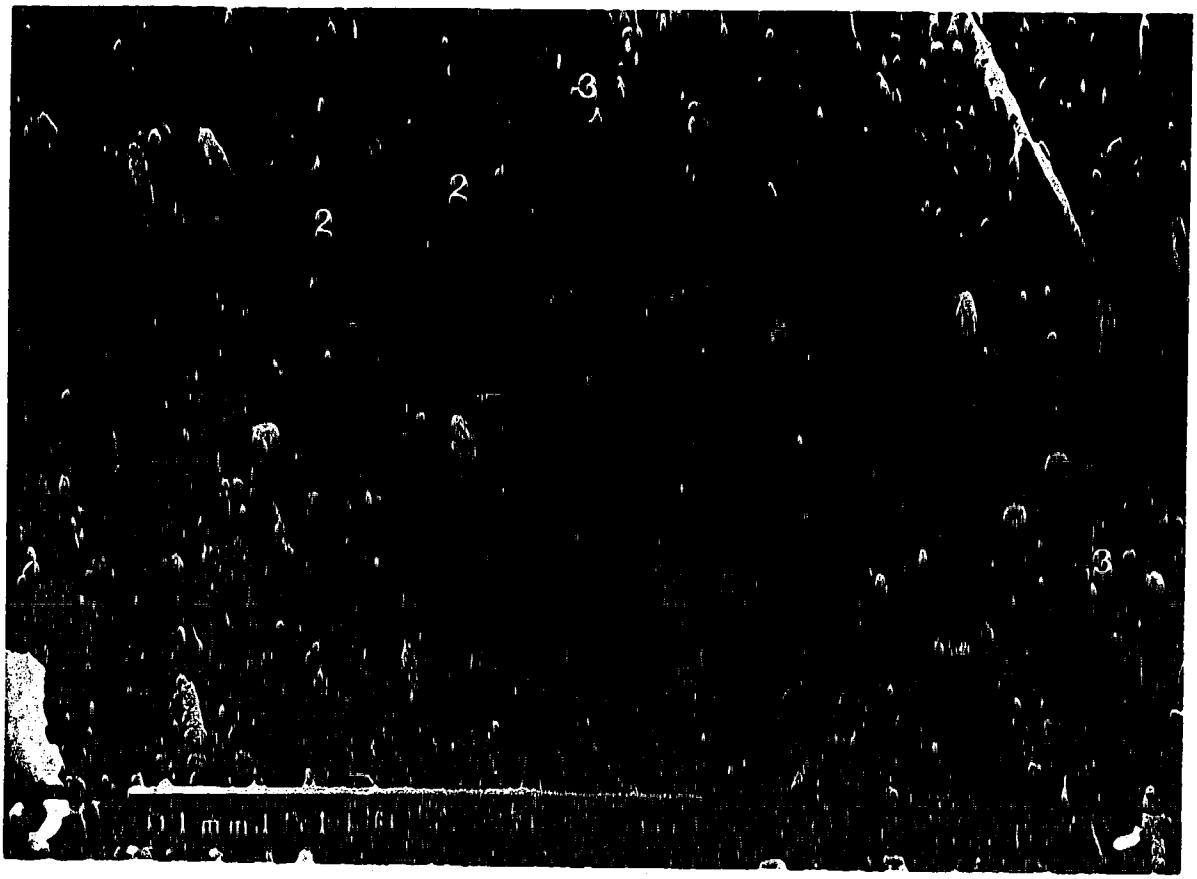


Plate 3. SEM view of 0.05% triton treated rat aorta. The blood flow was restored for 5 minutes after the treatment. Most of the surface shows a seriously injured and distorted EC area (LEC) covered with a layer of platelets. In areas where the EC was partially detached and displaced the partially exposed sub-endothelium (PES-EC) was covered with a few platelets. Areas where the EC were completely denuded leaving the sub-endothelium exposed with no platelets adhering on it were designated as De-EC(B).

3=LEC; 3a=PES-EC; 4b=De-EC(B).

(4) De-endothelialized area (De-EC): In this category, the EC was absent and thus the sub-endothelium was completely exposed, allowing platelets to adhere to a various extent (Plate 4). This category was designated as De-EC and marked number 4 in photographs. This surface was classified into two sub-categories according to the presence or absence of platelet adhesion:

(a) Sub-endothelium with adherent platelets was designated as De-EC(P) (Plate 4) and marked 4a in photographs.

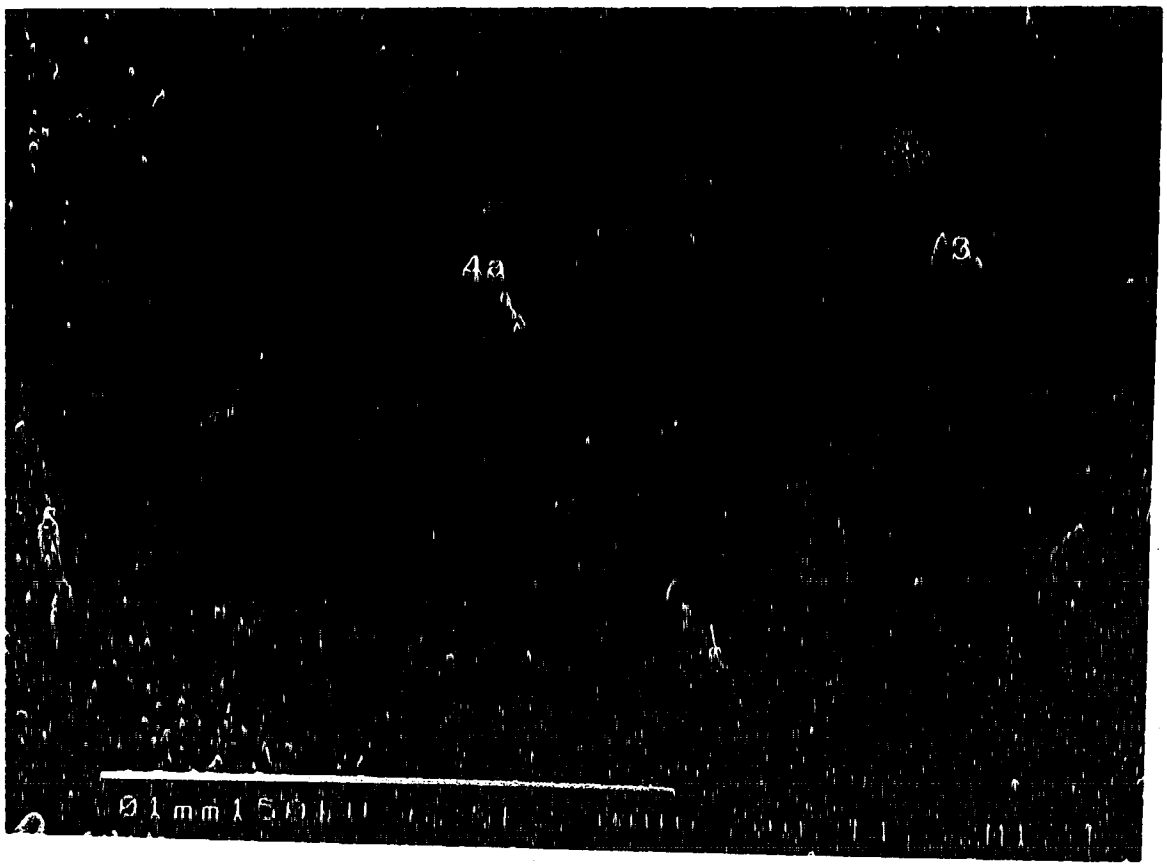
(b) Naked or bared sub-endothelium without platelet adhesion was called De-EC(B) (Plate 3) and marked 4b in photographs.

2.5.3. Categorization of Adherent Platelets

(1) Activated (or shape-changed) platelets: These are adherent platelets that had an irregular (spiny) shape and a brighter surface appearance in SEM with one or more visible pseudopods.

(2) Unactivated (or regular-shaped) platelets: These are adherent platelets that had only contacted the surface and were of regular shape without pseudopods.

Plate 4. The surface of 0.7% triton injured rat aorta fixed after 60-minute restoration of blood flow. The EC were seriously injured and lysed (LEC) or denuded with a layer of adherent platelets, most of which were activated (De-EC(P)). 3=LEC; 4a=De-EC(P).



2.5.4. Methods of Quantification

An overlay which marked 3 equally-sized square areas, each subdivided into 16 smaller squares, was used to select an area of each medium power photograph. The total scaled area was calculated according to the scale bar marked on the moderate power photographs. This was designed to give an equivalent area of 0.01 mm^2 of the specimen surface. The features of the photograph could be easily seen through the transparent film. Peripheral areas of the specimen were ignored to minimize the influence of artifacts produced by manipulation and handling during preparation for SEM.

2.5.5. Evaluation of EC Injury

The percentage of De-EC area was measured using the scaled transparent film applied to the low power photographs. This was calculated as follows:

Percentage of De-EC area = (measured De-EC area)/(total area of the photograph).

The incidence of different EC injury categories in each dosage group was determined according to the categorization method described above. This was done by identifying the individual categories in any of the three moderate magnification photographs for each specimen. The determined incidence of injury was expressed as follows:

(Number of specimens containing a certain type of injury)/(total number of specimens in each group).

The areas of individual injury categories were not measured because the border between the different injury categories was not clear cut.

2.5.6. Quantification of Platelet Adhesion

Quantification of platelet adhesion was carried out only in the 0.05% and 0.7% triton groups. First the average platelet density was measured using the scaled overlay (equivalent to 0.01 mm^2 of specimen area) over the three moderate magnification photographs. These were taken from unbiased locations on the injured surface. Secondly, the number of activated and unactivated platelets adhering to the surfaces of different injury categories were separately counted over the same photographs. This was performed by (1) identifying a size greater than the area of three EC of a particular category of EC injury, and (2) counting the number of adherent platelets in each identified area. Areas of a particular category of injury were not confluent but made up of local and small areas. The counts of platelets on the individual areas of a particular injury category were summed for each specimen and the density per 0.1 mm^2 was calculated. All the counting was performed by the author himself while blinded to the treatment groups.

2.6. Perfusion Apparatus for Metabolic Study

The perfusion apparatus for study of arterial metabolism was designed and used previously in this laboratory (Simatos, 1987). The perfusion apparatus consisted of the following: an eight reservoir unit built in a water bath and housed in an environment that permitted exposure of the perfusate to the 5% CO_2-95% O_2 atmosphere; an eight-vessel organ bath (90 ml volume each); and an eight circuit roller pump using 0.032 in. ID pump tubings. The perfusion circuit was connected as that recirculation of the perfusate through the arterial lumen, free of bubbles, was permitted while the gas tension, temperature, pressure in the artery and a flow rate of 1 ml/minute were maintained.

2.7. Preparation of Perfusate and PBS

In the perfusion studies, the perfusate was made up of MEM, 10% human lipoprotein deficient serum (LPDS) and 1% penicillin-streptomycin with pH 7.4 at 37^{o} C under 95% O2-5% CO2 atmosphere. Perfusate volume for each segment was 1 ml with addition of 300 mCi ^{3}H-acetate and 5 mCi ^{35}S-Methionine. PBS was prepared following the formula from Gibco.

2.8. Transfer of the Arteries to Perfusion Apparatus

After surgical preparation, the rabbit was placed on a small movable table and positioned over the perfusion apparatus which had been primed and readied for at least 1 hour. Segment removal was initiated by placing a self-closing surgical forcep at the proximal end of the isolated segment to occlude blood flow. A blunt 18G needle connected to a feed line was introduced (with the perfusate flowing) into the artery through a scissor-cut hole at the distal side of the occluding forceps. The tip (0.2 cm) of the needle was secured in the artery by a tie. The distal side of the segment was then cannulated with a sharp needle about 3-4 cm from the proximal cannula. The needle had been previously connected to the return line. The segment was now completely isolated from the blood circulation and perfused with medium. The cannulated segment was then secured in a tissue holder with its length maintained as in vivo. The segment was transferred to the organ bath. The control artery on the other side was removed in the same way. The animal was then euthanized by intravenous injection of KCl.

2.9. Tissue Analysis

After the perfusion period, each arterial segment (2-2.5 cm long) was removed from the apparatus and rinsed in PBS and

cut into two small segments. Individual segments were then homogenized with a tissue grinder in 0.5 ml of methanol. Approximately 1000-2000 cpm of 14C-cholesterol was added as an internal standard. Tissue lipid was extracted using the method of Folch (Folch et al., 1957). The lipid extract in chloroform was then evaporated under a stream of nitrogen and resuspended in a known volume of chloroform. An aliquot was used for the analyses below. Neutral lipid separation was carried out using TLC on Sil G TLC plates in a solvent system of hexane: ethyl ether: acetic acid (85:15:2). For polar lipid separation a solvent system of chloroform: methanol: acetic acid: water (50:25:7:3) was used. Lipid spots were identified by co-migration with known lipid standards run on adjacent lanes of the TLC plates. Individual lipid spots were cut from the plates and added to 15 ml of scintillation cocktail for evaluation of radio-label incorporation.

The evaluation of radio-label incorporation into tissue protein was initiated by sedimentation of an aliquot of lipid extract (1/5) in 15% trichloroacetic acid (TCA) with centrifugation. The pellet was washed 3 times with 15% TCA and dissolved in 1 N HCl. Scintillation cocktail was added and the radioactivity counted.

Total tissue protein was determined by the Lowry method. The radioactivity in lipid and protein was expressed as cpm/mg protein. Only polar lipid, cholesterol and protein data were obtained.

2.10. Experimental Design

2.10.1. Morphological Study

2.10.1.1. Quantification of EC Injury and Platelet Adhesion

In these experiments EC injury was produced by perfusing graded concentrations (0.01% to 2.0%) of triton solution. For concentrations of 0.01%, 0.05% and 0.7% there were at least 3 rats in each group for each reaction period. Only one rat was used for the 2% triton group. Two control rats were perfused with physiological saline.

2.10.1.2. Morphological Demonstration in Triton Foam Injury and Air-drying Injury

In an attempt to produce in the same artery, the full spectrum of defined graded injury, the triton injury model was modified by using triton foam instead of triton solution. Triton foam was made by violently shaking the diluted triton solution (0.1% and 1.0%). The triton foam was infused into the aorta in the same way as above. However, once the isolated segment was filled with triton foam, the infusion was stopped and the foam was kept in the artery for 1 minute. Six rats were used in this group. Four were subjected to 0.1% triton foam

treatment and two were subjected to 1.0% triton foam.

Air-drying injury experiments were carried out on both rabbit carotid artery and rat aorta. This physical injury was supposed to produce a more superficial injury than mechanical and chemical techniques. Six rabbits were used in this group. The air-drying times were 3-5 minutes.

2.10.1.3. Period of Blood-Vessel Wall Interaction

To allow the development of blood/vessel wall interaction in the triton injury experiment in rats, a 5 minute and a 60 minute period was used. A longer period was not used because of the difficulty of keeping the triton injured rats in good condition 60 minutes after injury. However, in the air-drying injured rabbit experiments, it was possible to allow the reaction time to last for as long as 3 hours. Therefore, the reaction times were 0.5-3 hours in the air-drying group.

2.10.2. Metabolic Study

2.10.2.1. Effect of EC injury in Incubated Arteries

The first experiments consisted of incubation of arterial segments in the same medium as used for the perfusion study. Three segments of carotid artery (1 cm each) were removed from each side. One of the two carotid arteries had been previously

air dried *in vivo* followed by restoration of blood circulation for 4 hours. Each segment was chopped into 3-4 sections and placed in a tissue culture dish containing 2 ml of culture medium. The culture dish was mounted on a shaker table and placed in an incubator. 500 uCi of ^{3}H-acetate was added after 1 hour incubation and the tissue was incubated for another 4 hours at 37^{o}C in 5% CO_2-95% O_2 atmosphere. The pulsed tissue was then removed and evaluated for incorporation of precursors.

2.10.2.2. Effect of EC Injury in Perfused Arteries

This series of perfusion experiments examined the effect of *in vitro* or *in vivo* EC injury on lipid metabolism. *In vivo* injury was produced by the air-drying technique described in the Methods section. Blood flow was then restored for 4 hours. *In vitro* injury was produced by either a mechanical or the air-drying technique. The *in vitro* mechanical injury was produced by advancing both needle cannulae toward each other through the lumen of the artery and then retracting them to their original position. Perfusion was maintained throughout this procedure. Complete de-endothelialization by this method has been successfully achieved and verified in a previous study by Simatos (1987). The in vitro air dry injury was produced as described. The main difference between *in vivo* and *in vitro* injury experiments was the possible involvement of blood cells (platelets and leucocytes) in the *in vivo* injury. The blood

cells were absent in the in vitro injury experiments. The in vitro mechanical injury technique was used in order to reproduce the metabolic response of the arterial wall to de-endothelialization described by Simatos (1987). After EC injury the arteries were perfused and pulsed with 3H-acetate and 35S-methionine, typically for 4 hours. In a few individual experiments perfusion was for 12 hours.

2.10.2.3. Evaluation of Tissue Viability During Perfusion

This set of perfusion experiments examined the viability of the perfused artery for up to 16 hours. In each experiment, a pair of carotid arteries, both of which had intact EC, were perfused in parallel and pulsed for various periods of time. Usually, one segment of the pair was perfused and pulsed for 2-4 hours longer than the other segment and the time course of one experiment overlapped that of another (i.e. the periods examined were 4-8, 8-10 and 12-16 hours).

2.10.2.4. Evaluation of Possible Contamination of Surrounding Membranous Tissue

The purpose of this study was to determine the metabolism in the arterial intima-media tissue where smooth muscle cells (SMC) are the major cell population if not the sole one. In arterial adventitia and adjacent connective tissues however,

the cell components are more complex and fibroblasts are the predominant cell type. The fibroblasts are multidirectional cells and are more active in cell replication when stimulated. Any contamination with such tissue might affect the apparent result on cell metabolism within the arterial wall.

As mentioned in the methods, the surrounding tissue had to be preserved on the arterial segment during the entire perfusion period to prevent damage to and leaking from the artery wall. The surrounding tissue was then removed completely from the artery before tissue analysis. In some experiments, this surrounding tissue was collected, weighed and subjected to tissue analysis. The precursor incorporation into this tissue was determined in the same manner as for arterial tissue. Because the surrounding tissue was always heavily blood stained, the tissue protein could not be determined. Results, therefore, are presented as total CPM and compared to total CPM for the host arterial tissue. The measured wet weights of individual artery segments were similar. The measured wet weight of surrounding tissue was always similar to that of the host artery. Therefore, the presentation of incorporation in terms of total CPM was the most reasonable alternative in the absence of tissue protein assay. The results were statistically analyzed by using pooled t-tests.

2.10.2.5. Tissue Perfusion in a Glass Chamber

Since the precursor incorporation results from the experiments using the perfusion apparatus as described were about 20 times lower than expected, we were concerned that the apparatus might release chemicals affecting the metabolic activity of the tissue as it was made of plastic material and was more than 2 years old. Therefore, a set of glass chambers was made which paralleled the function of the plastic reservoir and organ bath. Arterial tissue was perfused and pulsed using these glass chambers. The incorporation result was compared with those from the former experiments conducted in the plastic perfusion apparatus using pooled t-tests.

2.10.2.6. Metabolic Study in New Zealand White Rabbit Carotid

To test for a possible difference in lipid metabolism in the response to EC injury between different breeds of rabbits, one experiment using a New Zealand white rabbit was carried out using the same protocol as used in lop-eared rabbits with the in vitro air-dry technique.

2.10.2.7. Evaluation of Endothelial Cell Integrity of Perfused Artery by SEM

To evaluate the luminal surface of perfused arteries, both

the intact control carotid arteries and the *in vivo* air injured carotid arteries were perfused in the same perfusion apparatus and under the same conditions as used for the metabolic study except for the omission of precursors. After 4 hours perfusion, the arterial tissue was subjected to perfusion-fixation and prepared for SEM examination.

2.11. Statistical Analysis

All statistical analysis was performed by the Minitab program on the University of Alberta Mainframe Computer Operating System (MTS). The data of platelet adhesion was analyzed using unpaired Student's t-tests. In the metabolic study, paired data on control artery and injured artery was analyzed using paired Student's t-tests. Statistical significance was accepted if the p value was smaller than 0.05.

3. RESULTS

3.1. General Condition of the Rats

Estimated blood loss during the operative procedure and the measured blood pressure (BP) reduction during the experimental procedure are summarized in Figure 1. In control (physiological saline-treated) rats there was no drop in BP at either 5 or 60 minutes after perfusion. In all triton treated rats there was a BP drop which was more obvious 60 minutes after triton perfusion. Most rats lost less than 4 ml of blood during the entire experiment. Rats losing more than 8 ml of blood or in which there was heavy leaking during triton perfusion were discarded.

3.2. Morphological Study of the Relationship between Platelet Adhesion and EC Injury

3.2.1. Low Power Results with Triton Injury

The full range of injury categories described in section 2.5.2 could not be identified under low magnification (1.95-7.8 X 10). Denuded EC could be distinguished from nonde-endothelialized areas (Plate 5). There was no EC denudation in arteries treated with 0.01% triton. With triton dosage of

Figure 1. General condition of the rats examined at 5 and 60 minutes after EC injury. Blood pressure(BP) drop=(BP before injury)-(BP at the end of the experiment). Bars represent the mean ± S.E. of 3 experiments except for the 0.01% group at 5 minutes, the control, and the 0.7% groups at 60 minutes in which there were 2 experiments.

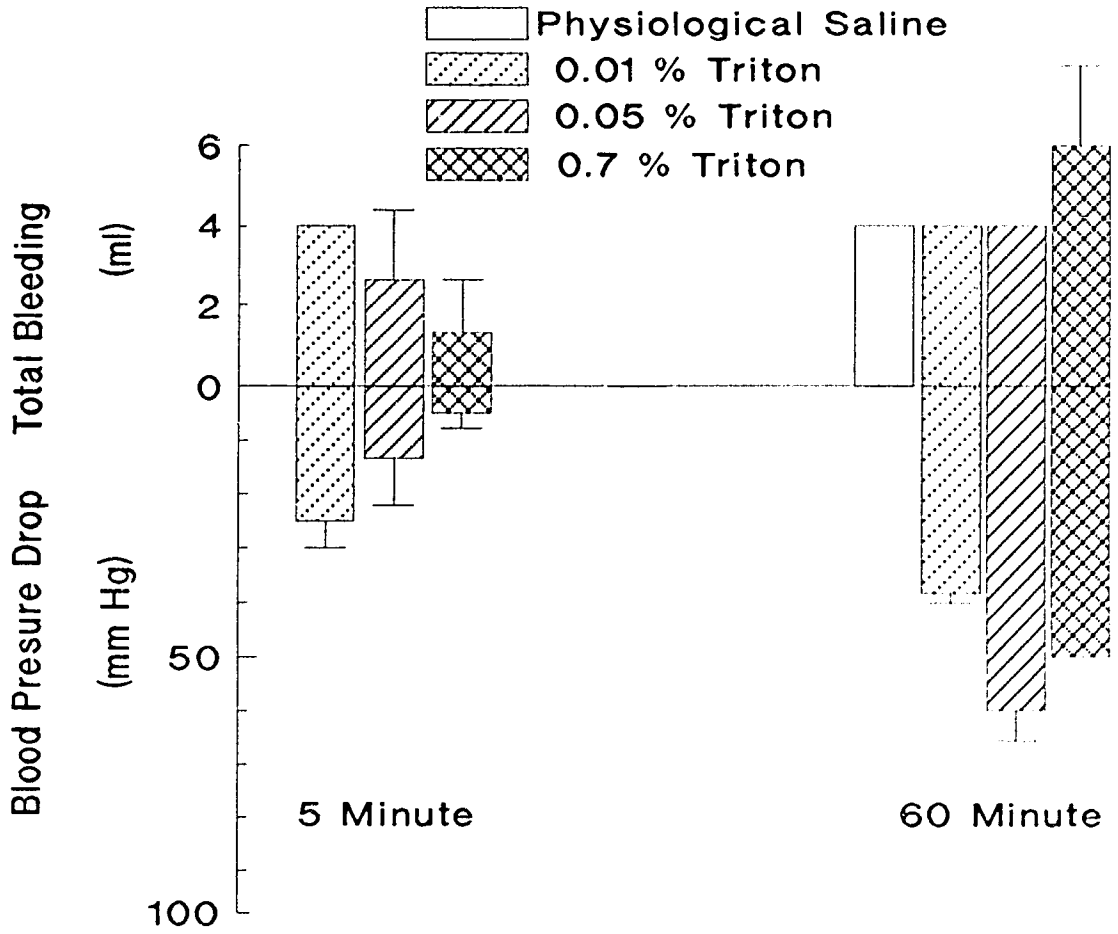
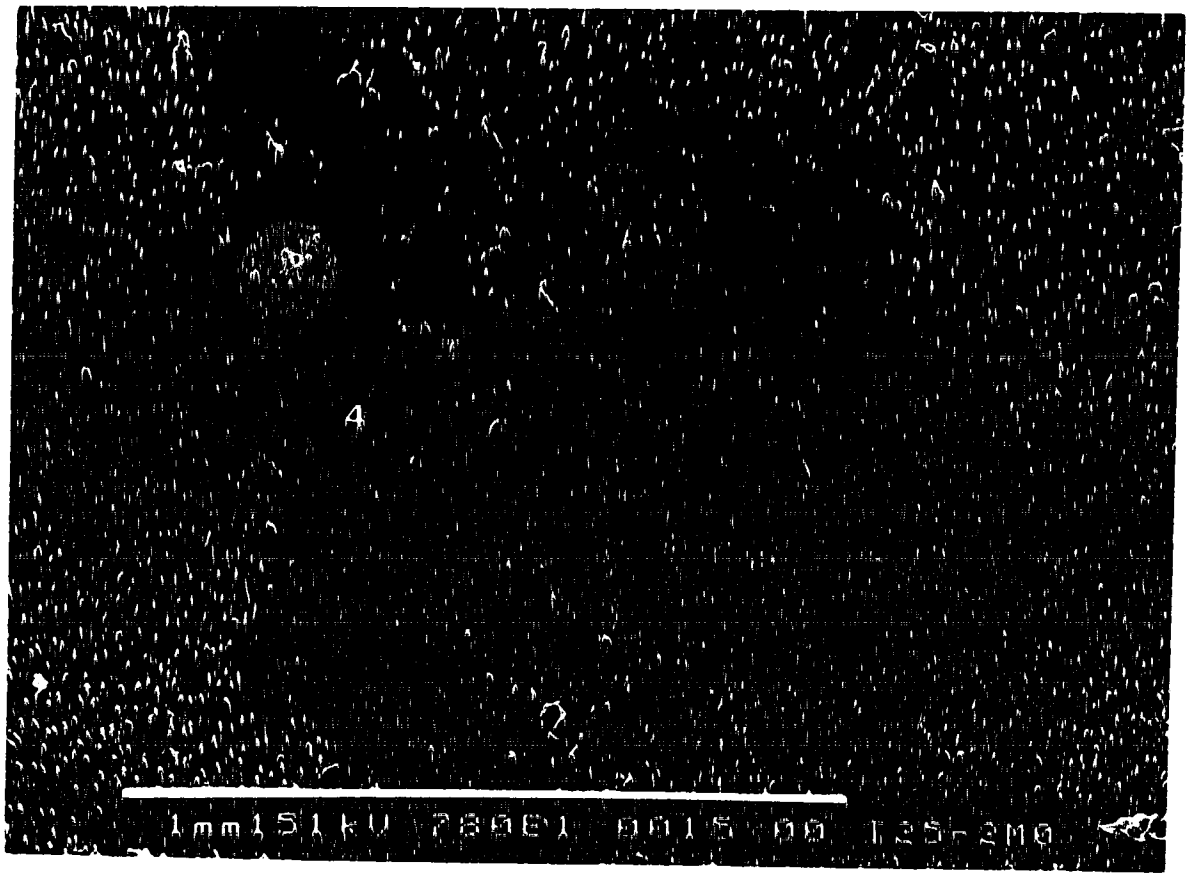


Plate 5. Low magnification view of 0.05% triton treated rat aorta. The blood flow was restored for 5 minutes after the treatment. Since individual injured EC can be well identified, an area with missing EC was designated as De-EC.

4=De-EC.



0.05% and above there was always some EC denudation but the amount was not related to dose. Figure 2 shows the measurements of the percentage of De-EC area in the low magnification photographs. The percentage of De-EC area in the 0.05% triton group was significantly greater than that in the 0.7% triton group at 60 minutes. There was no significant change in the percentage of the De-EC area with elapse of time from 5 to 60 minutes for both 0.05% and 0.7% triton groups.

3.2.2. Medium Power Results with Triton Injury

When viewed at moderate magnification (6.25×10^2) all the categories of EC injury could be recognized. The control arteries showed normal EC (Plate 1). Injury to the triton-treated rat aortas was not uniform. Specimens usually showed two categories of EC injury. In the 0.01% triton treated specimens the degree of EC injury was minimal. Much of the endothelium was apparently normal and injury was limited to IEC (Plate 6). Dosages of 0.05% and above produced more severe EC injury. There were no normal EC in specimens exposed to the higher concentrations of triton. Five minutes after treatment with triton the principal categories of injury were IEC and LEC. De-endothelialization was limited to one specimen exposed to 0.05% triton. In specimens examined 60 minutes after triton exposure there were no mildly injured EC (i.e. no IEC). The injury categories which predominated were LEC

Figure 2. Percentage of De-EC area of the injured rat aortas examined at 5 minutes and 60 minutes after graded triton injury. The results are the mean ± S.E. of 3 experiments. * significant difference between 0.05% and 0.7% triton groups at 60 minutes, P=0.05, D.F.=4, using a pooled t-test.

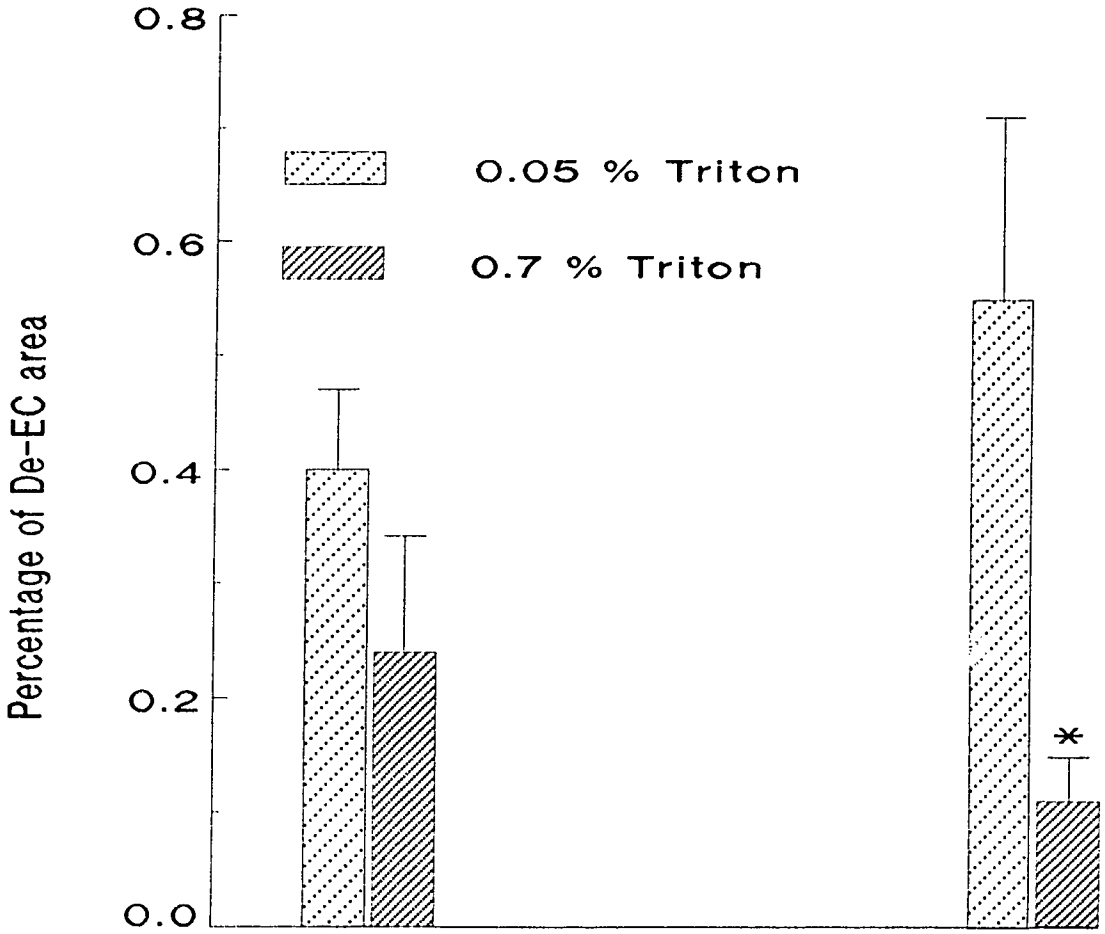
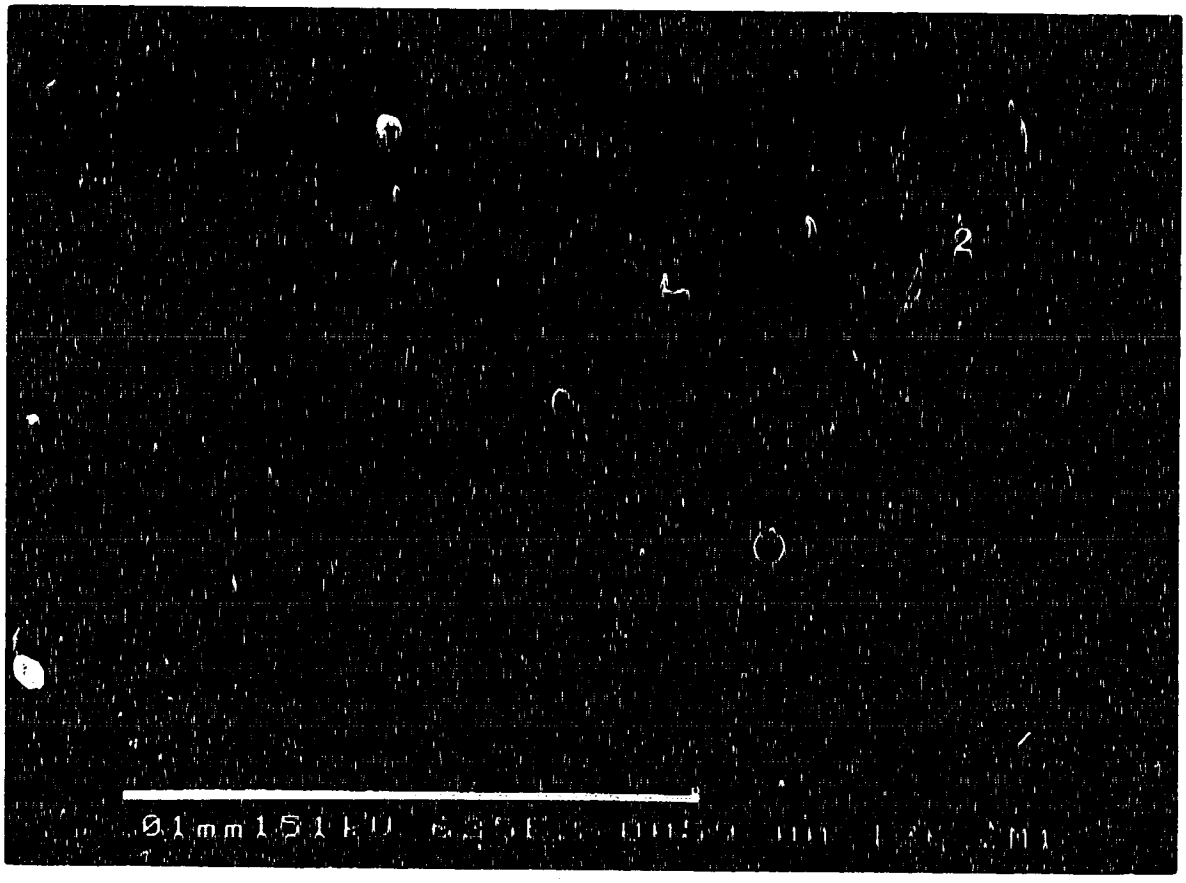


Plate 6. SEM view of 0.01% triton treated rat aorta. The blood flow was restored for 60 minutes after the triton treatment. The cell surface looks rough because of the damage of the cell membrane and possiblly the deposition of unformed blood material. The contour of injured cells is hardly recognized. The sub-endothelium was not exposed on this surface. No formed blood components were associated with the injured surface.

2=LEC.



and De-EC. The incidence of EC injury with triton dosage and time after exposure are summarized in Figure 3. The disappearance of mild injury and the predominance of more severe injury at dosages above 0.01% suggests that the injury process is still developing after 60 minutes. There was no suggestion of progression of mild injury caused by 0.01% triton. The one experiment using 2% triton with fixing at 60 minutes showed a similar injury pattern to that found with 0.05% and 0.7% triton.

3.2.3. Platelet Adhesion

3.2.3.1. Platelet Distribution

Platelet adhesion was not found in measurable amounts in 0.01% triton treated arteries. Distribution of adherent platelets was determined in rat aortas treated with 0.05% and 0.7% triton. An unbiased sampling of the moderate magnification photographs was used as described in section 2.5.1. The average platelet density, measured without considering the categories of EC injury, is shown in figure 4. There is no significant difference either between the 0.05% and 0.7% triton groups or between the two time intervals examined (5 minutes and 60 minutes). Two of the three experiments with 0.7% triton fixed at 60 minutes showed a much greater platelet density than any other experiments. This

Figure 3. Incidence of different types of EC injury examined at 5 (a) and 60 minutes (b). Bars represent the ratio of occurrence of each injury type in each group and number of experiments in each group. The number of specimens was distributed as one specimen in the 2% group at 60 minutes, 2 in the 0.01% group at 5 minutes and 3 in the other groups.

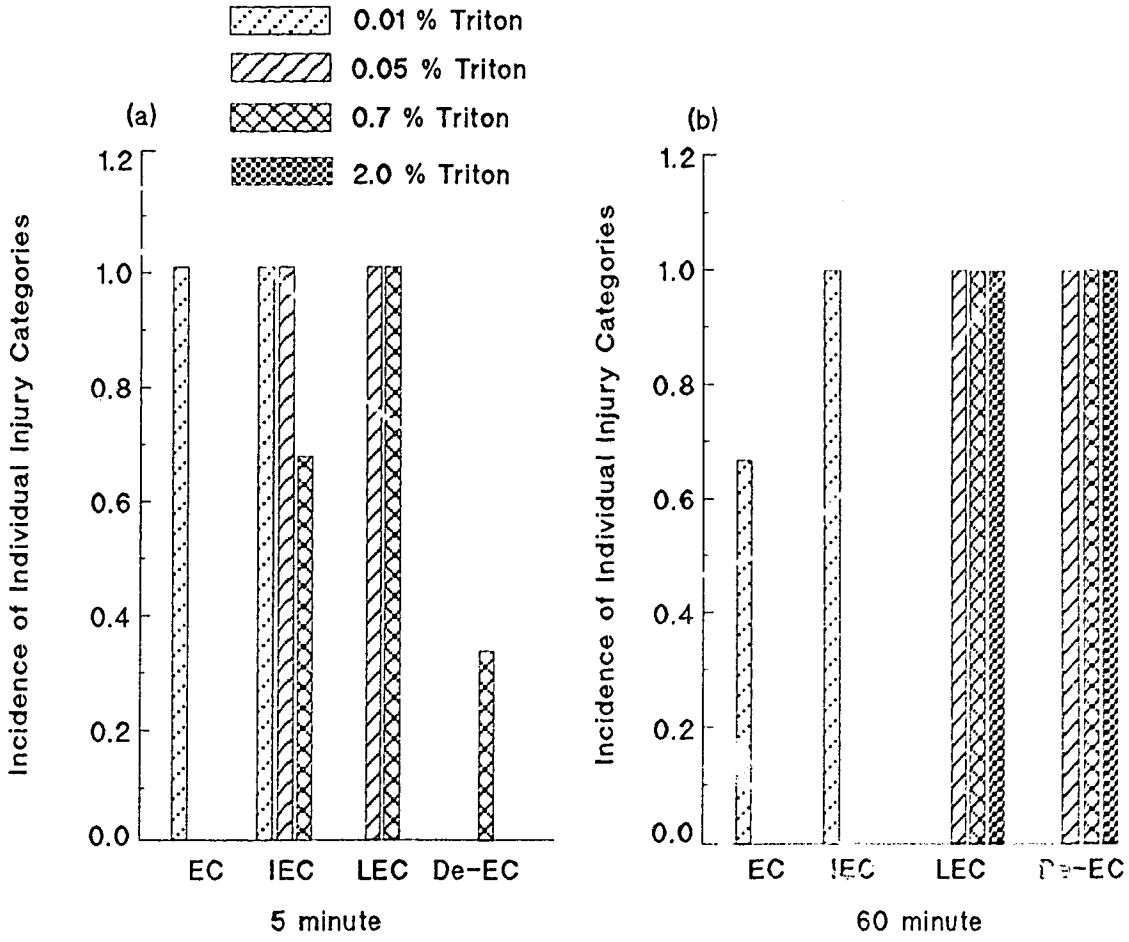
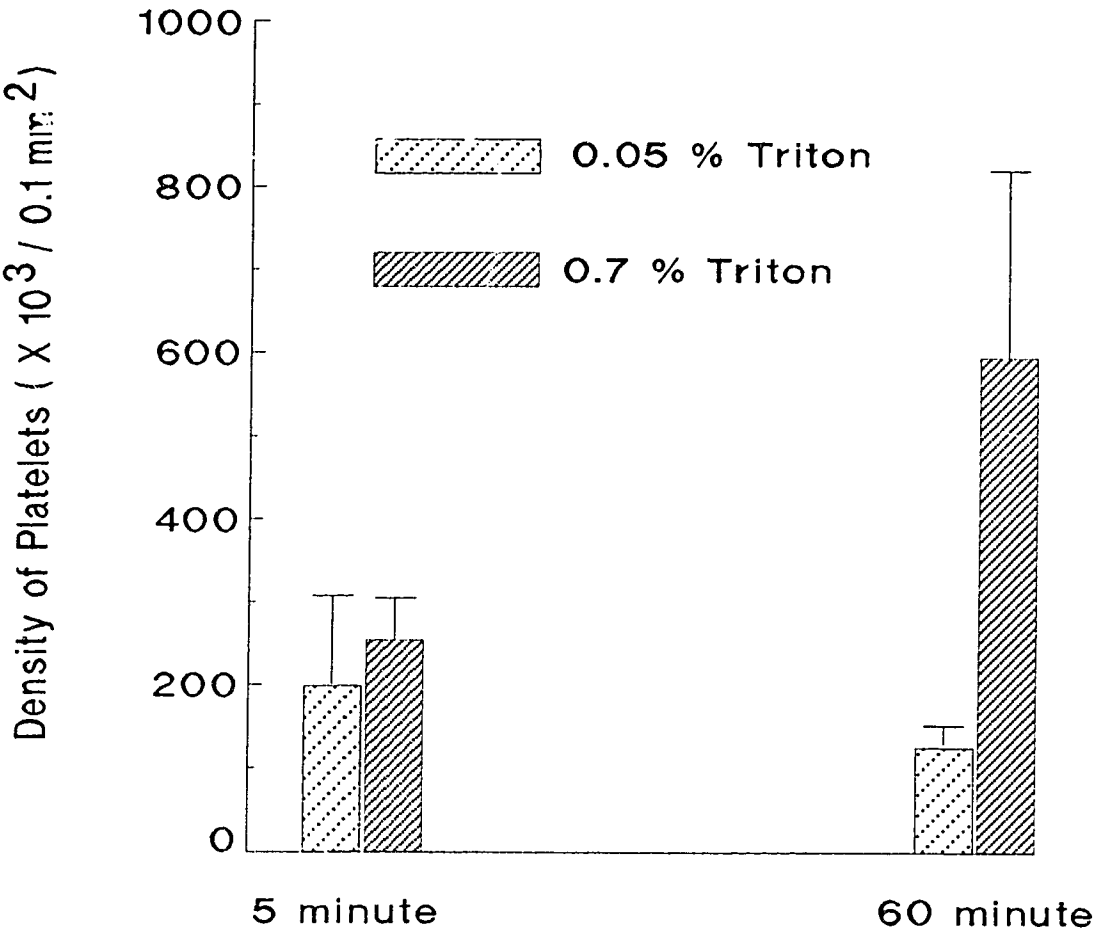


Figure 4. Density of adherent platelets on the surface of the graded triton-injured rat aortas examined after 5 and 60 minutes of restoration of blood flow. The platelet density was counted without considering the categories of EC injury. The results are the mean ± S.E. of 3 experiments. There was no significant difference using the pooled t-test.



probably is due to sampling error as the indicated trend is at variance with the reduced area of de-endothelialization caused by 0.7% triton compared to 0.05% triton (Figure 2).

Using the categorization of EC injury in section 2.5.2., the number of activated and unactivated platelets adherent to individual category areas were separately counted in the 0.05% and 0.7% triton treated arteries by using the medium power photographs. One to two measurements were made for each category area, if present, in each specimen. The IEC areas in these specimens were usually small and surrounded by LEC areas. The minimum size of IEC area used for platelet measurement was an area equivalent to the area of three or more EC. Peripheral regions were excluded because of transition to LEC. The total area of each category used for counting was equivalent to the area of 6-10 EC (mean=7).

Figure 5 shows the platelet density in individual types of EC injury areas. A trend to increased platelet density with increasing grade of injury is indicated at both 5 and 60 minutes after treatment. None of these differences were, however, statistically significant. A statistically significant difference of the platelet density was found in LEC of the 0.7% group between 5 and 60 minutes.

A subdivision of platelets into activated and unactivated forms was also carried out. The trend to increasing platelet density with increasing injury grade was paralleled by the activated platelets (Figure 6). Unactivated platelet density

Figure 5. Density of total platelets in different types of EC injury area. The arteries were injured by 0.05% and 0.7% triton solution and examined after 5 and 60 minutes of restoration of blood flow. The results are the mean ± S.E. of 1 specimen with De-EC (P) in 0.7% group at 5 minutes, 2 specimens with IEC in 0.05% group at 5 minutes and De-EC (P) in 0.05% group at 60 minutes, and 3 specimens with the others.
* There was a significant difference between the density in LEC area of 0.7% group at 5 minutes and that at 60 minutes. $P<0.05$ using pooled t-test (D.F.=4).

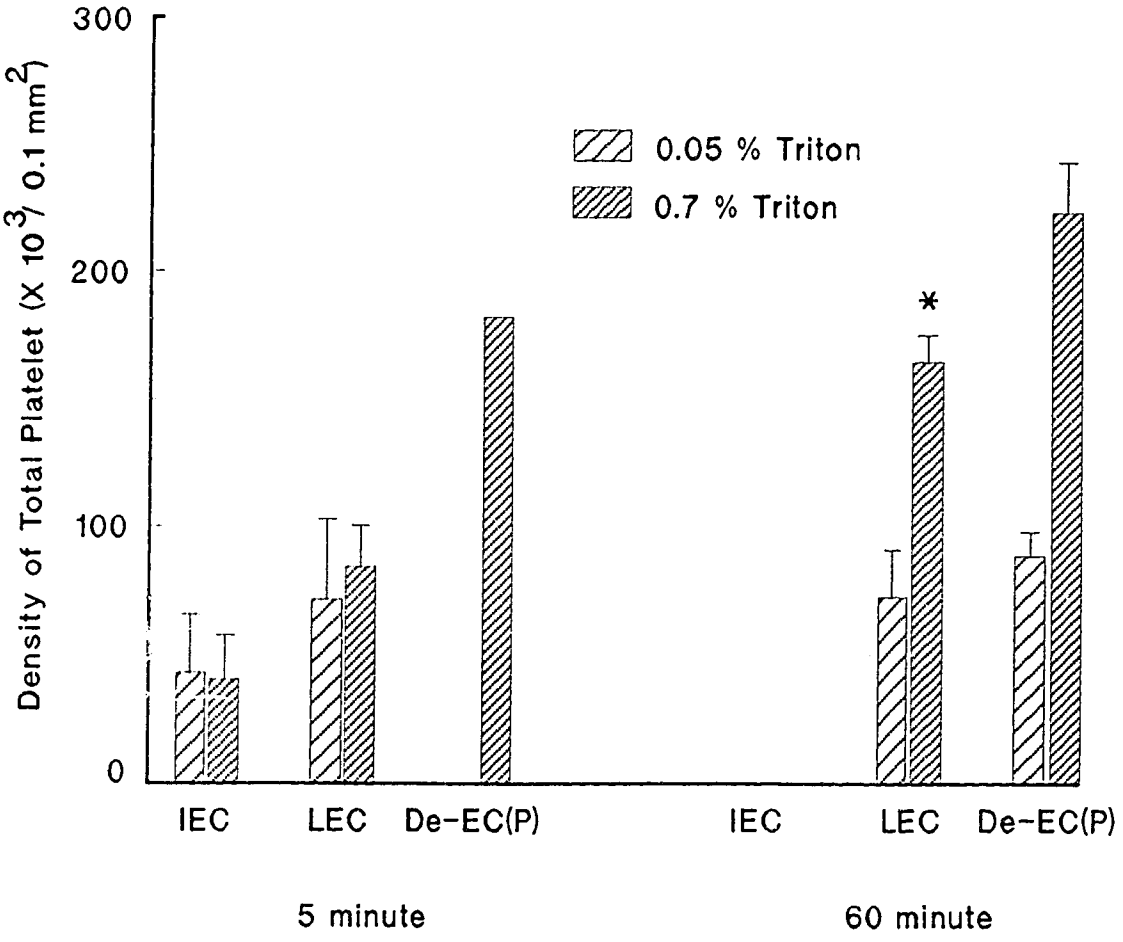
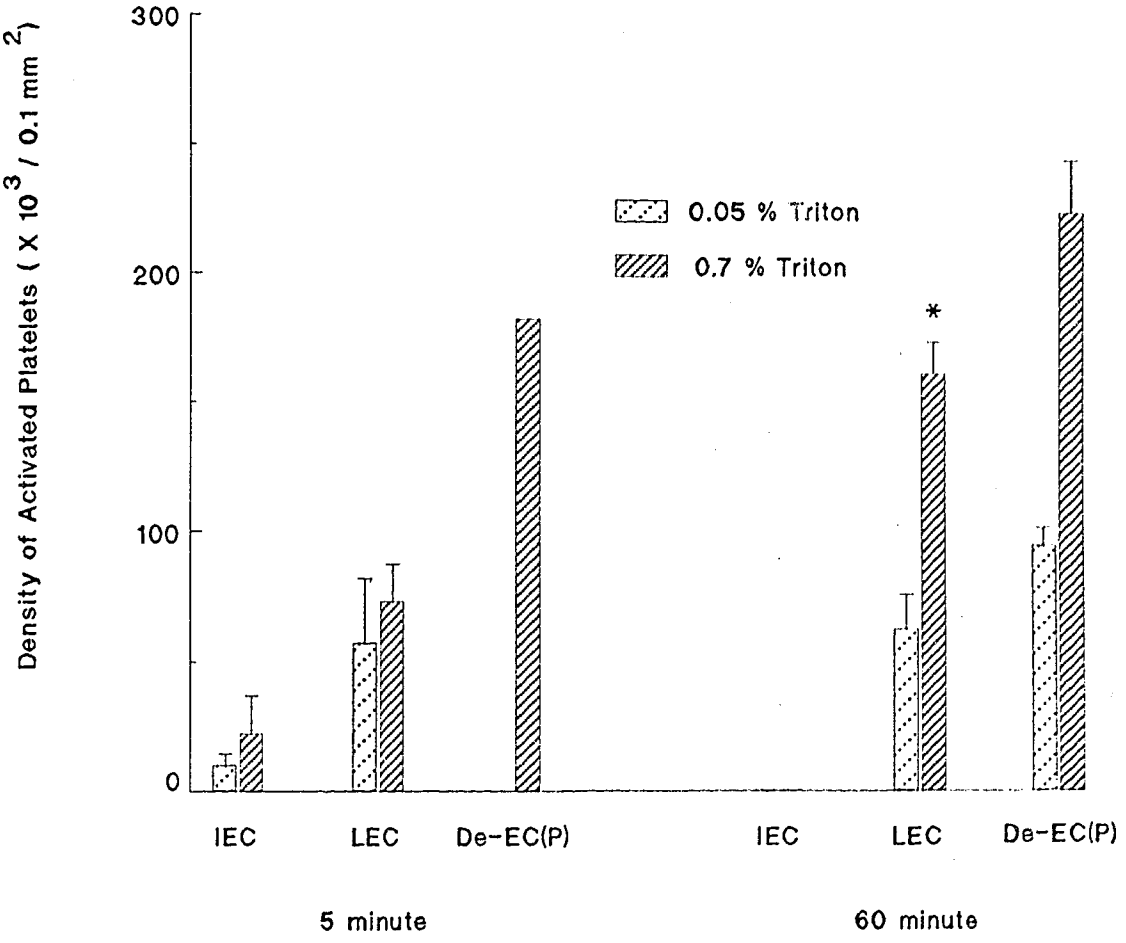


Figure 6. Density of activated platelets in different types of EC injury areas as described in Figure 5.

* There was a significant difference between the density in the LEC area of the 0.7% group at 5 minutes and that at 60 minutes. $P<0.05$ using pooled t-test (D.F.=4).



showed a tendency to decrease as grade of injury increased (Figure 7). No statistically significant differences were demonstrated.

The trend to increased total and activated platelet density with severity of injury is consistent with the greater exposure of sub-endothelium with grade of injury. To determine whether there was in fact a correlation between platelet adhesion and the degree of EC injury, the platelet densities for each category independent of dose and time were calculated and are summarized in Table 1. The densities of total and activated platelets in LEC was significantly greater than that in IEC but significantly less than that in De-EC. The trend to decreased unactivated platelets with increasing grade of EC injury remained but was not supported by statistics. In IEC areas the densities of unactivated platelets and activated platelets were not significantly different whereas in the LEC and De-EC areas the activated platelets represented the majority of the total ($P<0.01$).

3.2.3.2. Triton Foam and Air-drying Injury

The triton foam (0.1%, v/v) injury technique produced the full range of injury types from relatively normal EC to complete denudation of EC in the same specimen of rat aorta (Plate 7). Triton solution usually resulted in the presence of only 2 categories. There is a relatively sharp border among

Figure 7. Density of unactivated platelets in different types of EC injury area as described in Figure 5.

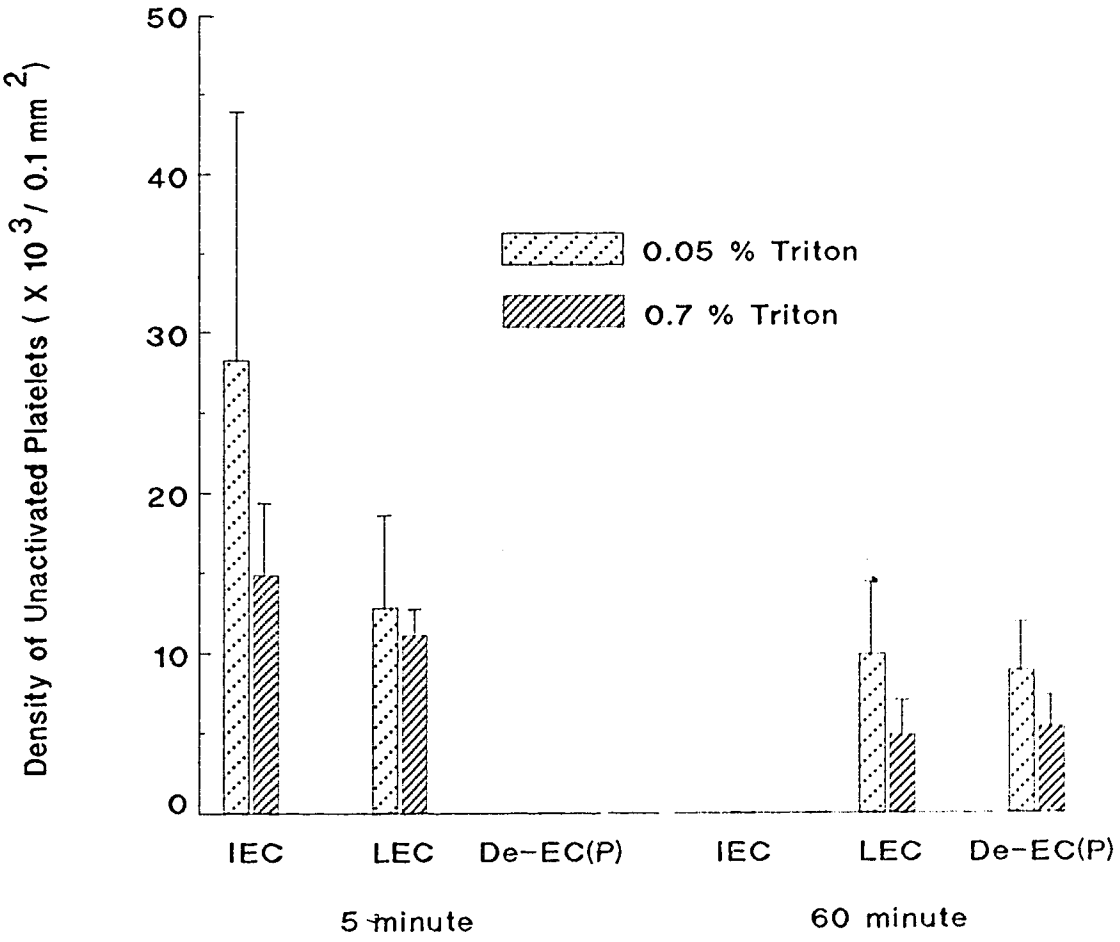


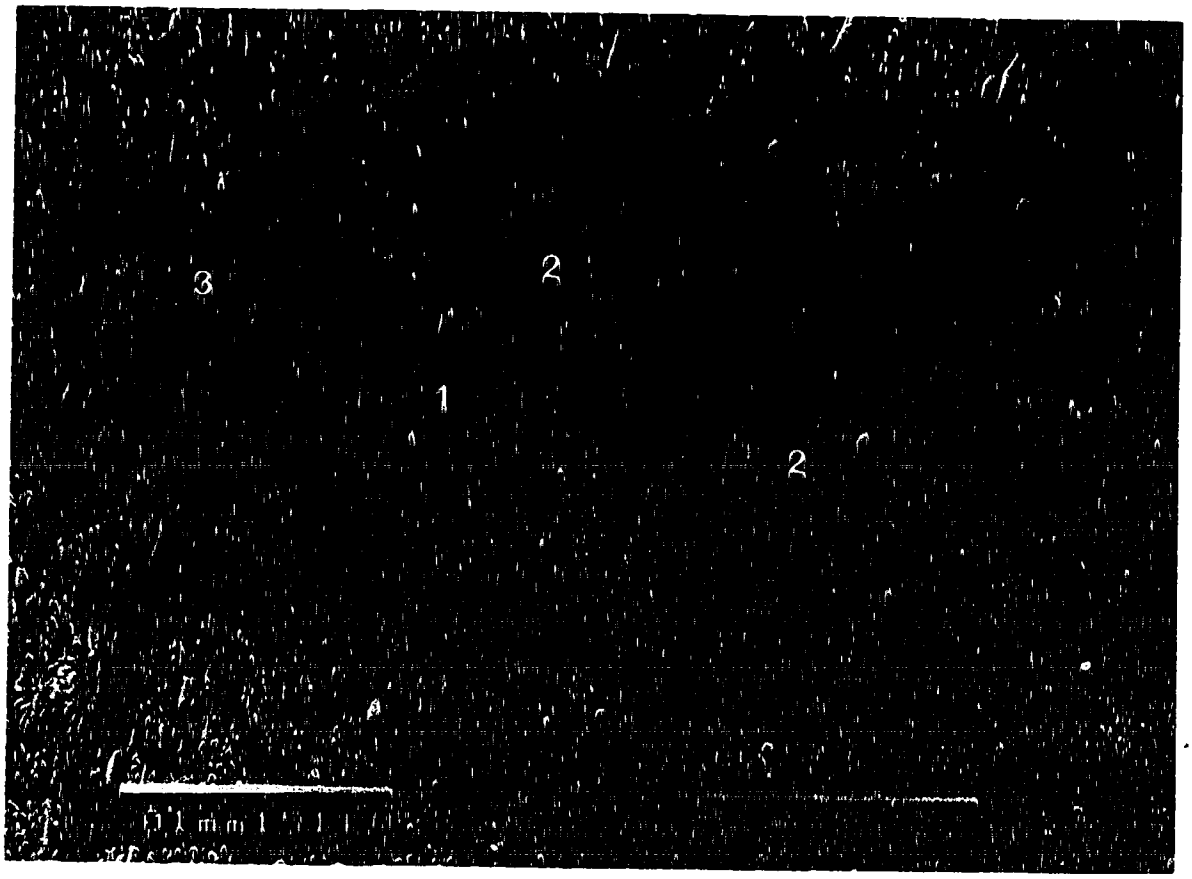
Table 1. Density of platelets in each category of EC injury independent of dose and time. The arteries were injured by 0.05% and 0.7% triton solution and examined after 5 and 60 minutes of restoration of blood flow. Mean values ± S.E. are listed in the table.

Significant differences from the platelet density in the LEC are expressed as * $P<0.05$ and ** $P<0.01$ using pooled t-tests.

Platelet Density	IEC	LEC	De-EC
	(n=5)	(n=12)	(n=5)
Total	4187±1368*	9798±1461	19449±2624**
Activated	1897±619**	8813±1464	18909±2672**
Unactivated	2281±925	967±190	539±203

Plate 7. SEM view of triton (0.1%) foam-injured surface of a rat aorta fixed after 60-minute restoration of blood flow. The dark areas represent relatively normal EC (EC). Mildly injured EC (IEC) can be found at the periphery of the normal EC islands beyond which there is a more seriously injured and lysed EC area (LEC) where the platelets tend to associate with the exposed sub-endothelium. Very few platelets are present on the IEC and normal EC area.

1=EC; 2=IEC; 3=LEC.



different categories of injury. Islands of relatively normal EC were found over the surface. Individual EC of the IEC category were found in an area between the relatively normal EC area and the injured/distorted EC area (LEC), as shown in Plate 7. More defined areas of IEC are shown in Plate 8. Observation on the platelet adhesion in Plates 7 and 8 shows that very few platelets adhered to the surface of relatively normal EC and IEC. In contrast, there was a large number of platelets associated with LEC. Quantification was not done in this group.

In the rabbit experiments air-dry-injury produced the full range of injury categories and subcategories. The most frequent categories, however, varied from IEC to De-EC. Occasionally, small areas of normal EC were preserved (data not shown). There was a sharp border between different types of injury. The pattern of platelet adhesion on the injured surface was very similar to that in the triton groups as shown in Plate 9. Very few platelets were associated with IEC. Large numbers of platelets, in contrast, were found on the denuded areas with sub-endothelium completely exposed (De-EC(P)). It is noted that in the boundary area between IEC and De-EC very few platelets adhered to the exposed sub-endothelium thus leaving bare areas (designated as De-EC(B) or PES-EC). Leucocyte attachment to injured surfaces was noted in this situation (Plate 10). This was not a feature in the triton injured rat aorta.

Plate 8. Detailed view of the same specimen of Plate 7. Very few platelets were associated with the injured EC (IEC) surface as long as underlying sub-endothelium was not directly exposed. 2=IEC; 3=LEC.

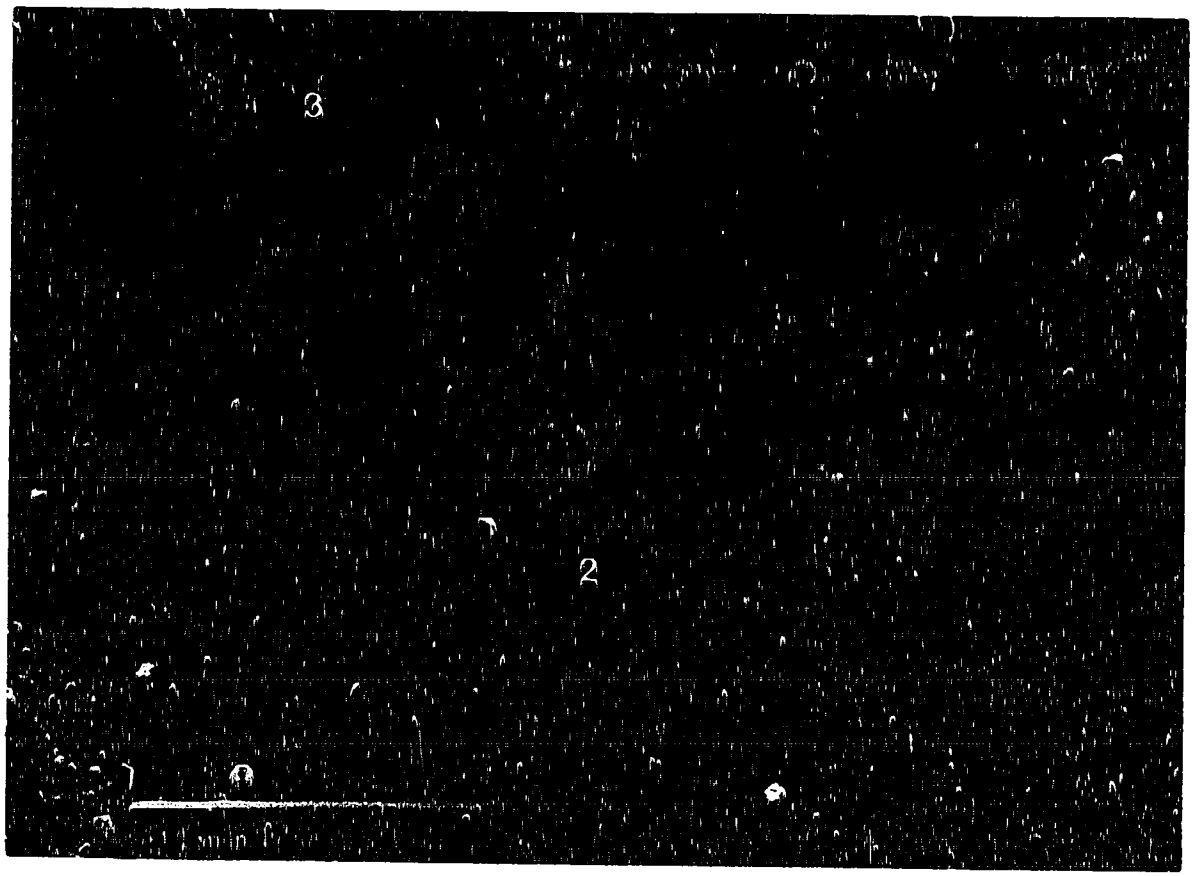
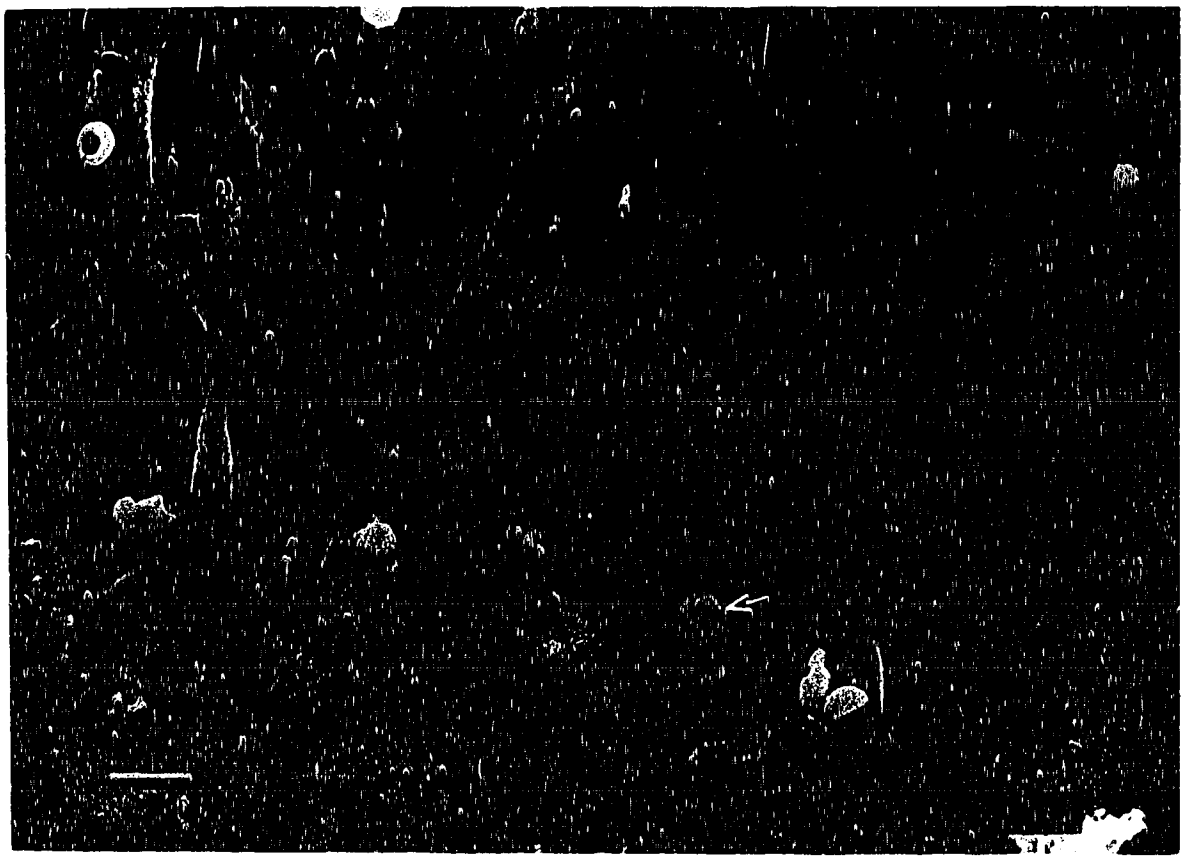


Plate 9. SEM view of the border between the mildly injured EC area and the de-endothelialized area of an air-dry injured rabbit carotid artery. Platelets preferred to adhere onto the completely denuded area. Very few platelets can be found on the injured EC (IEC) surface, even on the partially exposed sub-endothelium surrounding the injured EC sheet (PES-EC). Some leucocytes were associated with the denuded area (arrow). 2=IEC; 3a=PES-EC; 4a=De-EC(P).

Plate 10. SEM view of an air-dry injured surface of a rabbit carotid artery. The injured EC show rough surface and some of them were separated from each other, leaving gaps on the intercellular boundaries through which the leucocytes (arrow) tend to penetrate the arterial wall underneath. A few platelets are found on the partially exposed sub-endothelium (PES-EC).



3.2.3.3. Observation of Interactions of Platelets with the Injured Surface at High Magnification

Plate 11 shows the high magnification view of a single EC injured by triton. The underlying sub-endothelium was partially exposed through the crevices on the cell residue. A few platelets were present on the surface of the cell residue, but they always spread their pseudopods through crevices to attach to the partially exposed sub-endothelium. In contrast, a large number of platelets were associated with the exposed sub-endothelium adjacent to the cell residue.

3.2.3.4. Platelet Adhesion at Branch Orifices

Branch orifices were observed in some specimens. These branches had been ligated during the preparatory surgery. They therefore created blind sacs in which flow separation and stasis would be expected. In these regions direct platelet adhesion to injured EC occurred. The platelets adherent to naked nuclei of injured and lysed EC in these regions appeared aggregated rather than taking the form of a monolayer (Plates 12 and 13). They also appeared more distorted than in other regions.

Plate 11. High magnification view of a triton-injured EC. This EC was injured but preserved <u>in situ</u>. A few platelets appear associated with the cell residue but they always spread their pseudopods (arrow) to contact the partially exposed sub-endothelium through the crevices on the cell residue. 2=IEC; 3=LEC.

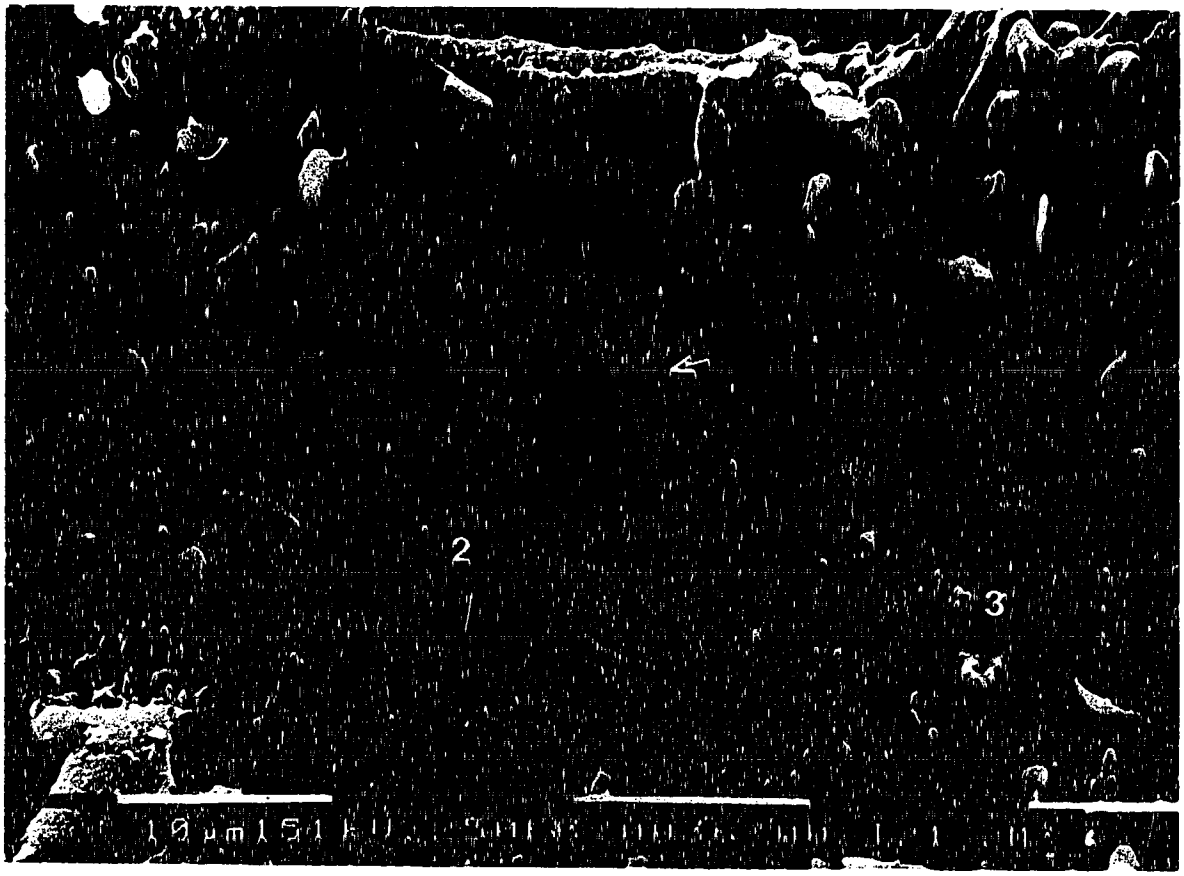


Plate 12. Triton injured surface in the region around a branching orifice (lareger arrow) in a rat aorta. Platelets were accumulated on the injured EC. These accumulated platelets seemed more associated with each other than associated with the EC residue forming platelet thrombotic plugs or aggregates (small arrow) (also see detail in the plate 13).

Plate 13. High magnification micrograph of injured rat aorta by 0.05% triton and fixed after a 5-minute restoration of blood flow. Many platelets are associated with the naked nuclei of the injured EC. These platelets tend to associate with each other, gathering in the distal portion of the nuclei and show more activated appearance than the neighbouring adherent platelets; they are called platelet aggregates (small arrow). Blood flow direction is indicated by a larger arrow. 3=LEC.

3.3. Labelled Precursor Incorporation

3.3.1. Tissue Incubation

In five paired experiments arterial tissue from intact arteries and from arteries injured by <u>in vivo</u> air drying were incubated with ^{3}H-acetate. As shown in Figure 8, there was no significant difference in ^{3}H-acetate incorporation into polar lipid and cholesterol between the intact and injured arteries.

3.3.2. Perfusion of Arterial Segments

In the arterial perfusion study, the effect of EC injury by different techniques on the incorporation of ^{3}H-acetate into lipids and ^{35}S-methionine into protein was tested.

(1) There was no significant effect of EC injury by in vitro mechanical damage on the ^{3}H-acetate incorporation into either polar lipid or cholesterol in three paired experiments (Figure 9). Precursor incorporation into protein was not examined in this set of experiments.

(2) <u>In vitro</u> air-drying injured arteries showed both a significantly decreased incorporation of ^{3}H-acetate into polar lipid (Figure 10 (a)) and a significantly decreased incorporation of ^{35}S-methionine into protein in three paired experiments (Figure 10 (b)).

Figure 8. Incorporation of ^{3}H-acetate into polar lipid (a) and cholesterol (b) in arteries with or without endothelium incubated in 10% LPDS in MEM. Data represents the mean ± S.D. from 5 experiments(3 segments each). De-endothelialization was produced by using the in vivo air-dry technique. There was no significant difference using a paired t-test.

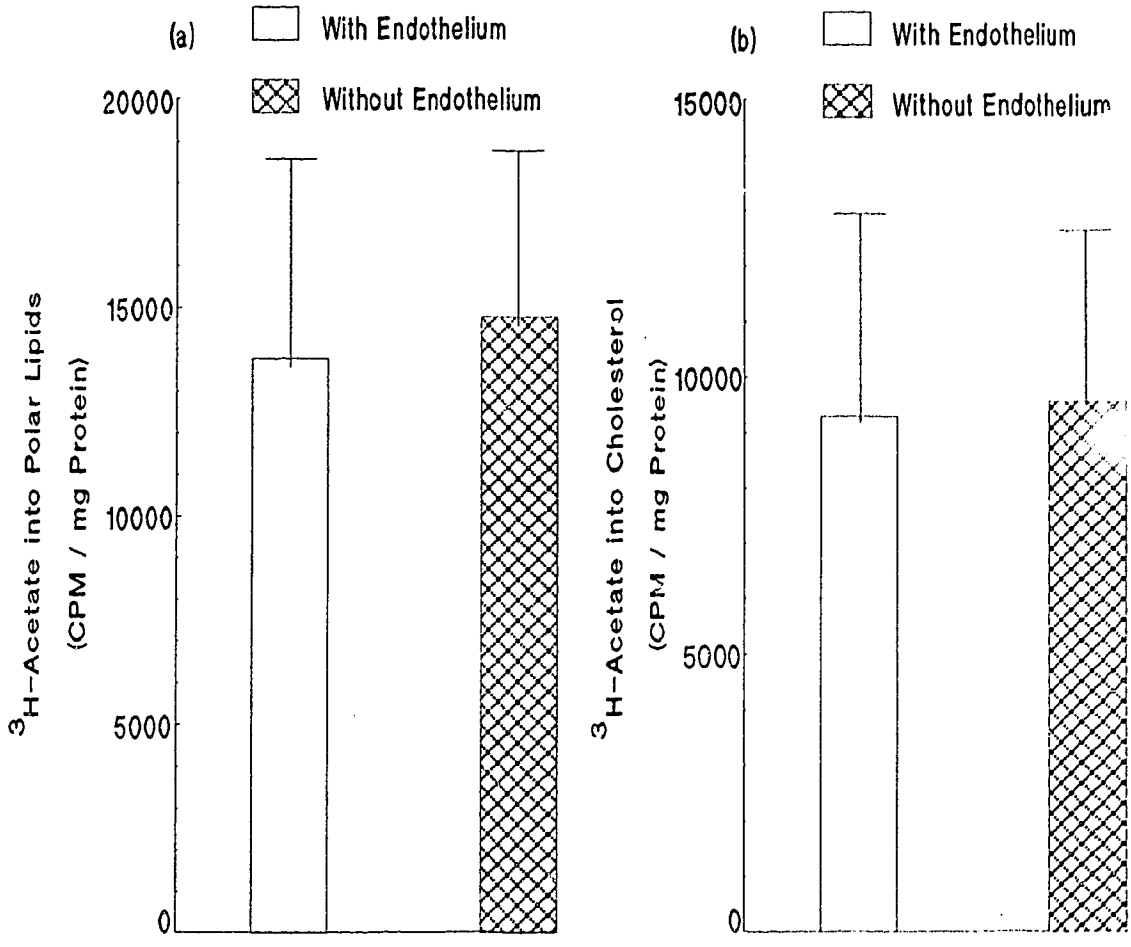


Figure 9. Incorporation of ^{3}H-acetate into polar lipid (a) and cholesterol (b) in arteries with or without endothelium perfused with 10% LPDS in MEM. Data represents the mean ± S.E. from 3 experiments(2 segments each). De-endothelialization was carried out by <u>in vitro</u> mechanical injury. There was no significant difference using a paired t-test.

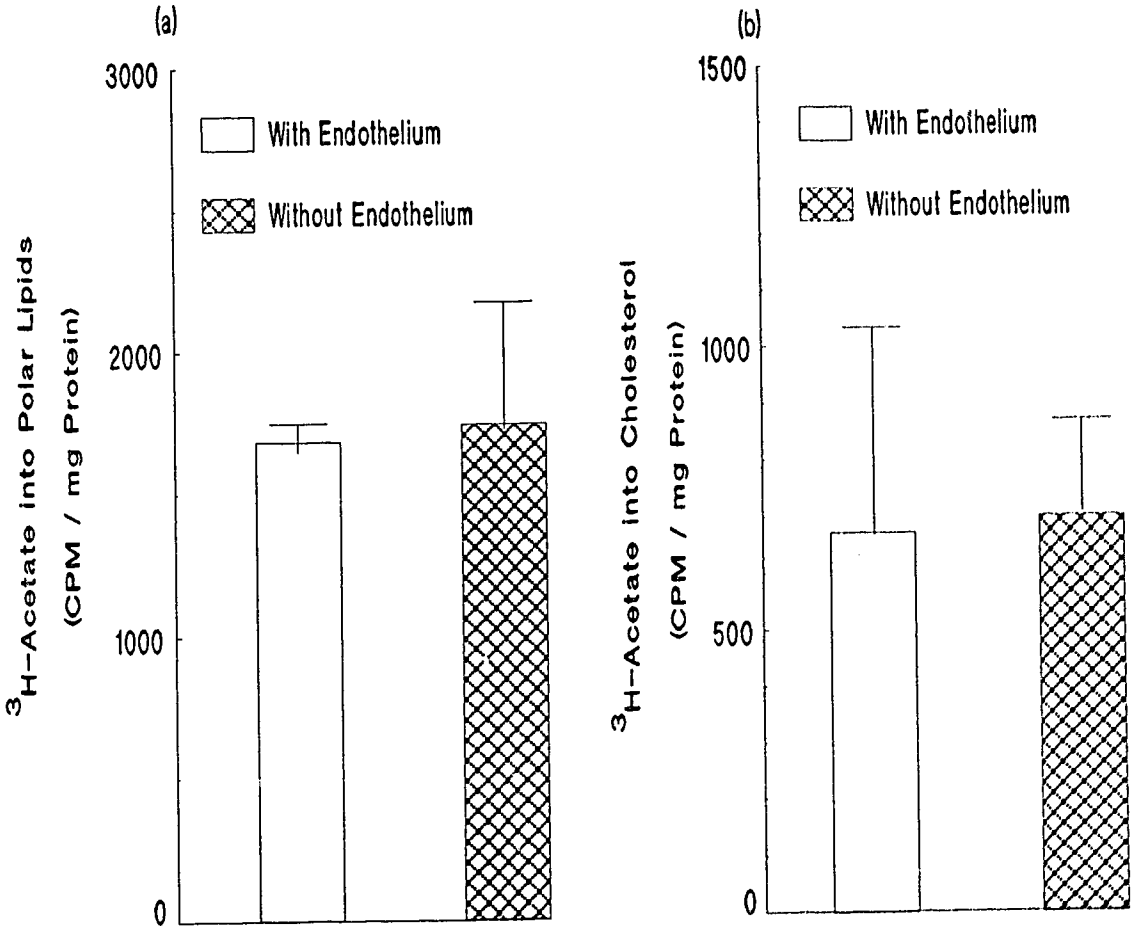
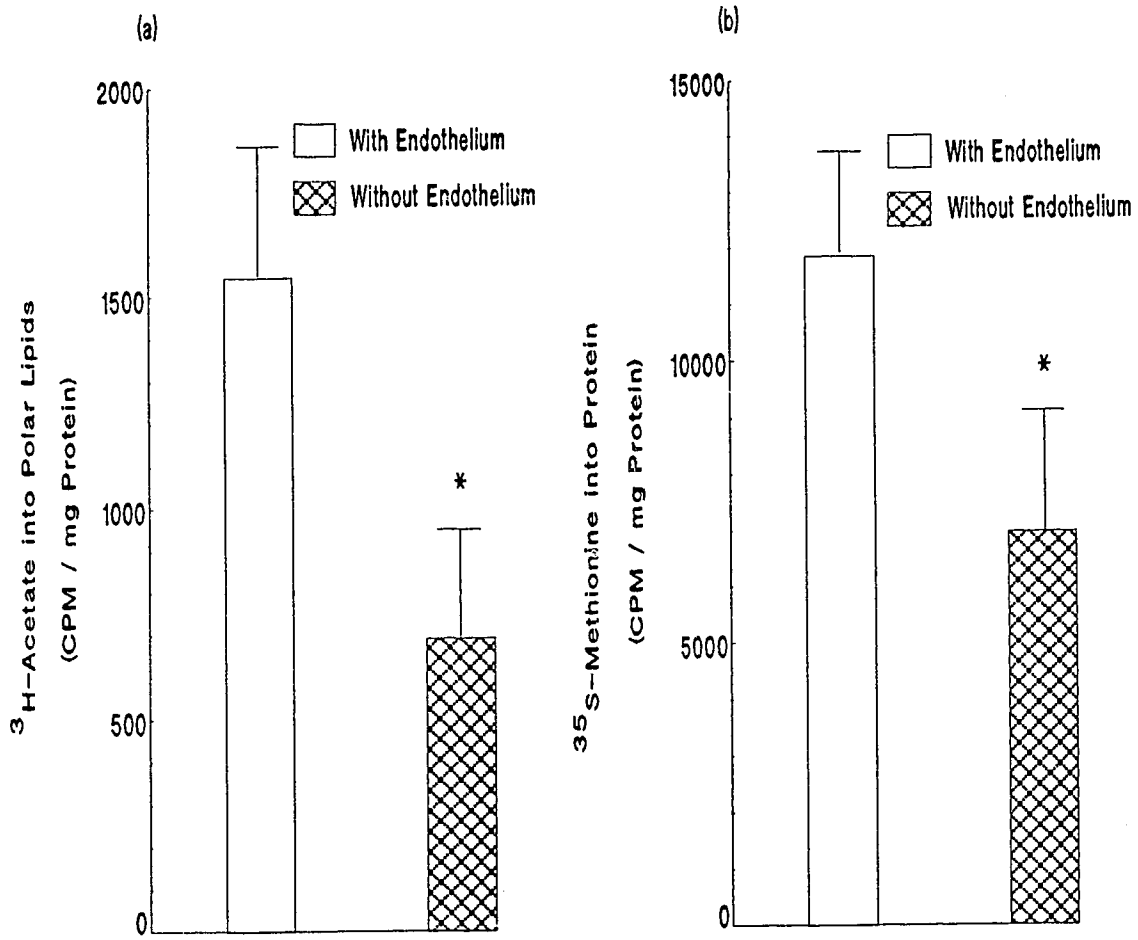


Figure 10. Incorporation of ^{3}H-acetate into polar lipid (a) and ^{35}S-methionine into protein (b) in arteries with or without endothelium perfused with 10% LPDS in MEM. Data represents the mean ± S.E. from 3 experiments(2 segments each). De-endothelialization was carried out by <u>in vitro</u> air-drying.
* Significantly different from control (arteries with endothelium), $P < 0.05$ using a paired t-test.



(3) Only one out of three *in vivo* air-drying injury experiments had results from both control and injured arteries. This single experiment showed an increased incorporation of ^{3}H-acetate into both polar lipid and cholesterol but not into protein after both 4 and 12 hour pulse periods. There was no change in ^{35}S-methionine incorporation (Table 2).

(4) Comparison of the perfused artery and incubated artery showed a greater incorporation of ^{3}H-acetate into the lipid of the incubated arteries (Figure 11).

3.3.3. Tissue Viability

Progressive incorporation of ^{3}H-acetate into polar lipid and of ^{35}S-methionine into protein was considered to indicate a viable tissue. Progressive incorporation of ^{3}H-acetate into polar lipid, cholesterol and tissue protein, and of ^{35}S-methionine into protein was demonstrated by removing half of the arterial segment at 4, 8 or 12 hours and continuing perfusion in the other half for a further 2 or 4 hour period. In each instance there was further incorporation of isotope during the extra 2 or 4 hours of perfusion (Figure 12 and 13).

Table 2. Incorporation of ^{3}H-acetate into lipid and protein, and ^{35}S-methionine into protein in intact artery or *in vivo* air-dry-injured artery. Tissue was perfused with 10% LPDS in MEM for 8 hours followed by a 4-hour or 12-hour pulse period. Results of the 4-hour period are for one segment from one experiment. Results of the 12-hour period are the mean ± S.D. of two segments from one experiment.

(a) ^{3}H-acetate Incorporation (CPM/mg Protein)

Labelling Period		Intact Artery	Injured Artery
4 hours	Polar Lipid	1986	3369
	Cholesterol	707	1426
	Protein	29508	34085
12 hours	Polar Lipid	8917±478	12213±2068
	Cholesterol	2593±368	3836±1199
	Protein	95886±4808	87353±1203

(b) ^{35}S-methionine Incorporation into Protein (CPM/mg Protein)

Labelling Period	Intact Artery	Injured Artery
4 hours	6100	7030
12 hours	20614±841	20545±499

Figure 11. Incorporation of ^{3}H-acetate into polar lipids (a) and cholesterol (b) in intact arteries perfused or incubated with 10% LPDS in MEM. Data represents the mean ± S.E.
* Significantly different from incorporation into perfused arteries, $P < 0.05$ using a pooled t-test(D.F.=7).

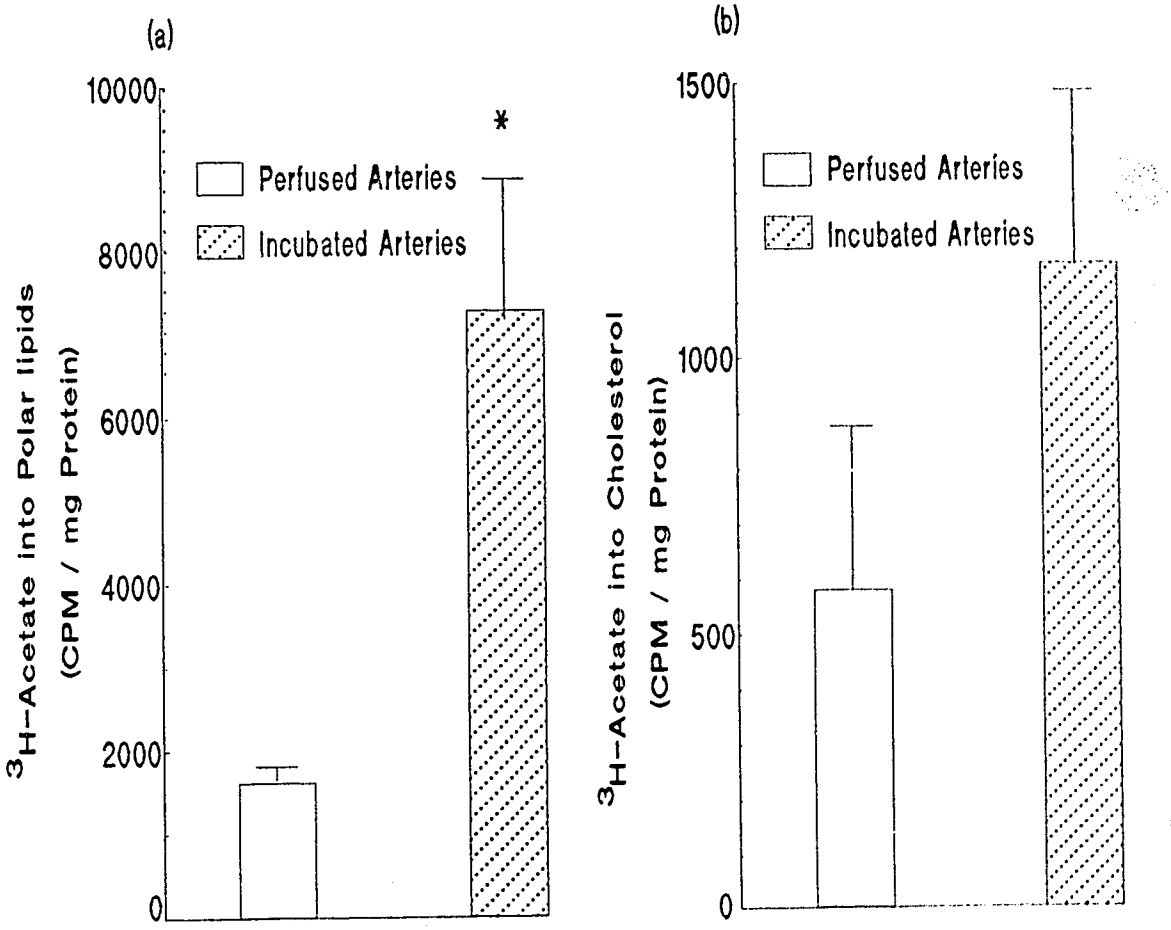


Figure 12. Accumulative incorporation of ^{3}H-acetate into polar lipid and cholesterol in perfused normal arteries after 4 to 16 hours of pulse. Each pair of joined points represents one experiment.

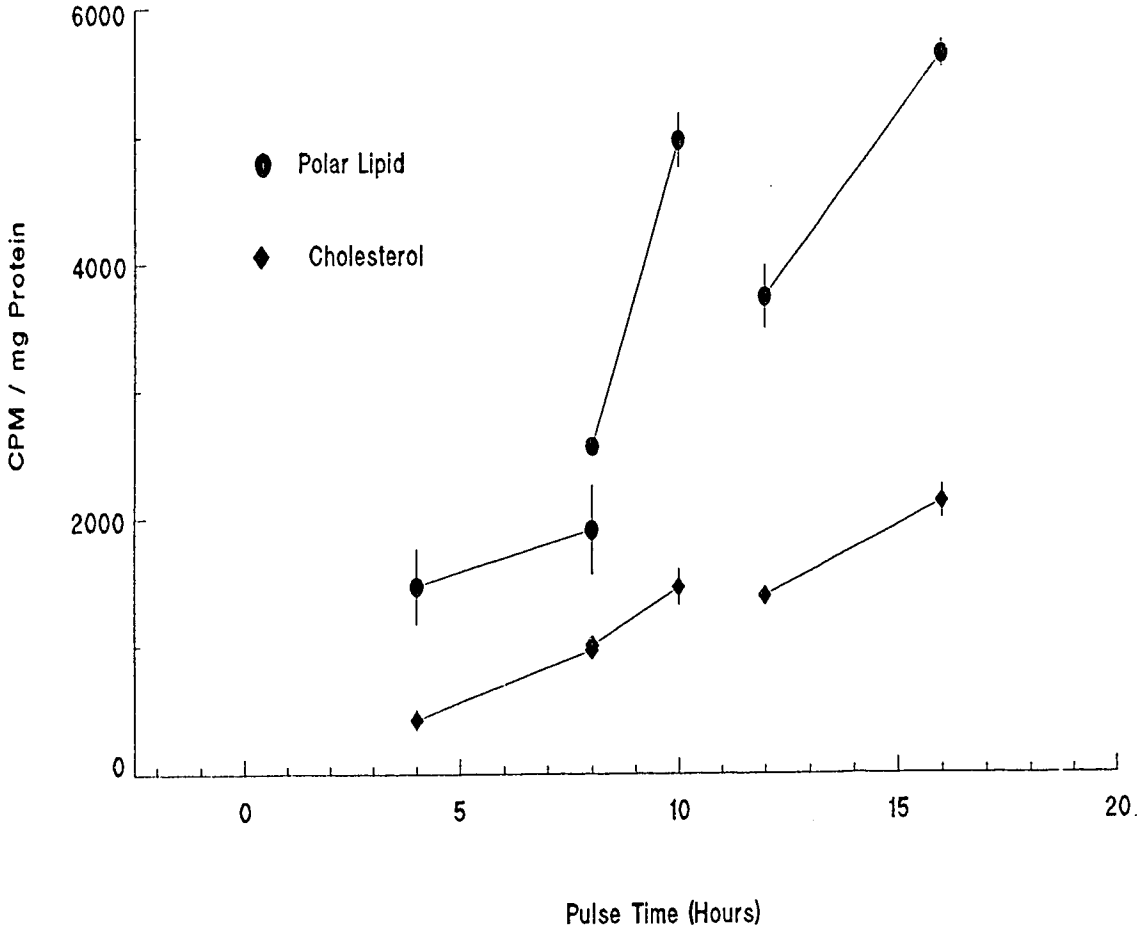
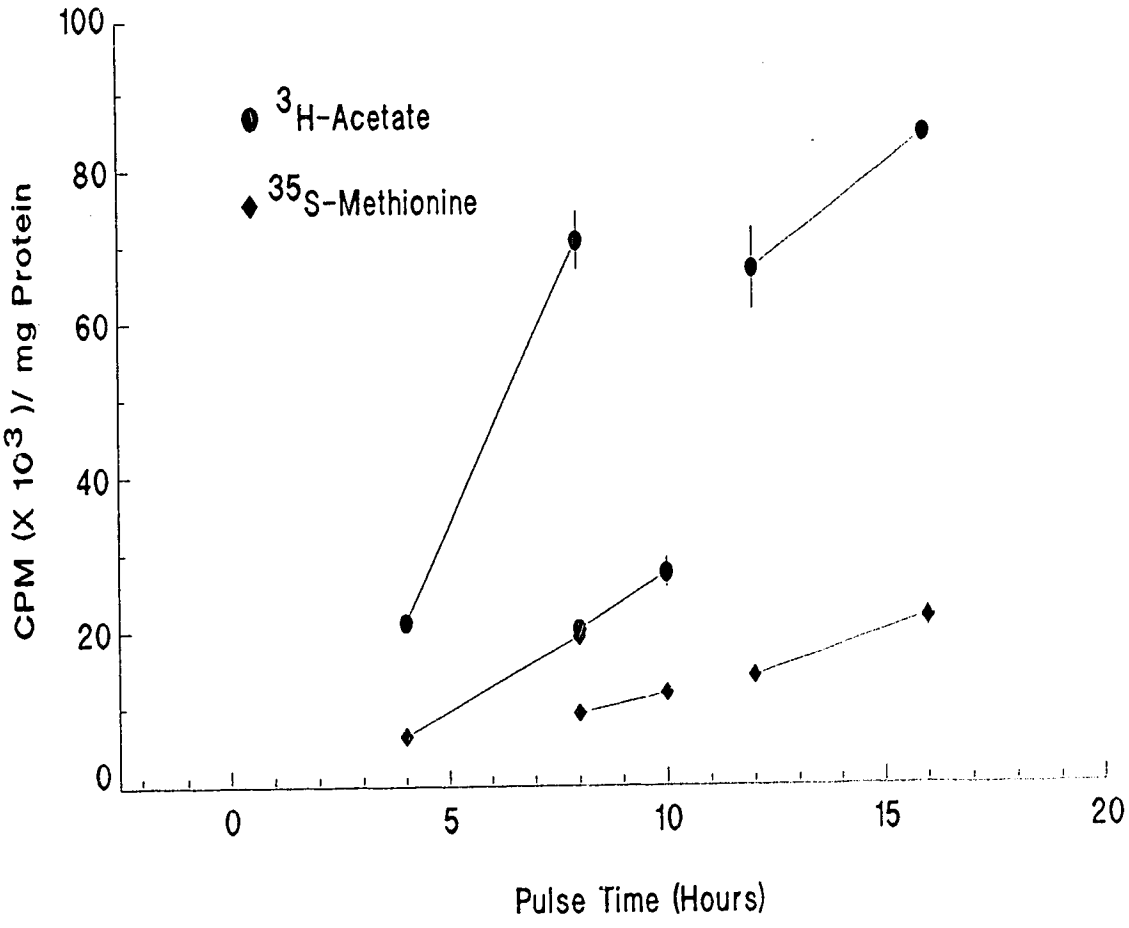


Figure 13. Accumulative incorporation of ^{3}H-acetate and ^{35}S-methionine into protein in normal arteries as described in the Figure 12.



3.3.4. Incorporation into Surrounding Tissue

Analyses of surrounding tissue showed a more than 5 times greater incorporation of ^{3}H-acetate into polar lipid (significantly different, D.F.=6) and 10 times greater incorporation into cholesterol (not significantly different, D.F.=4) compared to the incorporation in the host artery with similar wet weight (Figure 14). Comparison was also made between the surrounding tissue of intact artery and that of injured artery. This showed no significant difference using a pooled t-test (Figure 15).

3.3.5. Comparison of Chamber Types

Comparison of normal arterial tissues perfused in glass chambers or plastic chambers did not show a significant difference in the level of incorporation into polar lipid (Figure 16).

3.3.6. Incorporation Study in New Zealand White Rabbit

A single experiment in a New Zealand white rabbit is shown in Table 3. The intact artery had a comparable level of ^{3}H-acetate incorporation in lipid with the intact artery of lop-eared rabbits. The <u>in vitro</u> air-dry injured artery also showed decreased incorporation of ^{3}H-acetate into polar lipid,

Figure 14. Incorporation of ^{3}H-acetate into polar lipids (a) and cholesterol (b) in normal arteries and their surrounding tissue perfused with 10% LPDS in MEM.

* Significantly different from incorporation into arterial tissue, $P<0.05$ using a pooled t-test, D.F.=6.

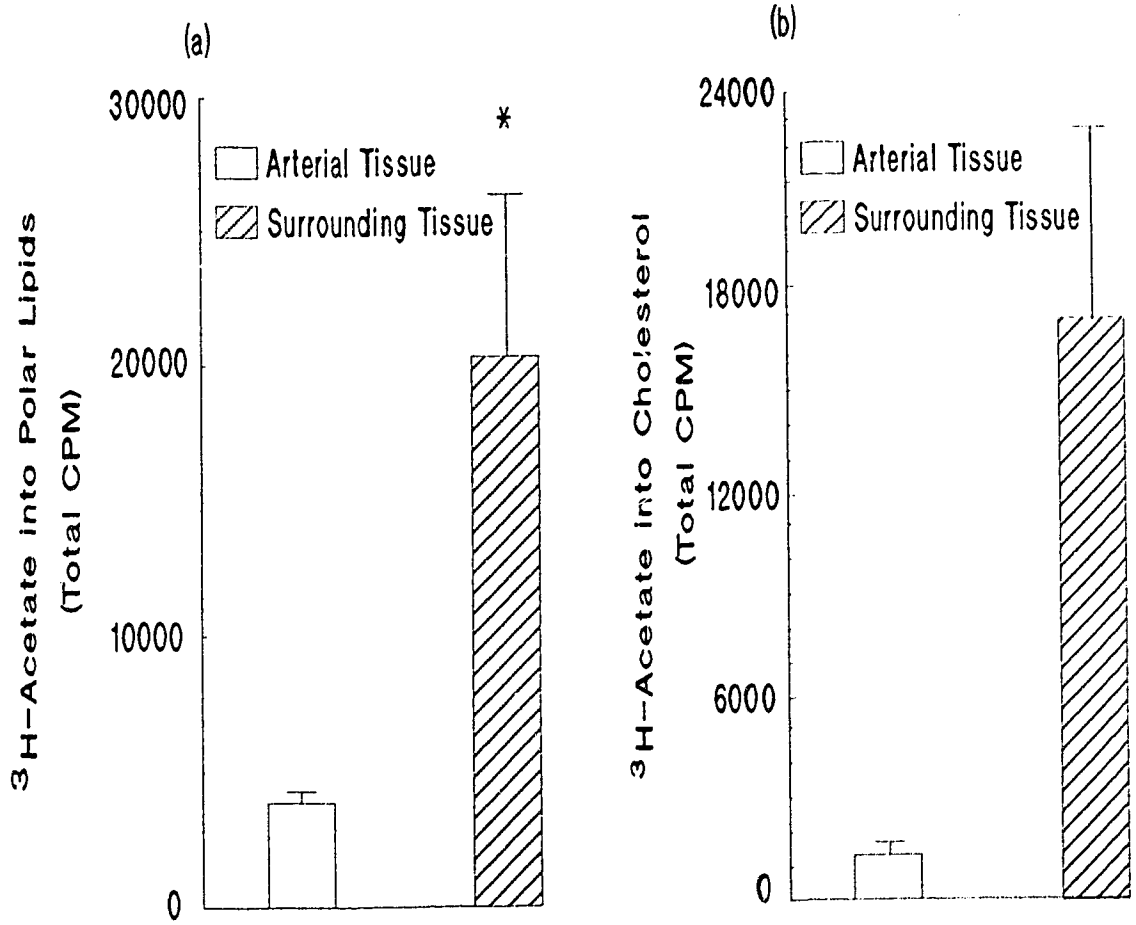


Figure 15. Incorporation of ^{3}H-acetate into polar lipids (a) and cholesterol (b) in surrounding tissue of perfused arteries with or without endothelium. Data represents the mean ± S.E. from 5 experiments. There was no significant difference using a pooled t-test (D.F.=8).

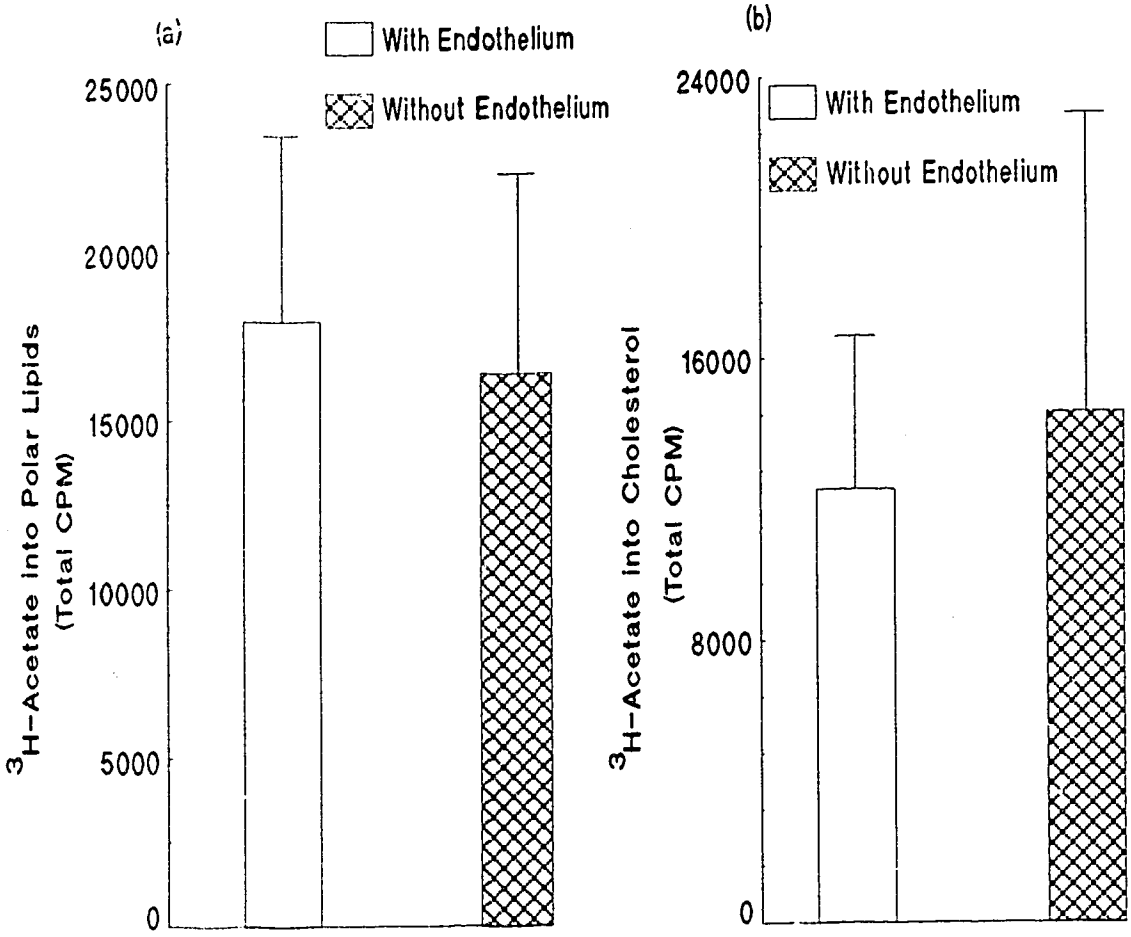


Figure 16. Incorporation of ^{3}H-acetate into polar lipids in arteries perfused in glass or plastic chambers. There was no significant difference using a pooled t-test (D.F.=5).

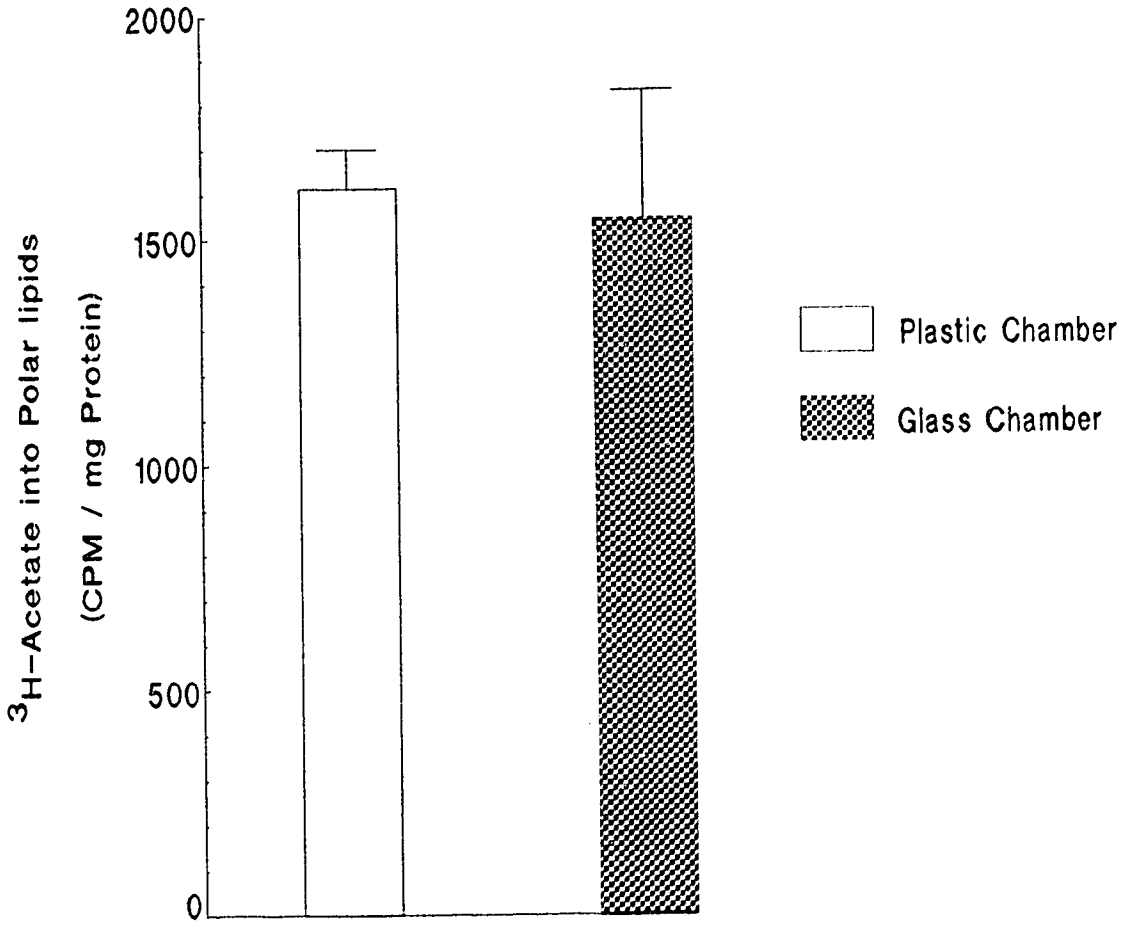


Table 3. Incorporation of ^{3}H-acetate into lipid and protein in intact or *in vitro* air-dry-injured artery of New Zealand white rabbit. Tissue was perfused and labelled for a typical 4-hour period. Results are mean ± S.D. of two segments from one experiment.

^{3}H-acetate Incorporation (CPM/mg Protein)		
	Intact Artery	Injured Artery
Polar Lipid	1322±162	688±189
Cholesterol	781±233	1149±40
Protein	12508±1755	6492±595

cholesterol, and protein similar to the lop-eared rabbits.

3.3.7. SEM of In Vitro Perfused Artery

SEM examination of the perfused intact artery showed a relatively normal appearance of the EC lining (plate 14). Platelet adhesion in the *in vivo* air-dry-injured artery was preserved during the 4 hour perfusion period (plate 15). *In vitro* perfused arteries previously subjected to *in vitro* air-dry and mechanical injury were not examined by SEM.

Plate 14. SEM of perfused rabbit carotid artery with intact EC. This segment of artery was perfused for 4 hours with 10% LPDS in MEM in the same perfusion system as used for metabolic study. The lumen of the artery shows a relatively normal appearance of EC lining.

1=EC.

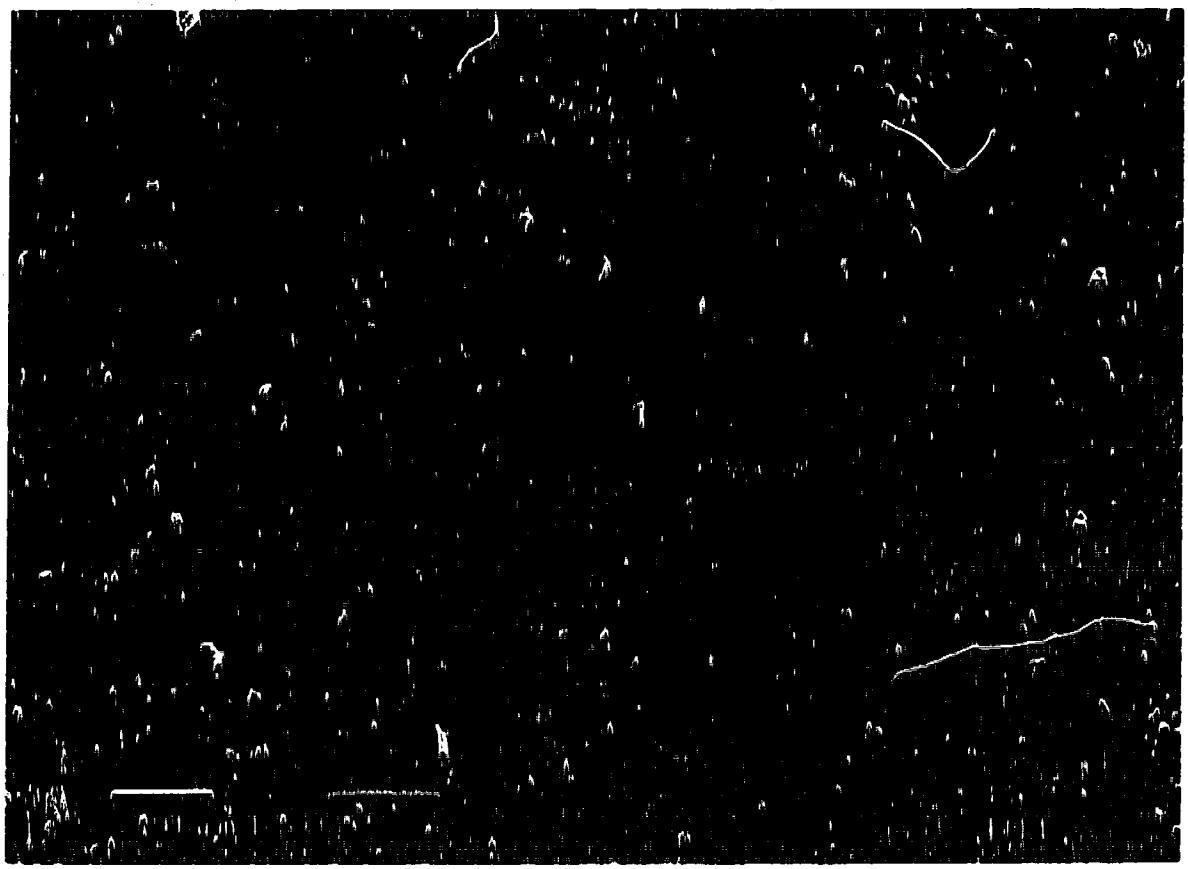
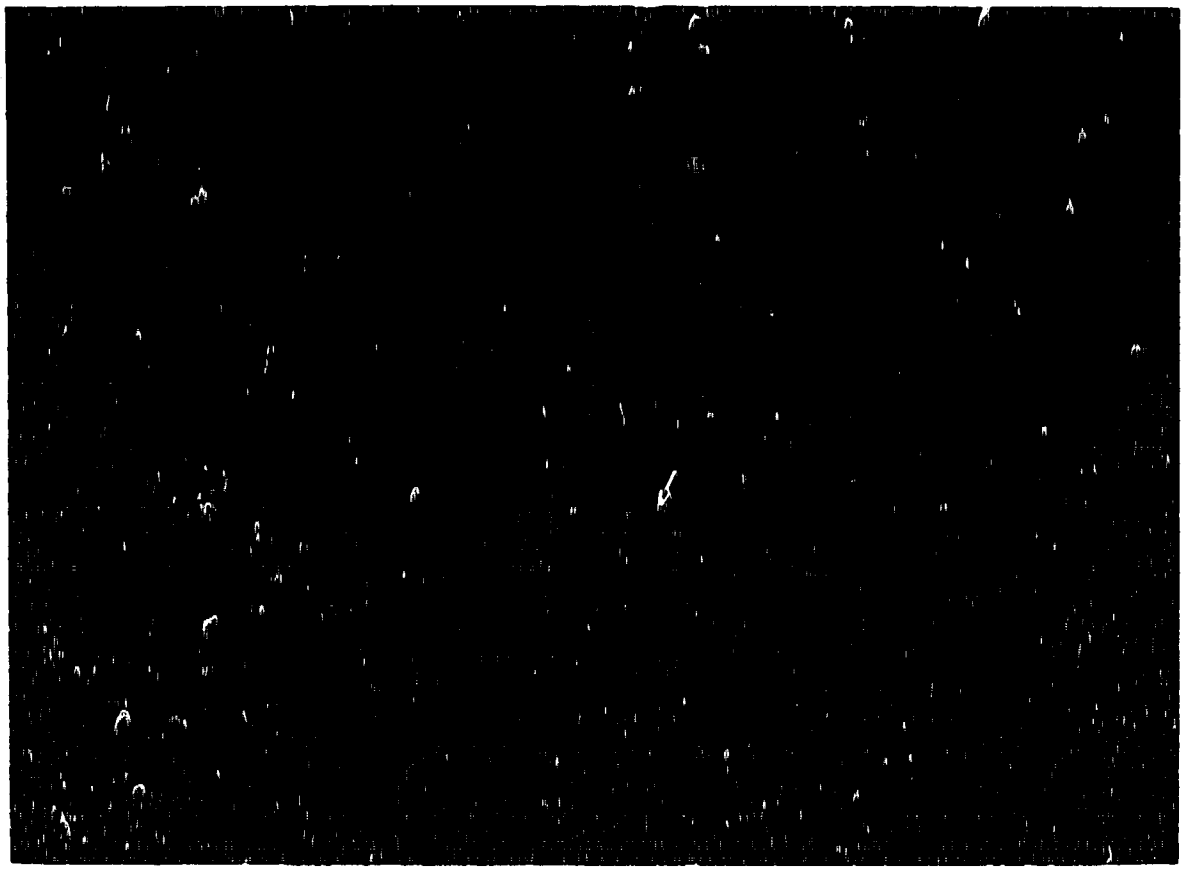


Plate 15. SEM of perfused carotid artery from the same animal as plate 14, but previously subjected to in vivo air-dry injury followed by a 3-hour restoration of blood flow. The luminal surface shows a complete de-endothelialization with platelets (arrow) adhering on the well preserved sub-endothelium. All platelets were spreading out.



4. DISCUSSION

4.1. Endothelial Injury

The original intent of this study was (1) to develop a method of producing graded *in vivo* arterial EC injury, (2) to use this animal model to study interactions of platelets with injured EC *in vivo*, and (3) to study the effects of EC injury on arterial wall metabolism *in vitro*. Two models of *in vivo* EC injury were produced. These are chemical (triton X-100 solution or foam) injury in rat aortas, and physical (air-dry) injury in rabbit carotid arteries.

Triton X-100 is a non-ionic detergent and surfactant, and has been used to solubilize a number of integral membrane proteins by forming soluble protein-detergent complexes. Triton X-100 can interact with the membrane lipid, causing a marked increase in the permeability of the cell to charged or neutral solutes and even macromolecules, and eventually, cell lysis (Heleniu and Simons, 1975). The acute injury produced by triton solution was not uniform. This uneven injury may be due to a differentiation of tolerance of EC in different regions of the artery to triton. Alternatively, it may be due to the effect of local rheology of blood flow on the autolytic process of the injured EC. In order to analyze the relationships between the EC injury and triton dosage, between

the injury and time, and between the injury and the platelet adhesion, EC injury was classified into different types. It should be pointed out that the categorization of EC injury was based on the degree of the morphological (pathological) changes of the injured cells [i.e. the mildest cell injury was defined as IEC, the most seriously injured cells were considered to be denuded (defined as De-EC), and the injured and distorted cells (defined as LEC) were the intermediate stage during the cell injury or lysis process]. Therefore, the defined grade of EC injury correlated with the degree of exposure of sub-endothelium.

Based on this categorization, an attempt was made to find if the EC injury was triton dose-related. Due to the difficulty in measuring the area of individual categories as mentioned previously, a comparison was only made between De-EC areas of the 0.05% and 0.7% triton groups (Figure 2). A greater percentage of De-EC area was found in the 0.05% triton group than the 0.7% triton group at both times (significant at 60 minutes). In other words, the higher the dose used, the more the injured endothelium was preserved <u>in situ</u>. Why a lighter dose would cause greater adhesion of the damaged EC is not readily apparent.

In terms of the incidence of individual injury categories, shown in Figure 3, 0.01% triton caused only mild EC injury (IEC) without platelet adhesion even 60 minutes after treatment. In contrast, dosages above 0.05% resulted in very

serious EC injury most of which was accompanied with massive platelet adhesion. Sixty minutes after the application of any of these dosages no normal EC or IEC remained. However, there was no difference in the pattern of incidence of EC injury between dosages above 0.05%. This suggests that a threshold dosage for acute morphological EC injury with platelet adhesion exists somewhere between 0.01% and 0.05% triton.

The results show that there is progression of the injury after the application period. The incidence of individual injury categories was determined and summarized in Figure 3. The disappearance of mild injury and the predominance of more severe injury with elapse of time suggests that the acute injury caused by triton treatment may undergo an irreversible degenerative process. This raises the possibility that there will be further progression after 60 minutes, possibly leading to full de-endothelialization as has been suggested by Langille and O'Donnell (1986).

The triton foam injury technique developed in this study produced a wide range of graded EC injury where relatively normal EC, IEC, and LEC are present adjacent to each other in the same artery.

For technical reasons the air-dry injury technique was found to be more suitable in rabbit carotid arteries. It was developed as an injuring technique for use in the metabolic study which required a set of paired arteries such as the common carotid arteries. The pattern of injury produced by

this method was similar to that produced by triton foam. It resulted in the full spectrum of EC appearance from relatively normal EC to De-EC in the same artery.

4.2. Interactions of Platelets with the Injured Arterial Wall

In the present study all the animals were heparinized before elucidating platelet adhesion. It has been recognized that the first platelet deposition on the sub-endothelium is not inhibited by heparin (Groves et al., 1979 and 1981; Dejana, 1987). This is also suggested by the fact that thrombin and fibrin do not contribute to this process (Groves et al., 1979; Packham and Mustard, 1984; Dejana, 1987). Similarly, absence of fibrin formation, as is the case with blood from fibrigenemic or haemophilic patients, does not alter platelet adhesion (Baumgartner et al., 1980).

Platelet adhesion was quantified in graded triton solution-treated rat aortas. There was not a significant difference of overall platelet adhesion between the 0.05% and 0.7% triton groups (Figure 6) even though there was an apparent increase of platelet adhesion in the 0.7% triton group compared to the 0.05% triton group at 60 minutes. There are two possibilities: a difference might exist between these groups but did not show significance because of the small group sizes and large intragroup variation, or there was no difference between dosages above the threshold concentration.

The purpose of this morphological study was to investigate the interactions between platelets and injured EC. Therefore, quantification of platelets in individual categories of EC injury was performed. This showed a tendency for lower platelet association with the mild injury areas and a greater association with more serious injury areas. This tendency existed for both dosages and for both time intervals after injury. When a comparison of platelet density for each injury category was carried out (independent of the dose used and the time interval after injury) significant differences were evident. These experiments, therefore, show that platelet density was correlated with the degree of EC injury. The fact that increasing degree of injury was associated with increasing exposure of sub-endothelium suggests that platelets are much less likely to adhere to EC, either normal or injured, than to sub-endothelium.

There was the suggestion that relatively less unactivated platelets were present with greater degree of EC injury. In LEC and De-EC areas where the sub-endothelium was partially or completely exposed, activated platelets predominated over unactivated platelets. In IEC areas, however, where exposure of sub-endothelium was minimal, the density of unactivated and activated platelets was similar. This suggests that platelet activation is dependent on the degree of EC injury and thus on the exposure of sub-endothelium.

The full range of EC injury was present in the same artery

injured by triton foam or air drying. This made it possible to assess the relationship between platelet adhesion and degree of EC injury under constant conditions. Factors affecting platelet adhesion such as the blood flow, blood composition and platelet function were identical for all injury grades in the same artery. The pattern of platelet adhesion followed a similar pattern to that seen in arteries injured by triton solution. Platelet density increased with more EC distortion and thus more exposure of sub-endothelium. There was little evidence of adhesion to either obviously injured cells or with the cell residue. Under high power magnification platelets apparently adherent to injured EC usually were observed to be attached to the sub-endothelium by extension of their pseudopods through gaps in the cell residue.

Based on the quantitative and qualitative observations on platelet distribution, an assumption of an EC injury degree-dependent platelet adhesion and activation is proposed. Both platelet adhesion and activation appear to result from the exposure of platelet reacting components (e.g. collagen fibres) in the sub-endothelium due to more damage and more distortion of EC. This is consistent with the general concept of preference of platelet adhesion to exposed sub-endothelial collagen which is the most potent stimulant for platelet activation.

Recognition of EC injury degree-dependent platelet adhesion is very important because it indicates that the

injured EC still acts as a structural barrier preventing platelet adhesion. These injured EC are unlikely to be able to produce and release as much anti-thrombogenic substance, if any, as the normal EC do. Normal EC do produce and release PGI_2 that prevents platelets from adhering to a foreign surface in vitro and there is an inverse correlation between PGI_2 production of the arterial wall and platelet adhesion after de-endothelialization (Eldor et al., 1981). It is still not known, however, how important the role of PGI_2 or other substances is in maintaining the non-thrombogenic property of the normal artery (Dejana, 1987). If the injured EC can act as a structural barrier, why not normal EC? This study would support the previous assumption that the EC lining plays a passive role in maintaining the non-thrombogenic property of a normal vessel (Gimbrone, 1987) in addition to any possible active role.

The only examples of platelet adhesion directly to injured EC were observed in regions around the orifices of ligated branches (Plates 12 and 13). Flow separation and stasis are likely present in such regions. These platelets are aggregated and may represent local platelet thrombus rather than platelet adhesion. They are more activated in appearance than the neighbouring adherent platelets and seem more associated with each other than associated with the EC residue. This is a similar arrangement to that described for platelet thrombi formed in vivo (Baumgartner and Muggli, 1976 and Adams, 1985).

It has been suggested that activated platelets tend to associate with each other (Sixma and DeGroot, 1986; Coller, 1987) and that development of a fibrin mesh-work, a result of activation of blood coagulation, facilitates platelet aggregation (Groves, et al., 1979). It is also recognized that platelet aggregation occurs on such a surface only when the blood flow is disturbed or reduced so that both materials released from platelets and activated platelets can accumulate (Mustard et al., 1987).

An interesting further point is that leucocytes were found associated with the air-dry-injured arterial wall in rabbits (Plate 10). This leucocyte-vessel wall interaction was consistent with the observation on the same animal model by Buchanan et al. (1987). However, in this present study leucocyte association with the triton-treated arteries in rat was not found. Individual experiments on both air-dry injured rats and triton injured rabbits showed the same result (i.e. leucocytes were again found on triton treated surfaces in rabbits and not found on air-dry surfaces in rats). Therefore, it is unlikely that the method of EC injury was responsible for the different leucocyte reaction. This suggests that there may be a species difference in leucocyte-injured vessel wall interaction.

4.3. Perfusion Study of Arterial Wall Metabolism

In the perfusion study, the effect of initial endothelial injury on lipid metabolism of the arterial wall was investigated. Paired common carotid arteries of rabbit were used for this study because (1) these identical arteries provided both control and experimental results from the same animal and (2) they have less major branches than any other elastic artery and therefore were more suitable for in vitro perfusion. The perfusion technique developed by Simatos (1987) in this laboratory was used and provided a means of retaining the structural integrity of the artery tissue. The preservation of a normal appearance of EC lining of perfused artery was confirmed by SEM (Plate 14). The maintenance of the viability of arterial tissue perfused in this apparatus was confirmed by cumulatively increased incorporation of precursors into the tissue up to 16 hours.

The increased incorporation of ^{3}H-acetate into polar lipid in de-endothelialized artery tissue was not reproduced in this study. The results were different from Simatos (1987) in two aspects. First, the levels of ^{3}H-acetate incorporation into polar lipid and cholesterol in perfused control arteries (with intact EC) were over 20 times lower in the present study. Secondly, loss of EC induced by <u>in vitro</u> mechanical injury did not produce a significant effect on ^{3}H-acetate incorporation into polar lipid. Therefore, the reason for much

lower incorporation levels was investigated. Degeneration of the plastic material of the apparatus has been ruled out by comparison with the incorporation result from tissue perfused in glass chambers. The preservation of tissue metabolic activity during 16-hour perfusion was confirmed by tissue viability study. There was no other comparable data available in the literature. Therefore, the problem remains unsolved.

Incorporation into incubated artery was greater than that into perfused artery. This suggests a better accessibility of the precursor to the incubated artery from both sides of the wall. A different metabolic status of the two preparations is another possibility.

Precursor incorporation into the tissue surrounding the artery was 10 times higher than into the arterial wall. This suggests that the in vitro lipid metabolism of the cells in the surrounding tissue is much more active than the SMC in the artery tissue. Therefore it should be noted that even a little contamination with surrounding tissue will affect the result attributed to SMC. In this study all incorporation studies were carried out on arterial segments completely freed of surrounding tissue contamination.

A significant decrease in incorporation into lipids and protein was observed in arteries injured by the in vitro air-dry technique. This decreased incorporation was probably due to the damage of this technique not only to the EC but also to deeper layers of the arterial wall including SMC. One

experiment using the in vivo air-dry injury animal model showed an increased incorporation of ^{3}H-acetate into both polar lipid and cholesterol during 4 and 12 hours perfusion and pulse (Table 2). This response is also different from that induced by Simatos (1987) who showed a serum-dependent increase in ^{3}H-acetate incorporation into polar lipid but not into cholesterol. Since in vivo injury was followed by a restoration of blood flow, the blood components must have interacted with the injured wall. At this initial stage of injury, interaction of platelets with the injured arterial wall was most impressive as demonstrated above. The adherent platelets on the injured arterial wall persisted for at least 4 hours during the perfusion as confirmed by SEM (Plate 15). They may undergo further activation and contraction releasing the contents from their cytoplasmic granules (Zucker and Nachmias, 1985) including platelet derived growth factor (PDGF). PDGF is a potent stimulater of growth of many cell types (e.g. fibroblasts, smooth muscle cells) (Ross, et al., 1974 and Ulutin, 1986). It may actively stimulate proliferation of neighbouring cells and thus be important in wound healing and atherogenesis according to the response-to-injury theory. In contrast to the results from in vitro injured arteries in the presence of serum, it is suggested that this increased metabolism of lipid in the in vivo injured arteries may reflect the stimulative effects of formed blood components especially the adherent platelets. This is not

conclusive and further study is required to confirm such a stimulative role of blood components and to identify the responsible material in the blood. Furthermore, it is not known whether this initial metabolic response represents an early event of atherogenesis or merely a healing process in response to the injury. However, this is the first attempt to study the effect of EC injury on arterial metabolism using the approach of the combination of the *in vitro* arterial perfusion and *in vivo* EC injury techniques. Such a combination of techniques will provide a promising approach to study the consequences of arterial EC injury or dysfunction which is now becoming more and more important in vascular pathology and pathophysiology.

5. CONCLUSION

In this study the initial responses to endothelial injury were investigated in terms of interactions of platelets with injured EC *in vivo* and incorporation of radio-labelled precursors into the arterial wall *in vitro*.

In vivo EC injury models were produced by perfusion of either triton X-100 in rats or dry air in rabbits. EC injury was classified and platelet adhesion was quantified by the SEM and their relationships were analyzed. The EC injury by triton solution at concentrations of 0.01-0.7% was not uniform. The acute injury was not stable and would lead to further progression. The lowest dosage required for EC injury and massive platelet adhesion was 0.05% of triton concentration and 1 minute of application time. *In vivo* platelet adhesion and activation on the arterial wall was EC injury degree-dependent. This is explained as the result of accessibility of platelet reacting components in the underlying sub-endothelium to circulating platelets. These relationships were further demonstrated and confirmed in triton foam- and air dry-injured arteries where the EC injury was widely graded in the same specimen. It was observed that platelets were rarely associated with the surface of obviously injured, *in situ* preserved EC as long as the underlying sub-endothelium was not exposed. This non-reactivity of injured or dysfunctional EC

to platelets is unlikely to be explained by an active role. Therefore, a structural barrier role of the EC lining in preventing circulating platelets from adhering to the normal artery is emphasized. Similarly, direct exposure of platelet reacting components in the sub-endothelium is crucial for initiating platelet responses to the injured artery. EC perturbation or dysfunction does not appear to initiate *in vivo* platelet adhesion directly without exposure of sub-endothelium.

The *in vitro* perfusion technique preserved not only the integrity of the EC lining but also the architecture and metabolic activity of the arterial wall. Such an approach represents a physiologic attempt to examine a complex cell system *in vitro*. In the metabolic study, there was no significant effect of *in vitro* mechanical de-endothelialization on the incorporation of ^{3}H-acetate into polar lipid and cholesterol in arterial tissue perfused and with MEM in the presence of serum (10% LPDS). The *in vitro* air-dry injury under the conditions used in this study reduced the metabolic activity of the arterial wall. The potential of surrounding tissue contamination in the study of metabolism of the arterial wall is emphasized.

6. REFERENCES

Adams, G. A. (1985). Platelet Adhesion: Past and Present. In: Longenecker, G. L. (ed). The Platelets: Physiology and Pharmacology. Academic. Orlando. pp. 15.

Ando, J., Nomura, H. and Kamiya, A. (1987). The Effect of Fluid Shear Stress on the Migration and Proliferation of cultured Endothelial Cells. Microvascular research. 33:62-70.

Barnes, M. J. (1986). Blood Vessel Wall Matrix Components Involved in Vessel Wall Thrombogenicity. In: Jolles et al. (eds). Biology and Pathology of Platelet-Vessel Wall Interactions. Academic Press. London. pp. 21-38.

Barnes, M. J. and MacIntyre D.E. (1979). Platelet-reactivity of Isolated Constituents of the Blood Vessel Wall. Haemostasis. 8: 158-170.

Baumgartner H. R. and Handenschild, C. (1972). Adhesion of Platelets to Sub-endothelium. Ann. NY. Acad. Sci. 201:22-36.

Baumgartner, H. R. and Muggli, R. (1976). Adhesion and Aggregation: Morphological Demonstration and Quantification *in vivo* and *in vitro*. In: Gordon, (ed). Platelet in Biology and Pathology. North-Holland. Amsterdam. pp. 23-60.

Baumgartner, H. R., Turitto, V. T. and Weiss, H. J. (1980). Effects of Shear Rate on Platelet Interaction with Subendothelium in Citrated and Native Blood. J. Lab. Clin. Med. 95: 208.

Bjorkerud, S. and Bondjers, G. (1973). Arterial Repair and Atherosclerosis after Mechanical Injury. Atherosclerosis. 18:235-255.

Booyse, F.M., Bell, S., Sedlak, B. and Rafelson, M. E.(1975). Development of an *in vitro* Vessel Wall Model for Studying Certain Aspects of Platelet-Vessel (Endothelial) Interactions. Artery. 1: 518.

Bourgain, R. H., Maes, L. and Andries, R. (1986). Arterial Thrombosis Studied *in vivo*. In: Jolles, G., Legrand, Y. J. and Nurden, A.(eds). Biology and Pathology of Platelet-vessel Wall Interactions. Academic Press. London. Orlando. pp. 337-349.

Brox, J. H., Osterud, B., Bjorklid, E. and Fenton, J. W. 2d. (1984). Production and Availability of Thromboplastin in Endothelial Cells. Br. J. Haematol. 57: 239-246.

Buchanan, M. R., Richardson, M., Haas, T. A., Hirsh, J. and Madri, J. A. (1987). The Basement Membrane Underlying the Vascular Endothelium Is Not Thrombogenic: In vivo and In vitro Studies with Rabbits and Human Tissue. Thrombosis and Haemostasis. 58: 698-704.

Bunce, D. F. (1965). Structural Difference between Distended and Collapsed Arteries. Angiology. 16: 53-56

Bunce, D. F. M.(ed) (1974). Atlas of Arterial Histology. Warren H. Green, St. Louis, Missouri. USA.

Clowes, A. W., Collazzo, R. E. and Karnovsky, M. J.(1978). A Morphologic and Permeability Study of Luminal Smooth Muscle Cells after Arterial Injury in the Rat. Lab. Invest. 39: 141-150.

Coller, B. S. (1987). Blood Elements at Surfaces: Platelets. Ann. NY. Acad. Sci. 516: 362-379.

Colucci, M., Balconi, G., Lorenzet, R., Pietra, A., Locati, D., Donati, M. B. and Semeraro, N.(1983). Cultured Human Endothelial Cells Generate Tissue Factor in Response to Endotoxin. J. Clin. Invest. 71: 1893-1896.

Curwen, K. D., Gimbrone, M. A. Jr. and Handin, R. I. (1980). In vitro Studies of Thromboresistance. Lab. Invest. 42: 366.

Davies, P. F. , M. A., Goode, T. N. and Bowye, D. E. (1976). Scanning Electron Microscopy in the Evaluation of Endothelial Integrity of the Fatty Lesion in Atherosclerosis. Atherosclerosis. 25: 125-130.

Davies, P. F., Remuzzi, A., Gordon, E. J., Dewey, C. F. Jr. and Gimbrone, M. A. Jr.(1986). Turbulent Fluid Shear Stress Induces Vascular Endothelial Cell Turnover in vitro. Proc. Natl. Acad. USA. 83: 2114-2117.

Day, A. J., Bell, F. P., Moore, S. and Friedman, R. (1974). Lipid Composition and Metabolism of Thromboatherosclerotic Lesions by Continued Endothelial Damage in Normal Rabbits. Circulation Res. 34:467-476.

DeGroot, Ph. G., Reinders, J. H. and Sixma, J. J. (1987). Perturbation of Human Endothelial Cells by Thrombin or PMA Changes the Reactivity of Their Extracellular Matrix towards Platelets. J. Cell. Biol. 104: 697-704.

Dejana, E. (1987). Endothelium, Vessel Injury and Thrombosis. Hematologica. 72: 89-94.

Dejana, E., Cazenave, J. P., Groves, H. M., Kinlough-Rathbone, R. L., Richardson, M., Packham, M. A. and Mustard, J. F.(1980). The Effect of Aspirin Inhibition of PGI2 Production on Platelet Adherence to Normal and Damaged Rabbit Aorta. Thromb. Res. 17: 453-464.

Dejana, E., Languino, L. R., Polentarutti, N., Balconi, G., Ryckewaert, J. J., Larrien, M. J., Donati, M. B., Mantovani, A. and Marguerie, G.(1985). Interaction between Fibrinogen and Cultured Endothelial Cells. Induction of Migration and Specific Binding. J. Clin. Invest. 75: 11-18.

Dewey, C. F. Jr., Gimbrone, M. A. Jr., Bussolari, S. R. and Davies, P. F. (1981). The Dynamic Response of Vascular Endothelial Cells to Fluid Shear Stress. J. Biochem. Eng. 103:177-185.

Drouet, L., Carrier, J. L., Rosa, J. P., Legrand, Y. F. and Caen, J. P. (1983). Platelet Deposition. In: Woolf, N.(ed). Biology and Pathology of the Vessel Wall. Praeger,. New York. pp. 81-100.

Drouet, L. and Caen, J. P. (1986). Antithrombotic Therapy. In: Jolles, G., Legrand, Y. J. and Nurden, A.(eds). Biology and Pathology of Platelet-vessel Wall Interactions. Academic Press. London. Orlando. pp. 385-406.

Eldor, A.,Falcone, D. J., Hajjar, D. P., Minick, C. R. and Weksler, B. B. (1981). Recovery of Prostacyclin Production by De-endothelialized Rabbit Aorta. J. Clin. Invest. 67: 735-741.

Faggiotto, A. and Ross, R. (1984). Studies of Hypercholesterolemia in the Non-human Primate. II. Arteriosclerosis. 4: 341-356.

Faggiotto, A., Ross, R. and Harker, L. (1984). Studies of Hypercholesterolemia in the Non-human Primate. I. Arteriosclerosis. 4: 323-340.

Falcone, D. J., Hajjar, D. P. and Minick, C. R.(1980). Enhancement of Cholesterol and Cholesterylester Accumulation in Re-endothelialized Aorta. Am. J. Pathol. 99: 81-104.

Fishman, J. A., Ryan, G. B. and Karnovsky, M. J. (1975). Endothelial Regeneration in the Rat Carotid Artery and the Significance of Endothelial Denudation in the Pathogenesis of Myointimal Thickening. Lab. Invest. 32: 339-351.

Flaherty, J. T., Ferrans, V. J., Pierce, J. E., Carew, T. E. and Fry, D. L. (1972a). Localizing Factors in Experimental Atherosclerosis. In: Likoff, W., Segal, B. L. and Inssull, W.(eds). Atherosclerosis and Coronary Heart Disease. Grune and Stratton. New York. pp. 40-83.

Flaherty, J. T., Pierce, J. E. and Ferrans, V. J.(1972b). Endothelial Nuclear Pattern in the Canine Arterial Tree with Particular Reference to Haemodynamic Events. Circulation Res. 30: 23-33.

Folch, J., Lees, M., Sloane-Stanley, G. H. (1957). Simple Method for the Isolation and Purification of Total Lipids from Animal Tissue. J. Biol. Chem. 226:497-509.

Gerrard, J. M. (1988). Platelet Aggregation: Cellular Regulation and Physiologic Role. Hospital Practice. 23: 89-98.

Gimbrone, M. A. Jr. (1986). Endothelial Dysfunction and the Pathogenesis of Atherosclerosis. In: Fidge, N. H. and Nestel, P. J. (eds). Atherosclerosis VII. Elsevier Science. New York. pp. 367-369.

Gimbrone, M. A. Jr. (1987). Vascular Endothelium: Nature's Blood- Compatible Container. Ann. NY. Acad. Sci. 516: 5-11.

Gimbrone, M. A. Jr. and Buchanan, M. R. (1982). Interactions of Platelets and Leucocytes with Vascular Endothelium. Ann. NY. Acad. Sci. 401: 171-183.

Groves, H. M., Kinlongh,-Rathbone, R. L., Richardson, M., Moore, S. and Mustard, J. F. (1979). Platelet Interaction with Damaged Rabbit Aorta. Lab. Invest. 40: 195-200.

Groves, H. M., Kinlough-Rathbone, R. L. and Mustard, J. F. (1981). Comparison of the Effects of Aspirin and Heparin on Platelet Accumulation on the Injured Neointima of Rabbit Aorta. Thromb. Haemost. 46: 200.

Groves, H. M., Kinlough-Rathbone, R. L. and Richardson, M., Jorgensen, L., Moore, S. and Mustard, J. F.(1982). Thrombin Generation and Fibrin Formation Following Injury to Rabbit Neointima. Lab. Invest. 46: 605-612.

Groves, H. M., Kinlough-Rathbone, R. L. and Mustard, J. F. (1986). Development of Non-thrombogenicity of Injured Rabbit Aorta Despite Inhibition of Platelet Adherence. Arteriosclerosis. 6(2):189-195.

Hajjar, D. P., Falcone, D. J., Fowler, S. and Minick, C. R. (1981). Endothelium Modifies the Altered Metabolism of the Injured Aortic Wall. Am. J. Pathol. 102: 28-39.

Hansson,G. K., Bondjers, G. (1980). Endothelial Proliferation and Atherogenesis in Rabbits with Moderate Hypercholesterolemia. Artery. 7: 316-329

Hansson, G. K. and Bondjers, G. (1987). Endothelial Dysfunction and Injury in Atherosclerosis. Acta. Med. Scand. (suppl) 715: 11-17.

Heleniu, A. and Simons, K. (1975). Solubilization of Membranes by Detergents. Biochem. Biophys. Acta 415:29-79.

Houdijk, W. P. M., Sakariassen, K. S., Nievelstein, P. F. E. M. and Sixma, J. J. (1985). Role of Factor VIII-von Willebrand Factor and Fibronectin in the Interaction of Platelets in Flowing Blood with Monomeric and Fibrillar Human Collagen Type I and III. J. Clin. Invest. 75: 531-540.

Jaffe, E. A. (1987). Cell Biology of Endothelial Cells. Hum. Pathol. 18: 234-239.

Kinlough-Rathbone, R. L., Pachhan, M. A. and Mustard, J. F. (1983). Vessel Injury, Platelet Adherence, and Platelet Survival. Arteriosclerosis. 3: 529-546.

Kruth, H. S. (1985). Sub-endothelial Accumulation of Unesterified Cholesterol: An Early Event in Atherosclerotic Lesion Development. Atherosclerosis. 57: 337-341.

Langille, B. L. and Adamson, S. L.(1981). Relationship between Blood Flow Direction and Endothelial Cell Orientation at Arterial Branch Site in Rabbits and Mice. Circulation Res. 48:481-488.

Langille, B. L. and O'Donnell, F. (1986). Reductions in arterial Diameter Produced by Chronic Decreases in Blood Flow Are Endothelium-Dependent. Science. 231:405-407.

Leeson, C. R. and Leeson, T. S.(eds) (1985). Atlas of Histology. Philadelphia. Saunders. pp.

Leonard, E. F. (1982). Rheology of Thrombosis. In: Coleman, R. W.(ed). Homeostasis and Thrombosis. Lippincott. Philadelphia. pp. 755-765.

Minick, C. R., Stemerman, M. B. and Insull, W. Jr. (1977). Effect of Regenerated Endothelium on Lipid Accumulation in the Arterial Wall. Proc. Natl. Acad. Sci. USA. 74: 1724-1728.

Minick, C. R., Stemerman, M. B. and Insull, W. Jr.(1979). Role of Endothelium and Hypercholesterolemia in Intimal Thickening and Lipid Accumulation. Am. J. Pathol. 95:131-158.

Moore, S.(1973). Thromboatherosclerosis in Normolipemic Rabbits: A result of Continued Endothelial Damage. Lab. Invest. 29:478-487.

Moore, S. (1979). Endothelial Injury and Atherosclerosis. Exp. Mol. Pathol. 31:182-190.

Morin, R. J.,Zemplenyi, T. and Peng, S. K. (1987). Metabolism of the Arterial Wall-Influence of Atherosclerosis and Drugs. Pharmacol. Ther. 32:237-283.

Morrel, E. M., Holland, J. H., Pritchard, K. A., Colton, C. K. and Stemerman, A. B. (1987). Endothelial Cell Perturbation and LDL. Ann. NY. Acad. Sci. 516:412-417.

Morrison, A. D., Berwick, L., Orci, L. and Winegard, A. I. (1976). Morphology and Metabolism of an Aortic Intima-media Preparation in Which an Intact Endothelium is Preserved. J. Clin. Invest. 57:650-660.

Mustard, J. F., Groves, H. M., Kinlough-Rathbone, R. L. and Packham, M. A. (1987). Thrombogenic and Non-thrombogenic Biological surfaces. Ann. NY. Acad. Sci. 516:12-21.

Nerem, R. M., Levesque, M. J. and Cornhill, J. F.(1981). Vascular Endothelial Morphology as an Indicator of the Pattern of Blood Flow. J. Biomech. Eng. 103:172-176.

Packham, M. A. and Mustard, J. F. (1984). Platelet Adhesion. In: Spaet, T. H.(ed). Progress in Homeostasis and Thrombosis. Grune and Stratton. New York. vol. 7. pp.211.

Pease, D. C. and Molinari, S. (1960). Electron Microscopy of Elastic Arteries: the Thoracic Aorta of Rat. J. Ultrastruct. Res. 3:469-483.

Peerschke, E. I. B. (1985). The Platelet Fibrinogen Receptor. Semin. Hematol. 22:241-259.

Pittilo, R. M., Mackie, I. J., Rowles, P. M., Machin, S. J. and Woolf, N. (1982). Effects of Cigarette Smoking on the Ultrastructure of Rat Thoracic Aorta and Its Ability to Produce Prostacyclin. Thrombo. Haemostas. 48:173-176.

Reidy, M. A. and Silver, M. (1985). Endothelial Regeneration VII. Lack of Intimal Proliferation after Defined Injury to Rat Aorta. Am. J. Pathol. 118:173-177

Rhodin, A. G. (1962). Structure of Vascular Wall in Mammals with Reference to Smooth Muscle Component. Physiol. Rev. 42:48-87.

Rhodin, A. G. (1974). Arteries. In: Histology. Oxford University Press. New York. pp 340-346

Roessner, A., Schmitz, G. and Sory, C. (1987). What's New in the Pathology of Atherosclerosis. Path. Res. Pract. 182:694-698.

Ross, R. (1986). The Pathogenesis of Atherosclerosis-An Update. New Eng. J. Med. 314:488-500.

Ross, R. and Glomset, J. A. (1976). The Pathogenesis of Atherosclerosis. N. Eng. J. Med. 295:369-377 and 420-425.

Ross, R.,Glomset, J., Kariya, B. and Harker, L. (1974). A Platelet-dependent Serum Factor That Stimulates the Proliferation of Arterial Smooth Muscle Cells in vitro. Proc. Natl. Acad. Sci. USA. 71:1207.

Ross, R., Glomset, J. A. and Harker, L. (1978). The Response to Injury and Atherogenesis: The Role of Endothelium and Smooth Muscle. Atherosclerosis. Rev. 3:69-75.

Ryan, U. S. (1987). Activation of Endothelial Cells. Ann. NY. Acad. Sci. 516:25-38.

Schwartz, S. M., Stemerman, M. B. and Benditt, E. P. (1975). The Aortic Intima. II. Repair of the Aortic Lining after Mechanical Denudation. Am. J. Pathol.81:15-42.

Schwartz, S. M., Reidy, M. A. and Hansson, G. K. (1983). Injury at the Vascular Surface. In: Schettler, G.(ed). Fluid Dynamics as a Localizing Factor for Atherosclerosis. Springer-Verlag. Berlin, Heidelberg. pp. 188-199.

Simatos, G. A. (1987). Ph.D. Thesis. Physiology, University of Alberta.

Simionescu, N. and Simionescu, M. (1977). The Blood Vessels. In: Leon Weiss and Greep. (eds) Histology. McGraw-Hill Book Co. New York. pp. 373-420.

Sixma, J. J., Sakariassen, K. S., Stel, H. V., Houdijk, W. P. M., In der Maur, D. W., Hamer, R. J., de Groot, P. G. and van Mourik, J. A. (1984). Functional Domains in VWF, Recognition of Discrete Tryptic Fragments by Monoclonal Antibodies That Inhibit Interaction of VWF with Platelets and With Collagen. J. Clin. Invest. 74:736-744.

Sixma, J. J. and DeGroot, P. G. (1986). Interaction of Blood Platelets with the Vessle Wall. In: Jolles, G., Legrand, Y. J. and Nurden, A.(eds). Biology and Pathology of Platelet-vessel Wall Interactions. Academic Press. London. Orlando. pp.39-51.

Sixma, J. J., Patricia, F. E. M. et al. (1987). Adhesion of Blood Platelets to the Extracellular Matrix of Cultured Human Endothelial Cells. Ann. NY. Acad. Sci. 516:39-51.

Sprague, E. A., Steinbach, B. L., Nerem, R. M. and Schwartz, C. J.(1987). Influence of a Laminar Steady-State Fluid-Imposed Wall Shear Stress on the Binding, Internalization, and Degradation of LDL by Cultured Arterial Endothelium. Circulation. 76(3):648-656.

Steen, V. M. and Holmsen, H. (1987). Current Aspects on Human Platelet Activation and Responses. Eur. J. Hematol. 38:383-399.

Steinberg, D. (1979). Research Related to Underlying Mechanisms in Atherosclerosis. Circulation. 60:1559-1565.

Stel, H. V., Sakariassen, K. S., DeGroot, P. G., van Mourik, J. A. and Sixma, J. J. (1985). VWF in the Vessel Wall Mediates Platelet Adherence. Blood. 65:85-90.

Stemerman, M.B. (1974). Vascular Intimal Components: Precursors of Thrombosis. In: Spaet, T. H.(ed). Progress in Homeostasis and Thrombosis. vol 2. Grune and Stratton. New York. pp. 1-47.

Stemerman,M.B. (1981). Effects of Moderate Hypercholesterolemia on Rabbit Endothelium. Atherosclerosis. 1:25-32.

Stemerman, M. B. (1982). Anatomy of the Blood Vessel Wall. In: Colman, R. W.(ed). Homeostasis and Thrombosis. Lippincott. Philadelphia. pp. 525-544.

Stemerman, M. B. and Ross, R. (1972). Experimental Arteriosclerosis. I. Fibrous Plaque Formation In Primates, an Electron Microscope Study. J. Exp. Med. 136:769-789.

Stemerman, M. B., Colton, C. K. and Morrel, E. M. (1984). Perturbation of the Endothelium. In: Progress in Homeostasis and Thrombosis. vol. 7. Spaet, T. H.(ed). Grune and Stratton. New York. pp. 289-324.

Stemerman M. B., Morrel, E. M., Burke, K. R., Colton, C. K., Smith, K. A. and Lees, R. S.(1986). Local Variation in Arterial Wall Permeability to LDL in Normal Rabbit Aorta. Arteriosclerosis. 6:64-69.

Stern, D. M., Bank, I., Nawroth, P. P., Cassimeris, J., Kisiel, W., Fenton, J. W. 2d., Dinarello, C., Chess, L. and Jaffe, E. A.(1985). Self-regulation of Procoagulant Events on the Endothelial Cell Surface. J. Exp. Med. 162:1223-1235.

Turitto, V. T. and Baumgartner, H. R. (1979). Platelet Interaction with Sub-endothelium in Flowing Rabbit Blood: Effect of Blood Shear Rate. Microvasc. Res. 17:38-54.

Turitto, V. T. and Baumgartner, H. R. (1982).Platelet-surface Interactions. In: Colman, R. W.(ed). Homeostasis and Thrombosis. Lippincott. Philadelphia. pp. 364-379.

Turitto, V. T., Weiss, H. J., Zimmerman, T. S. and Sussman (1985). Factor VIII/VWF in Sub-endothelium Mediates Platelet Adhesion. Blood. 65:623-631.

Ulutin, O. (1986). Atherosclerosis and Homeostasis. Seminars in Thrombosis and Homeostasis. 12:156-174.

Weibel, E. R. and Palade, G. E. (1964). New Cytoplasmic Components in Arterial Endothelial Cells. J. Cell Biol. 23:101-112.

Weiss, H. J., Turitto, V. T. and Baumgartner, H. R. (1978). Effect of Shear Rate on Platelet Interaction with the Sub-endothelium in Citrated and Native Blood. 1. Shear Rate - dependent Decrease of Adhesion in von Willebrand's Disease and Bernard- Soulier Syndrome. J. Lab. Clin. Med. 92:750-764.

Weiss, H. J., Turitto, V. T. and Baumgartner, H. R. (1986). Role of Shear Rate and Platelets in Promoting Fibrin Formation on Rabbit Sub-endothelium. Studies Utilizing Patients with Quantitative and Qualitative PLatelet Defects. J. Clin. Invest. 78:1072-1082.

Weiss, H. J., Baumgartner, H. R. and Turitto, V. T. (1987). Regulation of Platelet-fibrin Thrombi on Sub-endothelium. Ann. NY. Acad. Sci. 516.:380-397.

Wekster, B. B. (1987). Platelet Interactions with the Blood Vessel Wall. In: Colman, R. W.(ed). Homeostasis and Thrombosis. Lippincott. Philadelphia. pp. 804-815.

White, J. G. (1987). Platelet Structural Physiology: The Ultrastructure of Adhesion, Secretion, and Aggregation in Arterial Thrombosis. Cardiovasc. Clin. 18(1):13-33.

Zucker, M. B. and Nachmias, V. T. (1985). Platelet Activation. Arteriosclerosis. 5:2-18