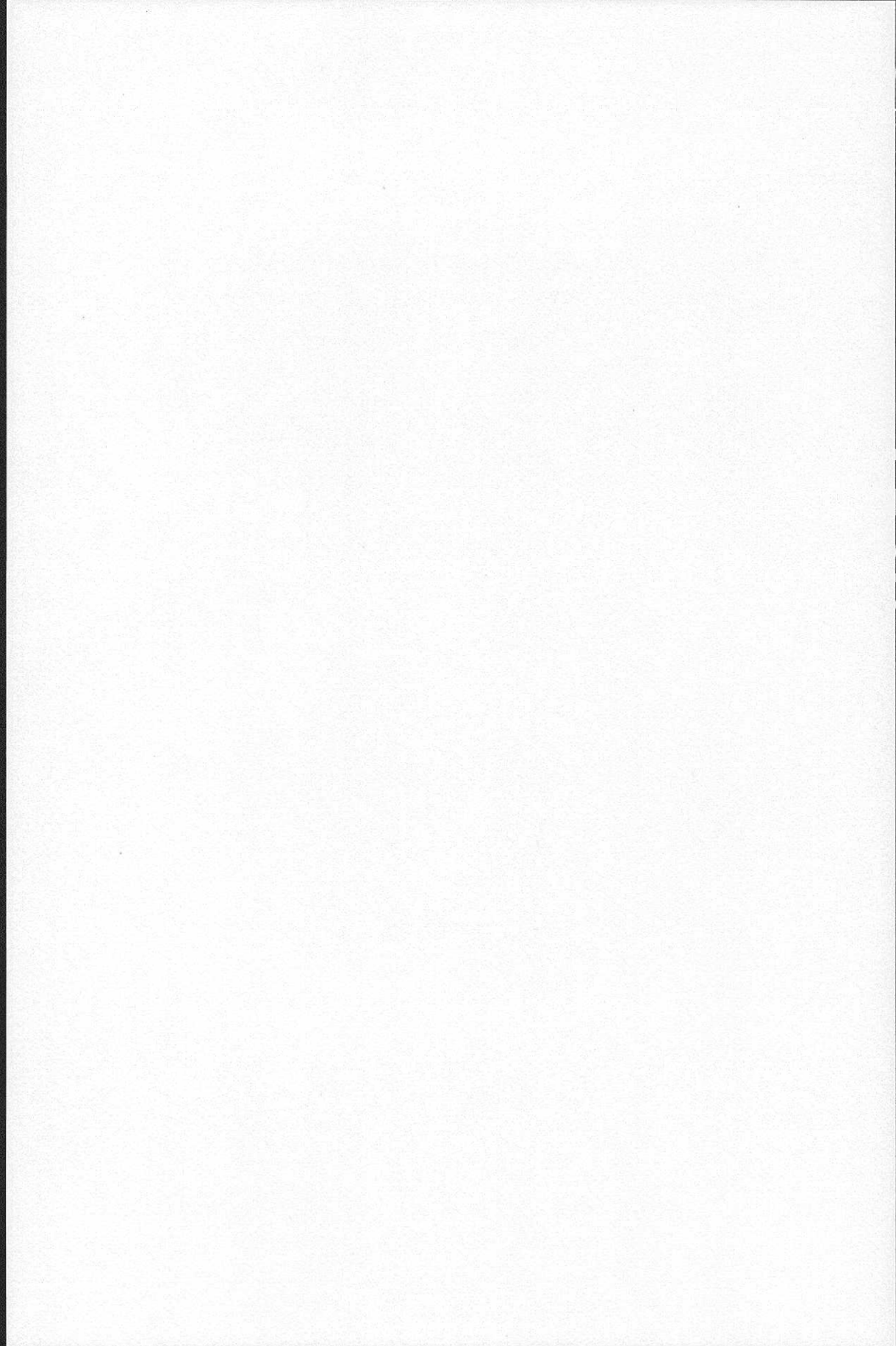# The Arterial Surface Ultrastructure

Effects of cholesterol feeding and mechanical injury

by
Anders Bylock

Göteborg 1981

# The Arterial Surface Ultrastructure

## Effects of cholesterol feeding and mechanical injury

**Avhandlingen baseras på följande arbeten:**

I. Bylock A., Björkerud S., Brattsand R., Hansson G.K., Hansson H.-A., Bondjers G.: Endothelial structure in rabbits with moderate hypercholesterolemia, a scanning electron microscopy study. Acta Path. Microbiol. Scand. sect. A. 85:671−682, 1977.

II. Bondjers G., Brattsand R., Bylock A., Hansson G.K., Björkerud S.: Endothelial integrity and atherogenesis in rabbits with moderate hypercholesterolemia. Artery 3(5):395−408, 1977.

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IV. Risberg B., Bylock A.: Vascular trauma induced by clamping—correlation between surface ultrastructure and fibrinolytic activity. Acta Chir. Scand. 147:25−32, 1981.

V. Bylock A., Bondjers G.: Immediate reactions of the arterial wall, following mechanical trauma. A scanning and transmission electron microscopy study. Acta Path. Microbiol. Scand. sect. A. 89:313−323, 1981.

VI. Bylock A., Jonasson L., Bondjers B.: Arterial reactions to experimental mechanical injury. Studies on the surface ultrastructure of the resulting intimal thickenings up to one year after injury. Submitted for publication.

# ABSTRACT

BYLOCK, A., THE ARTERIAL SURFACE ULTRASTRUCTURE. Effects of cholesterol
feeding and mechanical injury. Page 1-53.
Department of Histology, University of Göteborg, Box 330 31, S- 400 33  Gö-
teborg, Sweden. Thesis defended December 19, 1981.

For a number of years, endothelial injury has been considered to be a poten-
tially important factor for the development of intimal thickenings and the
formation of thrombi on the arterial wall. The objects of these investi-
gations were to study surface ultrastructural changes occuring after de-endo-
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the endothelial lining in experimental hypercholesterolemia in rabbits.
Scanning electron microscopy served as the basic method for the studies and
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mission electron microscopy were other methods that were used. The signs of
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was developed, providing an enhanced contrast and membrane delineation. Im-
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exclusion test allowed a macroscopic evaluation of endothelial integrity
as well as localization of endothelial injury on the cellular level.

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studied in aortae of rabbits. Endothelial cells were desquamated and adhe-
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injured areas. 7 days after injury, a characteristic cell type was observed
at de-endothelialized parts of the vessels. This, so-called "pseudoendothe-
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In contrast to the intima covered by normal endothelial cells, these areas
permitted uptake of Evans blue. Immunoglobulin G-immunoreactive material was
also seen under the pseudoendothelium. The pseudoendothelial cells did not
contain detectable amounts of coagulation factor VIII and Weibel-Palade bo-
dies were absent. These two findings, taken together, make an endothelial
origin of this cell type unlikely. It is suggested that these cells were
derived from smooth muscle cells of the arterial wall. They did not, how-
ever, resemble either of the two defined phenotypic states of such cells:
the contractile or the synthetic. The possible existence of a third pheno-
typic state, the pseudoendothelial, is discussed.

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cal clamp, was studied. Signs of endothelial injury and desquamation were
seen, accompanied by an adhesion of platelets, erythrocytes, leukocytes and
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was found to be reduced. A return to pre-injury levels was noted when re-
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mia.
ISBN: 91-7222-439-8. Correspondence to Anders Bylock, Dept of Histology, Uni-
versity of Göteborg, Box 330 31, S- 400 33  Göteborg, Sweden.

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## ABBREVIATIONS

SEM:  Scanning electron microscopy
TEM:  Transmission electron microscopy
IgG:  Immunoglobulin G
$PGI_2$:  Prostaglandin $I_2$, also called prostacyklin
SMC:  Smooth muscle cell
PDGF: Platelet derived growth factor
HRP:  Horseradish peroxidase
PMN:  Polymorphonuclear granulocyte

## COVER MICROGRAPHS

Front cover: A mononuclear cell located on the luminal membrane of a process extending from a "pseudoendothelial cell" found on an area which one year earlier had been de-endothelialized by mechanical injury. Rabbit aorta. Transmission electron micrograph. Staining en bloc with uranyl acetate and counterstaining with bismuth subnitrate. 8300 X. For details, see paper VI.

Back cover: The border between an area covered by "pseudoendothelial cells" (right) and normal endothelial cells (left). Note the "liplike" structure connecting the endothelial cells lying along the border. Scanning electron micrograph. Four weeks after induction of mechanical trauma. 1600 X. For details, see paper VI.

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For a number of years, endothelial injury has been considered to be a potentially important factor for the development of intimal thickenings and the formation of thrombi on the arterial wall. The objects of these investigations were to study surface ultrastructural changes occuring after de-endothelialization induced by mechanical injury and to study the integrity of the endothelial lining in experimental hypercholesterolemia in rabbits. Scanning electron microscopy served as the basic method for the studies and a preparational scheme, particularly suitable for arteries, was developed in order to provide optimal reproducibility and resolution. Light microscopy, interference contrast microscopy, fluorescence microscopy and transmission electron microscopy were other methods that were used. The signs of endothelial injury seen by the different methods were compared. A new method for the staining of specimens for transmission electron microscopy was developed, providing an enhanced contrast and membrane delineation. Immunofluorescence was used for the detection of IgG and factor VIII. A dye-exclusion test allowed a macroscopic evaluation of endothelial integrity as well as localization of endothelial injury on the cellular level.

The effects of direct mechanical trauma to the endothelial lining was studied in aortae of rabbits. Endothelial cells were desquamated and adhering platelets, leukocytes, erythrocytes and fibrinous strands were seen on injured areas. 7 days after injury, a characteristic cell type was observed at de-endothelialized parts of the vessels. This, so-called "pseudoendothelial" cell, could be seen covering  some sites even one year after injury. In contrast to the intima covered by normal endothelial cells, these areas permitted uptake of Evans blue. Immunoglobulin G-immunoreactive material was also seen under the pseudoendothelium. The pseudoendothelial cells did not contain detectable amounts of coagulation factor VIII and Weibel-Palade bodies were absent. These two findings, taken together, make an endothelial origin of this cell type unlikely. It is suggested that these cells were derived from smooth muscle cells of the arterial wall. They did not, however, resemble either of the two defined phenotypic states of such cells: the contractile or the synthetic. The possible existence of a third phenotypic state, the pseudoendothelial, is discussed.

In femoral arteries of rats, the response to the application of a surgical clamp, was studied. Signs of endothelial injury and desquamation were seen, accompanied by an adhesion of platelets, erythrocytes, leukocytes and fibrinous strands. The fibrinolytic activity of the vessel was measured and was found to be reduced. A return to pre-injury levels was noted when re-endothelialization was completed, 3 weeks after clamping.

In rabbits with dietary induced moderate hypercholesterolemia, intimal thickenings were found in the aortae, predominantly at branching points.On such lesions, a discontinuous intimal lining with swollen endothelial cells and IgG-containing cells, could be noted. Signs of injury were most frequent along the borders of the thickenings. Leukocytes were occasionally seen adhering to the thickened areas.

The possible significance of endothelial injury for the development of atherosclerotic lesions is discussed in the light of these and other studies.

Key words: Endothelial injury, mechanical arterial injury, scanning electron microscopy, transmission electron microscopy, dye-exclusion test, IgG, coagulation factor VIII, bismuth, fibrinolytic activity, hypercholesterolemia.

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## CONTENTS

# I:    INTRODUCTION

## I:1  ATHEROSCLEROSIS

Atherosclerosis is one morphological variant within the group of diseases
called arteriosclerosis. An atherosclerotic lesion is formed by an accumu-
lation of cells, connective tissue and lipids in the intima of an elastic
or muscular artery. The intimal thickening causes, in the late stages, a re-
duction of the cross-sectional area of the artery, an event which leads to
an impaired blood flow. This may cause a disturbed nutrition of peripheral
tissues, among which the myocardium and the brain are the two most impor-
tant from a clinical point of view. Infarctions as well as more slowly de-
veloping symptoms of impaired function may thus develop as an effect of
atherosclerosis (Herrick 1912, Schwartz and Mitchell 1962, Baroldi 1965,
Chapman 1968, Roberts and Buja 1972), even if local factors also may be sig-
nificant (Waldenström 1976).

Several risk factors have been postulated for atherosclerosis. Besides
age and sex, tobacco smoking, high blood pressure and hyperlipidemia have,
due to statistical relationships, been considered to be major risk factors
(Stamler 1972, Kannel and Gordon 1973, Wilhelmsen et al. 1973, The Pooling
Project  Research Group 1978).

A statistical relationship between a disease and a risk factor does,
however, not necessarily mean that a direct cause-effect connection exists.

Many hypotheses on what causes atherosclerosis have been proposed. Vir-
chow (1856) observed that atherosclerotic lesions were not randomly distri-
buted over the arterial surface. The lesions occurred predominantly in areas
where disturbed blood flow could be expected, i.e. in the aortic arch and
close to branching points. From these and many other studies a "response-
to-injury" hypothesis has emerged, according to which injury to cells of
the inner surface of arteries is important for atherogenesis (Björkerud and
Bondjers 1971, Ross and Glomset 1976). Anitschkov (1912, 1913) discovered
that feeding rabbits cholesterol led to a development of intimal thickenings
in arteries. This served as the basis for the "lipid-filtration" hypothesis.
Filtration of lipids from plasma into the vessel wall is, in this case, con-
sidered to be the main event (Page 1954).

Many other theories and modifications of the above mentioned have been
proposed, but the knowledge on the mechanisms underlying the development
of atherosclerotic lesions is still incomplete. Only a better understanding

of these processes can result in an efficient search for therapeutical aids
against this important disease. Further studies on the cellular response to
some of the risk factors were considered necessary. Some comments will first
be made on the properties of the cells and tissue components involved in
atherogenesis.

I:2 THE STRUCTURE OF THE NORMAL ARTERIAL WALL

The main components of the arterial wall are elastin, collagen, glycos-
aminoglycans, glycoproteins and cells. The elastic tissue forms the "skele-
ton" of the wall. It consists of interconnected tubes, which in sections
through the wall appear like laminae, lying 6 to 19 μm apart.

The internal elastic membrane and the tissue components on its luminal
side constitute the tunica intima. In newborn humans, this compartment solely
consists of endothelial cells lying on the internal elastic membrane. Hypo-
thetically, smooth muscle cells, SMCs, are considered to penetrate through
this membrane, populating the subendothelial space. Due partly to the in-
crease in cell number, the tunica intima may, in adults, reach a thickness
of over 1 mm at certain sites (review: Robertson 1977).

The compartment extending from the outer part of the internal elastic
membrane to the external elastic membrane is called the tunica media. In
this part of the wall, the so-called "musculoelastic system is found (Wo-
linsky and Glagov 1967). This word is used to describe the interacting sys-
tem of elastic lammellae and SMC´s. This system, together with the collagen net-
work, are the main stress-bearing components of the wall.

The tunica adventitia is found outside the external elastic membrane.
This layer is externally vascularized by vasa vasorum, small vessels that
also penetrate the outer layers of the tunica media. The tunica adventitia
consists of ordinary connective tissue. Its most common cell type is the
fibroblast.

In the gelatinous matrix that surrounds the organized components of
the wall, several families of macromolecules can be found. Glycosaminogly-
cans (acid mucopolysaccharides, GAGs) and proteoglycans are probably the
most important (review: Radhakrishnamurthy et al. 1981). It has been sugges-
ted that such stationary molecules may interact in a complex manner with
the transport of other molecules through the wall (Iverius 1972, Hoff et al.
1975, Camejo et al. 1975).

In large arteries, the intima and the media depend upon diffusion over
long distances for their nutrition. The growth conditions in the center

of the wall may be impaired if the distance exceeds about 0.5 mm, the "critical depth" according to Geiringer (1951) (see also: Adams 1964, Wolinsky and Glagov 1967).

I:3 THE ENDOTHELIUM

The endothelium is a layer of flat, polygonal cells constituting the lining of the inner surface of blood vessels. The endothelial cells are 25 to 50 μm long, 10 to 15 μm wide and have a thickness of about 0.1 to 3 μm over their cytoplasmic parts and 5 to 10 μm in the nuclear area (reviews: Simionescu and Simionescu 1977, Majno and Joris 1978, Thorgeirson and Robertson 1978).

The long axis of the endothelial cells is oriented in the direction of the blood flow as judged from light microscopy (Fry 1968), Häutchen preparations (Silkworth and Stehbens 1975) and scanning electron microscopy studies on vessels (Reidy and Bowyer 1977) and on vascular casts (Reidy and Levesque 1977). A deformation from the longitudinal shape takes place in areas of secondary flow, i.e. close to branching points (Reidy and Bowyer 1977, Langille and Adamson 1981).

The endothelial cells contain all the usual cytoplasmic organelles (reviews: Majno and Joris 1978, Thorgeirson and Robertson 1978). Scattered, rounded mitochondria are seen as well as some rough endoplasmic reticulum. Free ribosomes are also found within the cytoplasmic matrix. The golgi complex is located in the vicinity of the nucleus. In capillaries the endothelium contains vesicles with a diameter of 6 to 7 nm (Simionescu and Simionescu 1977, Johansson 1979). They show fusion with each other as well as with the cellular membranes (Frökjär-Jensen 1980). Results from serial sectioning have indicated that this fusion leads to communicating canaliculi (Simionescu et al. 1975). A rod-shaped cytoplasmic component, the Weibel-Palade body (Weibel and Palade 1964), can be found in endothelial cells. It is thought to be specific for this cell type and it consists of a bundle of microtubules enveloped by a unilaminar, tight membrane. The organelle is up to 3 μm long and about 0.1 μm thick. It is present in highly variable numbers in different parts of the vascular bed and in different species. Its function is unknown.

Contractile proteins like actin and myosin have been demonstrated in endothelial cells (Becker and Nachman 1973). Thin (diameter 4 to 7 nm) filaments are considered to represent the actin (Gabbiani et al. 1975), while

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the myosin is seen as thick (diameter about 15 nm) filaments (Moore et al. 1977). Intermediate (diameter about 10 nm) filaments of uncertain composition have also been described (Blose and Chacko 1976).

The endothelial cells are the first cells to be influenced by changes both in hemodynamic forces and in serum composition. They are considered to form a barrier which is capable of regulating the conditions in the arterial wall,within certain limits (Bondjers and Björkerud 1973 a and b).

The cells are connected with each other by well organized tight junctions and gap junctions (communicating junctions)(Hüttner et al. 1973, Simionescu et al. 1976). Landis and Pappenheimer (1963) suggested that routes allowing penetration of substances over this barrier exist, even if the endothelium is intact. In experimental studies on capillary endothelium, tracers of different size have been used to demonstrate these routes. Large molecules, such as lactoperoxidase (MW 82.000) and ferritin (MW 460.000 to 750.000) could be found solely within the vesicles mentioned above (Stein and Stein 1972, Johansson 1978b). Smaller molecules, like horseradish peroxidase, HRP (MW 40.000), have been localized also to spaces between endothelial cells (Florey and Sheppard 1970, Hüttner et al. 1973) but this latter finding has not been reproducible in all experimental systems used (Johansson 1978b). The vesicular system has also, on the basis of experiments using labelled lipoproteins, been suggested to be important for the transport of such molecules (Stein et al. 1973).

The luminal membrane of the endothelial cell is covered by a carbohydrate-rich layer, the cell-coat or the endo-endothelial layer (Luft 1966, 1976, Weber et al. 1973, Gerrity et al. 1977). It has a varying thickness ranging from below 1 nm to about 80 nm. The intact endothelial cell has a negative interfacial charge on its luminal side. This has also been thought to be of importance for the barrier functions of the intimal lining. Adhesion of formed elements of the blood as well as of fibrin is considered to be counteracted by this negative charge (review: Sawyer et al. 1973). The relevance of the electrical properties alone for the formation of thrombi has, however, been subject to dispute since thrombogenic components like collagen have charge densities comparable to those of the endothelium (Danon and Skutelsky 1976).

Endothelial cells produce prostaglandin $I_2$, (prostacyklin), which is very potent inhibiting platelet aggregation (Gryglewski et al. 1976, Moncada et al. 1976, Weksler et al. 1977, review: Smith 1980). Plasminogen activators

(PA) (Todd 1959), the antihemophilic factor, factor VIII (Jaffe et al. 1973, Hoyer et al. 1973) as well as the angiotensin converting enzyme (Ody and Junod 1977) are all produced by endothelial cells. The presence of factor VIII and the angiotensin converting enzyme have been used for the identification of endothelial cells in culture (Ody and Junod 1977).

The rate of turnover and growth of endothelial cells in vivo has been studied with labelled thymidine. The average proportion of cells undergoing mitosis during 24 hours has been found to be between 0,7 and 0,9% in the guinea-pig (Payling-Wright 1972) and between 0,3 and 1,5% in the rat (Schwartz and Benditt 1973).

1:4 NORMAL AND ABNORMAL VARIATIONS IN THE STRUCTURE OF THE HUMAN ARTERIAL
WALL

Even in the young human, focal changes in the tunica intima can be seen. One of these, the "fibromuscular intimal thickening", consists of an accumulation of smooth muscle cells, intercellular substances and fibers. It is generally considered to be an essentially normal structure (review: McGill 1974). "Fatty streaks", mainly consisting of lipid-laden cells, can also be found. It is not known whether these represent  early stages of atherosclerosis, or not, and if they are reversible (review: McGill 1974). The "fibrous plaque" is a lesion which is first seen in the third decade of life. It is preferentially found at ostia of brances and in the aortic arch. Extracellular lipid  surrounded by collagen, elastin and necrotic smooth muscle cells,are its main components. It is a common opinion, that this kind of lesion may continuously increase in size (McGill 1974). It may even release the contents of the core, an event which may lead to thrombosis, ulceration or even the formation of aneurysms.

# 2 AIM OF THE PRESENT STUDY

The aim of the present study was:

To investigate the significance of endothelial injury for the development of intimal lesions in experimental atherosclerosis.

To improve the methods for preparation of arterial specimens for scanning electron microscopy.

To develop a method for staining of specimens allowing an improved delineation of cellular membranes and intracellular structures as seen in transmission electron microscopy.

# 3    MATERIALS AND METHODS

## 3:1    ANIMALS

Male albino rabbits of the Swedish country strain (paper I and II) or
of the New Zealand white strain (paper III, V and VI) were used. They were
fed fibre-enriched standard pellets (Maintenance Food, Astra-Ewos, Söder-
tälje). Their weight   at the beginning of the experiments were between 2,0
and 2,5 kg. They had been kept at the department for at least two weeks in
order to avoid stress reactions.

Sprague-Dawley rats of both sexes were used in some studies (paper IV).
They weighed between 200 and 300 g and were fed ordinary rat-pellets (Ast-
ra-Ewos, Södertälje).

## 3:2    ANAESTHESIA

In the experiments on hypercholesterolemic rabbits (paper I and II),
the animals were anaesthetized by intravenous injection of sodium pentobar-
bital (Mebumal[R], ACO, Stockholm, Sweden).

In all other experiments on rabbits (paper III, V and VI) inhalation of
$O_2$, $N_2O$ and fluothan (Halothan[R], Hoechst, Frankfurt, West Germany) was used
The rats (paper IV) were anaesthetized by inhalation of ether.

## 3:3    EXPERIMENTAL SYSTEMS FOR INDUCTION OF ARTERIAL INJURY

### 3:3:1 DIETARY INDUCTION OF INTIMAL THICKENINGS

Fibre-enriched standard rabbit pellets (Maintenance Food, Astra-Ewos,
Södertälje) were used as basic food (papers I and II). For induction of hy-
percholesterolemia, 0,1 to 0,3% cholesterol was added (Brattsand 1977). The
serum cholesterol levels were kept stable in each animal by adjusting the
cholesterol of the diet. The majority of the hypercholesterolemic rabbits
were kept at serum cholesterol levels between 200 and 400 mg % (5,0 to 10,0
mmol/l) for a period of about 18 months.

### 3:3:2 MECHANICAL INDUCTION OF LESIONS

' In rats, injury to the femoral artery was induced by the application of
a Diffenbach clamp for 30 sec (paper IV). The clamp was placed transversely

over the vessel.

In paper V and VI, a microsurgical instrument (Björkerud 1969) was used. It consisted of a nylon catheter (external diameter: 1,0 mm) in which a nylon monofilament (diameter: 0,35 mm) was located. The catheter was closed at one end by heating and a 6 mm long opening was made at a distance of 5 mm from this tip. By application of a pressure to the free end of the monofilament, it could be forced to extend through the slot, thus forming a half circle. At this location, industrial diamonds (size 100/120 MDA) immersed in a plastic glue, were applied. Only the tips of these stones extended above the level of the glue.

The femoral artery of rabbits was exposed during anaesthesia and after local infiltration with lidocain 0,1% (Xylocain[R], Astra, Södertälje). The vessel was opened and a mixture containing 0,5 ml lidocain 0,1% and 0,5 ml papaverin (ACO, Stockholm, Sweden) was slowly injected into the lumen in order to prevent contraction of the vessel due to mechanical stimulation. The catheter was introduced into the lumen and injuries were induced at four levels of the aorta by rotation of the catheter while the monofilament was pressed out. The distance between the injuries was 15 to 20 nm. These operations were performed under sterile conditions. The lesions were classified as superficial circumferential injuries of a large area (transverse injuries) (Björkerud and Bondjers 1971).

## 3:4 PREPARATION OF SPECIMENS

### 3:4:1 EXSANGUINATION AND THE DYE EXCLUSION TEST

In all experiments, the thorax of the animal was opened during general anaesthesia. A catheter was introduced into the apex of the left ventricle of the heart. It was fixed in this position and great care was taken not to touch any part of the aorta. Ringer´s glucose solution (paper I, II, III, IV and V) or Ringer´s glucose solution containing 10 mM HEPES (M-2 hydroxy ethylpiperazine-N-2-ethane-sulphonic-acid) pH 7,35 (Flow lab., Irvine, Scotland) (paper VI) was used for rinsing the vessels from blood. The first step of flushing continued for 5 (I), 3 (II) or 2 (IV, V and VI) min at a pressure of 100 mm Hg. In paper III, the effects of varying this time interval were studied. When performing the dye-exclusion test (paper II, III, V and VI) the rinsing was followed by perfusion with 0,1% Evans blue, dissolved in the glucose solution, for 1 min at 14 mm Hg. The vessels were then again rinsed for 1 to 2 min at 100 mm Hg. Fixation followed (see below).

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The dye exclusion test was used for the identification of areas of endo-
thelial injury or of areas covered by pseudoendothelium. Such parts of the
arteries were stained blue. In the microscope, injured endothelial cells
could be identified due to their uptake of the dye (for further details and
comments, see: Björkerud et al. 1981).

3:4:2 PREPARATION FOR SEM

The vesselsdescribed in paper I and some specimens in paper II were fixed
for one hour in 4% formaldehyde (freshly prepared by alkaline hydrolysis of
paraformaldehyde) (KEBO, Stockholm, Sweden) in a sodium cacodylate buffer
(Karnovsky 1965), pH 7,35. Otherwise,(paper II (some specimens), III, IV, V
and VI) a combined fixative was used, containing both formaldehyde, 2%, and
glutar  aldehyde, 2,5%, (Ultrastructural Grade,Polaron, Watford, England)
in a sodium cacodylate buffer, pH 7,35 (Karnovsky 1965). The arteries were
fixed in situ at a pressure of 100 mm Hg for one hour if only formaldehyde
was used and for 20 min if the combined fixative was used. After being dis-
sected out, the specimens were immersed in the same fixative over night. In
paper III, the results of a comparison between different methods are de-
scribed. After dehydration in a graded series of ethanol solutions, amyl
acetate (Analytical grade, Merck AG., Darmstadt, West Germany) was used as
intermediate fluid. This step was followed by critical point drying as de-
scribed by Anderson (1951) using $CO_2$ as transitional fluid. The specimens
were then glued onto specimen holders with silver or carbon conductives and
coated with copper in a vacuum  evaporator (JEOL, Tokyo, Japan) (paper I,
II and parts of paper III) or coated with gold in a sputter coater (Edwards
S 150, Edwards High Vacuum, Crawley, Sussex, England) (paper IV, V, VI and
parts of paper III). A cooled specimen stage as well as a fractionated sput-
tering procedure was used in paper IV, V and VI. This means that the high
tension was applied for three one min long periods. Thus heating of the spe-
cimens could be reduced. For additional comments, see paper III.

3:4:3 PREPARATION FOR TEM

After perfusion and fixation in situ as described above for SEM, speci-
mens were excised for TEM and immersed for two to four hours in a fixative
similar to that used during the perfusion. They were then kept in 0,15 M
sodium cacodylate over night. Postfixation in 2% osmium tetroxide (Analyti-
cal Standards, Kungsbacka, Sweden) followed for one hour, with better re-

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sults obtained at $4^0$ C than at room temperature (see also paper VI). A modification of the ordinary preparation procedure was tried on some specimens (paper VI). These were stained en bloc by immersion in 1% uranyl acetate in distilled water after osmification, in order to improve contrast. All tissue blocks were dehydrated in a graded series of ethanol solutions.

Some of the specimens studied in paper V and VI were embedded in Epon[R] 812 (Polaron, Watford, England) after immersion in propylene oxide. Insufficient penetration into the tissue led, however, to cracking during ultrathin sectioning. Therefore, Spurr´s resin (Taab lab., Reading, England) was tried due its lower viscosity (Spurr 1969). Immersion in propylene oxide was, in the latter case, not necessary. Sectioning was facilitated when Spurr´s resin was used. Semithin (1 μm) and ultrahin (50 to 80 nm) sections were made on LKB Ultrotomes[R] type III and V (LKB, Stockholm, Sweden) using a Jumdi diamond knife (Juniper, Stockholm, Sweden). The semithin sections were stained with Richardson´s methylene-blue-azur II solution (Richardson et al. 1960) and studied in a light microscope. The ultrathin sections were laid on copper grids with a hexagonal meshwork. Specimens not stained en bloc were laid on droplets containing saturated solutions of uranyl acetate and lead citrate for 5 min each (Reynolds 1963). The specimens that had been stained en bloc with uranyl acetate were counterstained with an alkaline bismuth solution (Ainsworth and Karnovsky 1972). The bismuth subnitrate staining solution was made by dissolving 400 mg sodium tartrate in 10 ml 2 N sodium hydroxide. The alkaline tartrate solution was added dropwise to 200 mg bismuth subnitrate (K and K lab. Inc., Plainview, N.Y., USA) with constant stirring. Upon the addition of 6-8 ml sodium tartrate, the solution began to clear and with the further addition of sodium tartrate (10 ml total), all of the bismuth was chelated and the final solution clear. Prior to use, a sample stock solution was diluted 1:50 with distilled water. For further comments on the histochemical properties of bismuth, see Riva (1974) and Locke and Huie (1977).

3:5  SCANNING ELECTRON MICROSCOPY

Most of the specimens described in paper I, II and some in paper III, were studied in a JEOL JSM 35 scanning electron microscope (JEOL, Tokyo, Japan). A Zeiss Novascan electron microscope was used as a complement. All specimens in papers IV, V and VI as well as some in paper III were examined in a JEOL 100 CX temscan electron microscope (JEOL, Tokyo, Japan) equipped with a scanning electron microscopy unit. This microscope was al-

so equipped with an anti-contamination device cooled with liquid nitrogen. The accelerating voltage used was in all cases 15 to 25 kV.

## 3:6   TRANSMISSION ELECTRON MICROSCOPY

The ultrathin sections were studied in a JEOL 100 CX electron microscope in transmission mode. Accelarating voltage was 60 to 80 kV.

## 3:7   CHOLESTEROL ANALYSIS

Serum cholesterol levels in the rabbits studied in the experiments described in paper I and II were determined manually with the enzymatic method described by Röschlau et al. (1974). The tissue cholesterol contents in the rabbit aortae (paper II) was determined fluorometrically (Bondjers and Björkerud 1971) after separation on thin layer chromatography following extraction of lipids by choloroform: ethanol (2:1) and washing. Both free and esterified cholesterol was determined and the specimens were freeze-dried and  weighed.

## 3:8   HISTOCHEMICAL DETERMINATION OF FIBRINOLYSIS (IV)

The vessels were rinsed with Ringer´s solution, carefully dissected, soaked in the same solution and immediately frozen in carbon-dioxide snow. Plasminogen activator activity (PA) was determined using a slightly modified version of Todd´s histochemical method (Todd 1959, Risberg 1975).

Eight μm thick sections were made in a cryostate and transferred to glass slides. Duplicate samples were covered with a plasminogen rich fibrinogen film and incubated in a moist chamber at $37^0$ C for 30-45-60 and 90 min. After processing, areas of fibrinolysis were identified as unstained zones in the bluish film. The activity was estimated semiquantitatively according to the following scheme:

Grade 1: no fibrinolysis visible (0 points)
- " - 2: punctuate zones of fibrinolysis (1 point)
- " - 3: larger but not confluent zones of fibrinolysis (2 points)
- " - 4: confluent zones of fibrinolysis (3 points)

The point score from each vessel was recorded at the sum of all points registered from the different incubation times. In separate specimens, plasminogen-free films were used in order to exclude non-specific proteolytic activity.

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## 3:9    QUANTITATION OF THE NUMBER OF EVANS BLUE-POSITIVE CELLS

Aortae were subjected to the dye exclusion test as described above (paper II). They were rinsed with Ringer´s glucose solution and fixed at 100 mm Hg in a buffered formaldehyde solution  (Karnovsky 1965). Embedding in starch hydrolysate (Björkerud 1972) followed and the specimens were studied in a dissecting microscope and in an interference contrast microscope. The number of Evans blue-positive cells per unit surface area was counted.

## 3:10    IMMUNOFLUORESCENCE

### 3:10:1 DETECTION OF IgG

Following the dye-exclusion test the specimens were frozen in hexane immersed in liquid nitrogen.

Paper II: Four μm  thick cryostate sections were prepared, fixed in 95% ethanol and air dried in $-20^0$C. The sections were incubated with antiserum to rabbit immunoglobulin G (Nordic Immunology, London, Great Britain) which was conjugated with FITC (fluorescein isotiocyanate), dilution 1:20. Washing in phosphate-buffered saline followed and after this the sections were mounted in Pertex (Histolab., Bethlehem trading, Göteborg, Sweden).

Paper VI: 10 μm thick cryostate sections were made, fixed in 95% ethanol and washed in phosphate-buffered saline (PBS) at a pH of 7,6. Treatment with 0,1% Evans blue (Merck AG, Darmstadt, West Germany) dissolved in PBS, pH 7,6, followed for 5 min in order to eliminate the green autofluorescence. The sections were then incubated with unconjugated goat-anti-immunoglobulin G(Fc) (Nordic Immunology, London, Great Britain), dilution 1:20. Repeated rinsing in PBS, pH 7,6, followed for one hour and after this the sections were incubated with FITC-conjugated rat-anti-goat-immunoglobulin G (Meloy lab., Springfield, Virginia, USA), dilution 1:20. A final rinse was made in PBS at pH 8,0. The sections were mounted in Kaisers glycerin-gelatin (Merck AG, Darmstadt, West Germany). Specimens incubated with antibodies absorbed to IgG as well as specimens where the first incubation had been excluded served as controls.

### 3:10:2    DETECTION OF FACTOR VIII (paper VI)

The rabbit aortae were treated according to the dye-exclusion test and frozen as described above. 10 μm thick cryostate sections were made, fixed

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in 95% ethanol and washed in phophate buffered saline (PBS), pH 7,6. Treatment with 0,1% Evans blue dissolved in PBS followed in order to reduce autofluorescence. The sections were incubated with unconjugated goat-anti-human-factor VIII antibodies (Nordic Immunology, London, Great Britain), dilution 1:20. Rinsing in PBS followed for one hour and,after this, incubation with FITC-conjugated rat-anti-goat-immunoglobulin G (Meloy lab., Springfield, Virginia, USA), dilution 1:20, took place. A final rinse in PBS, pH 8,0, followed, and the sections were mounted in Kaisers glycerin-gelatin. As controls, specimens which had not been subjected to the first step of antibodies or sections  treated with goat-anti-human IgG(Fc) (Nordic Immunology) as the first antibody, were used.

3:11   FLUORESCENCE MICROSCOPY

The specimens were examined in a fluorescence microscope using interference filters which gave excitation at 450-500 nm wavelength and barrier filters which allowed transmission of emitted fluorescence at 528-560 nm for detection of FITC (paper II and VI).
For detection of Evans blue fluorescence (paper II), filters that allowed excitation at 536-556 nm and transmitted fluorescence with a wavelength above 590 nm, were used.

3:12   STATISTICAL METHODS

Wilcoxon´s test was used for calculation on differences in fibrinolytic activity between vessels from rats of different groups (paper IV).

# 4    RESULTS AND DISCUSSION

## 4:1    ELECTRON MICROSCOPY IN STUDIES OF ARTERIES

### 4:1:1 SCANNING ELECTRON MICROSCOPY (SEM)

SEM is particulary well suited for studies of the surface structure of
the intima of large blood vessels. High resolution can now be obtained,
and the results are reproducible (Bowyer et al. 1977, paper III, Gerrity
and Naito 1980 b).

When these investigations started, a scheme for preparation of speci-
mens for SEM was lacking. This resulted in presentation of diverging data
by different groups, even regarding the normal surface morphology (discus-
sions: Clark and Glagov 1976, Bowyer 1977). SEM-studies subsequently be-
came subject to discredit. Therefore we, as well as other groups (Clark
and Glagov 1976, Bowyer 1977) considered it necessary to improve the tech-
nique for preparation.

Clark and Glagov (1976) discovered and reported upon several pitfalls.
They suggested that other investigators had taken crystals, mounting glue,
serum proteins, vapor bubbles and glycerol for normal tissue constituents.
Changes in osmolality, physical manipulation like freezing and heating,
shrinkage due to drying and solvent exchange could all cause distortion of
the details of cell and tissue surfaces. Heating and charging during view-
ing were also mentioned as sources of artefacts.

A basic preparational scheme was later published and this led to more
uniform results between different groups(Bowyer et al. 1977). This scheme
has been further modified (III) and comments will be made on some critical
steps.

The preparative procedure can be divided into six steps: 1: Rinsing and
staining prior to fixation. 2: Fixation. 3: Drying. 4: Mounting. 5: Coating.
6: Exposure to the electron beam.

It was found that, if the time interval before fixation exceeded 5 min,
microvillous projections occurred on the surface of some endothelial cells
(III). The number of cells showing such protrusions increased with increa-
sing time. Oxygenation of the rinsing solution did not alter the appearance
of the surface within detectable limits, when compared with when brief rin-
sing with Ringer´s solution  was immediately followed by fixation. Neither
did the addition of a one min long period of staining with Evans blue after
rinsing. It was still necessary, however, to keep the time before fixation

below 5 min, as has earlier been mentioned.

In pilot studies it was found, that fixation at physiological pressure was necessary when preparing large arteries for SEM (III). This was also emphasized by other investigators (Davies and Bowyer 1975, Clark and Glagov 1976, Swinehart et al. 1976). If arteries were fixed at atmospheric pressure, longitudinal ridges occurred. These caused a wave-like surface which was difficult to study in the SEM (III). This artefact may be the result of retraction of elastic tissue as well as of contraction of SMC´s of the media. If the specimens had been excised from the rabbit within 24 hours after induction of mechanical injury, wrinkling of the arteries was observed even after pressure fixation, however. It was suggested, that a contraction of the vessel had occurred as a response to the mechanical injury and that this could not be overcome by the high fixation pressure(paper V).

As fixative, a sodium-cacodylate-buffered formaldehyde solution was used in the first experiments (I and II). A combined fixative which contained both glutar aldehyde and formaldehyde dissolved in the same kind of buffer was used in all other experiments (III, IV, V and VI). When formaldehyde was used alone, perfusion under pressure had to last for at least one hour in order to avoid the longitudinal ridges. When the fixatives were used together, the time could be reduced to 20 min, still avoiding these artefacts. The addition of glutar aldehyde thus seemed to improve the preservation of the artery in its dilated condition. This may possibly be due to the cross-linking properties of this dialdehyde (review: Hayat 1970). Formaldehyde was not excluded from the solution since it has been considered to penetrate more rapidly through tissue blocks.

The combined fixative (Karnovsky 1965) is very hyperosmolalic per se but isotonic with regard to the buffer included. The results obtained with this technique (III, IV, V and VI) demonstrated good preservation and high reproducibility even at high levels of magnification (10 000 X to 30 000 X). Variations in the concentration of formaldehyde o  glutar aldehyde (III) did not affect the results. This spoke in favour of the hypothesis that the osmolality of the buffer vehicle was more important than that of the fixative in regulating the preservation of cell volume (review: Hayat 1970). The fixatives used, provided a good stabilization of the surface cells since the specimens became strong enough to withstand dehydration in solutions of

very varying osmolality. Postfixation with osmium tetroxide did not alter
the results within detectable limits. A slightly improved stability under
the electron beam was, however, noted.

Dehydration and drying procedures were found to be critical. Freeze-
drying resulted in bad reproducibility due to the deposition of large a-
mounts of an amorphous material on the surface of the specimens. This was
particularly pronounced when the tissue had been fixed with glutar alde-
hyde (comments in paper I and III).

Following air-drying after immersion in absolute ethanol or acetone, dis-
continuities and crater-like structures often occurred (I and III). It was
also found that the frequency of such defects varied according to the time
used for the subsequent coating prodedure. Since the specimens became sub-
jected to heat during this step, it was considered probable that air-dried
specimens were more sensitive to such physical stress. It is possible that
the air-dried vessels were insufficiently dried since moist left in the
specimens resulted in the same artefacts (III).

Critical-point drying provided, in our hands, the best reproducibility
and resolution. The stability of the specimens under the electron beam was
also superior and deposits on the surface of the specimens were rare (III).

When the dry pieces were to be mounted on specimen holders other arte-
facts were found to occur if too much glue was used (III). The liquid base
of the glues as well as the conductives used became absorbed by the hygro-
scopic specimens. A disturbed surface morphology could be noted if the li-
quid penetrated through the tissue. This was most pronounced if the speci-
mens had been air- or freeze-dried.

The use of non-conductive glues was abandoned since it was found that
the conductive material reduced  the amount of charging in the electron
microscope and a higher signal/noise ratio  could thus be obtained (III).

Coating of specimens was initially performed in a vacuum evaporator.
Heated copper was used as source for metal atoms (I, II and parts of pa-
per III). Craters and holes were found to occur in parallel with prolonged
exposure of the specimens to this coating procedure (III). The decreased
reproducibility was suggested to be an effect of high temperature. Coating
was later performed in a sputter coater, equipped with a water-cooled spe-
cimen holder (IV, V, VI and parts of paper III). The time used for coating
was also minimized and divided into three  periods. With these modifica-

tions, the number of artefacts was reduced.

A gold cathode was used in the sputter coater. In the SEM, gold coating gave a higher signal/noise ratio as compared to copper. Thus, better resolution and/or reduced thermic stress could be achieved in the SEM. It was therefore possible to further improve reproducibility and prolonged observation on small areas could be performed without overheating the specimen (III).

The optimal method for preparation described in this thesis (III, VI) led to a clear demarcation of cellular borders. In the latter experiments (III, IV, V and VI), the level of resolution was high, allowing magnification up to about 30 000 X. Silver staining, a method used by many other groups (Collatz-Christensen and Garbasch 1973, de Bruijn et al. 1974, Davies and Bowyer 1975, Reidy and Bowyer 1977) in order to delineate cell borders, was not found necessary.

It should also be added that the power of resolution obtained in routine work showed a remarcable variability between different microscopes.

4:1:2 TRANSMISSION ELECTRON MICROSCOPY (TEM)

TEM served in this thesis as a complement to SEM (paper V and VI). It made it possible to compare extra- and intracellular signs of injury and thus it provided an integrated view on the three dimensional arrangement of injured cells and formed elements of the blood over the arterial surface.

Limitations for TEM alone were both the small size of each specimen and the difficulties occurring when the frequency of luminal structures was to be estimated, a particularly important question in studies of this kind. This led to problems of sampling since large areas had to be excluded. The combination of SEM and TEM therefore proved to be of great value.

A strong contrast and a good preservation of endothelial plasma membranes is crucial when early cell reactions to injury are to be studied. This was, however, difficult to achieve using conventional methods for preparation of specimens for TEM.

The use of bismuth as a counterstain was originally recommended for enhancement of the electron density of ferritin particles (Ainsworth and Karnovsky 1972). In studies not included in this thesis it was found, however, that it also could be used for conventional specimens (Bylock and Hansson, in preparation). The combination of counterstaining with bismuth and stai-
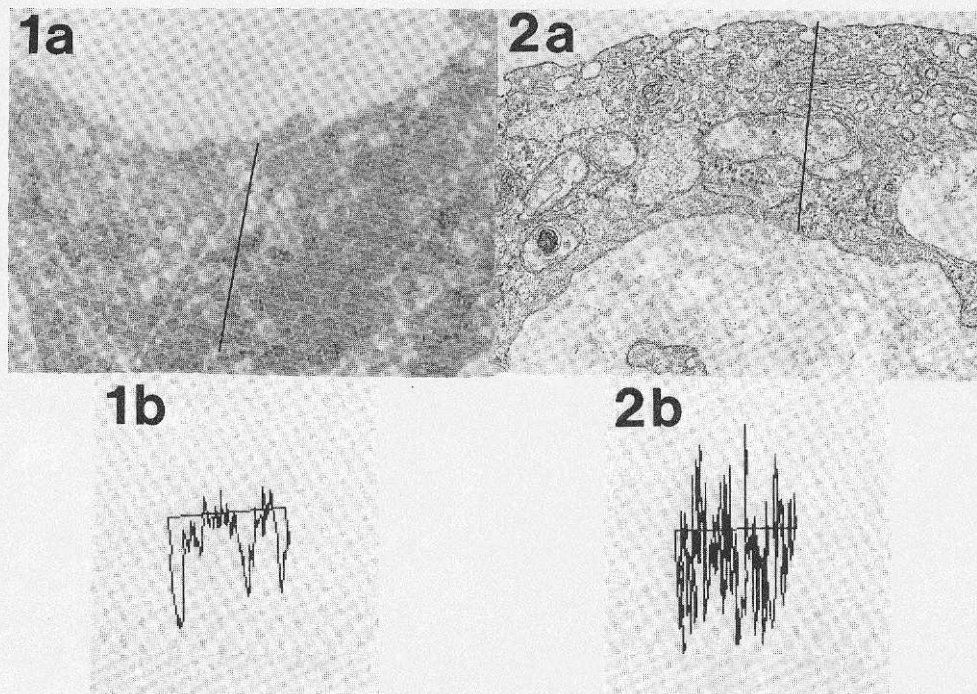
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FIGURE LEGENDS

These pictures and diagrams are examples on the improvement in contrast
and delineation of membranes and filaments achieved using the new method
for perparation described in paper VI. An optical densitometer has been
utilized, allowing a semiquantitative estimation of the degree of contrast.

Fig. 1a. The endothelium of a white, not injured area of a rabbit aorta.
The intercellular space and some vesicles can be discerned. The membranes
show a low degree of contrast in relation to the cytoplasmic contents. This
specimen was prepared according to the conventional method, using uranyl acetate
and lead citrate. The line indicates the area measured by the densitometer
(see fig. 1b). Transmission electron micrograph. 20 000 X.

Fig. 1b. In this diagram, the result of a photometric evaluation of the
density of the photographic plate used in fig. 1a, is shown. The total
length of the curve is 646 arbitrary units. Along the y-axis, the degree
of optical density is demonstrated.

Fig. 2a. An endothelial cell of a white, not injured area of a rabbit
aorta. From the same animal as fig. 1. Membranes and ribosomes are densely
stained while the cellular matrix has a low electron density. This specimen
was stained en bloc for one hour immediately after osmification, using 1%
uranyl acetate (see Materials and Methods , 3:4:2), The ultrathin section
was counterstained with bismuth for 50 min.( The swelling of the rough endo-
plasmic reticulum and the mitochondrion are signs of injury, probably occu-
ring as a response to a prolonged time lapse (30 min.) between rinsing and
fixation in this particular experiment). The line indicates the area measu-

*red by the densitometer (see figs. 2b). Transmission electron micrograph.
16 000 X.*

   *Fig 2b. This diagram shows the results of a photometric evaluation of
the photographic plate (fig.2a). The total length of this curve is 2670
arbitrary units. The y-axis shows the degree of optical density. To be
compared with 1b.*

ning en bloc using uranyl acetate gave an increased electron density and
an improved delineation of endothelial cell membranes and filaments (VI).
The cytoplasmic matrix remained virtually unstained if lead citrate was ex-
cluded as a counterstain and this also resulted in enhanced contrast. Den-
sitometric evaluation allowed a semiquantitative estimation of the degree
of contrast obtained after the different alternatives investigated (Bylock
and Hansson, in preparation) (see also figs la, b, 2a, b).

   The final procedure also included postfixation  in osmium tetroxide at
$4^0$ C, instead of at room temperature, which resulted in a reduced apparent
thickness of cellular membranes.

   Staining en bloc with uranyl acetate as well as counterstaining with bis-
muth alone both resulted in low electron density of all cellular structures.

## 4:2 STUDIES ON THE INTIMAL SURFACE OF UNMANIPULATED ARTERIES

   Normal endothelial cells have served as control specimens throughout the
experiments. In the scanning electron microscope they showed the characte-
ristics described in the introduction.

   At areas remote from branching points, SEM-studies showed that the nor-
mal endothelial cell had a flat, polygonal shape and a nucleus which bulged
gently into the lumen. Cell borders were seen as rows of protruding flaps
or microvilli. Interdigitation of these structures was occasionally seen.
The cells of rabbits and rats appeared similar.

## 4:3 DETECTION OF INTIMAL INJURY

   In studies on intimal injury, the possibility of studying large speci-
mens would be an advantage. Information about minor ultrastructural changes
both within cells as well as on their surface should also be possible to ob-
tain. No single method corresponding to this has yet been found. Several dif-
ferent methods suitable for detection of injuries to the intimal lining have

therefore been used in the studies included in this thesis. The methods have also been correlated to each other in order to provide a more complete view over the characteristics of an injured surface.

A dye-exclusion test (II, III, V and VI) was performed in order to make it possible to detect areas not covered by an intact endothelial lining already during dissection.

The dye was introduced into the vessel after exsanguination (Björkerud and Bondjers 1972). An intact endothelial lining excluded most of the dye but binding to exposed proteins within injured endothelial cells as well as to tissue below such cells could occur. The dye seemed to become trapped within the basement membrane or bound to the elastic tissue if access to these tissue components was gained. Blue areas correlated also to parts of the vessel covered by pseudoendothelial cells (II, III, V and VI).

In control vessels, an uptake could also be detected. This was seen preferentially at areas near ostia of branches, i.e. the sites where the early lesions of the hypercholesterolemic rabbits were found (I and II). This may suggest a connection between endothelial injury and the initial formation of dietary induced experimental atherosclerosis.

On the cellular level, an intracellular uptake was also seen. To detect this, whole mount specimens were studied by interference contrast microscopy according to Nomarski (1955) (paper II). After excitation by ultraviolet light, Evans blue emitted red fluorescence. In this way, also fluorescence microscopy could be used for detection of cells and sites having accumulated the dye. Swelling of endothelial cells correlated to Evans blue uptake. Swollen cells could be detected by interference contrast microscopy and, to some extent, by conventional light microscopy. Elevation of endothelial cells has also by other authors been suggested to be a sign of injury. Both light microscopy (Fry 1968, Björkerud and Bondjers 1972), SEM (Reidy and Bowyer 1977) and SEM on vascular casts (Reidy 1979) are methods by which such changes have been detected, for example at areas of high hemodynamic stress.

The presence of immunoglobulin G, IgG, within endothelial cells is evidence for cellular injury (paper II, see also: Hansson et al. 1979, 1980). Lately, also immunoelectron microscopy has been used to confirm this (Hansson et al. 1980). It is probable, that these molecules have gained access to the cytoplasm of the injured cells in vivo, since the blood was removed from the vessel as a first step in the preparation procedure. Postmortal

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artefactual injury could in this way hardly be considered to result in up-
take of IgG (II and VI). IgG could be found, not only in injured cells, but
also within the intima of dietary induced lesions and in the tissue under-
lying pseudoendothelial cells (paper II and VI). It may be suggested, that
this was due to an increased leakage of plasma constituents at such sites.

TEM has proven to be of great value, particularly for the detection of
the first signs of injury (V and VI). It also enabled us to identify cells
involved in the healing process after mechanical trauma (V and VI). The
gradation of endothelial injury chosen  followed that described by Tsâo
(1970). According to this, minimal injury was recognized by the occurence
of extensive subendothelial swelling and an increase in the number of mem-
brane bound vesicles in endothelial cells. In such damaged cells, myelin
whirls could be detected. More severe injury was characterized by dilata-
tion of the rough  endoplasmic reticulum, cytoplasmic vacuolization and
swelling of mitochondria. This stage was followed by a loss of mitochond-
rial christae and by irregularities in the perinuclear space. Condensation
of cytoplasmic structures, swelling of the perinuclear space and associated
nuclear pyknosis was then found. At last, cellular lysis occurred, allowing
access of detectable amounts of markers to the cytoplasm as well as to the
subendothelial space.

Scanning electron microscopy, SEM, has been the basic method for detec-
tion of intimal changes throughout the experiments included in this thesis.
A gradation of cellular injury similar to that described for TEM has not
been possible to implement. Swelling of the cytoplasm and the occurrence of
holes in the cell membrane were two SEM findings that correlated to the
characteristics for severe injury, obtained by other methods (II, IV, V and
VI).
The most severe degrees of injury were detected by SEM as large ruptures
through the cellular membranes, loss of adherance to the wall and finally
cellular desquamation (IV, V and VI). Following this, platelets, polymor-
phonuclear granulocytes (PMNs) and monocytes adhered to such areas. The
presence of such cells on the surface could therefore also be taken as a
sign suggesting endothelial injury.
The main advantage with SEM was found when the three dimensional arrange-
ment of components present on injured areas was to be studied on relatively
large specimens.

In the studies on the reactions following clamping (paper IV), it was found that endothelial injury and desquamation was accompanied by a reduction of the fibrinolytic activity in sections of the vessel wall. A regrowth of the endothelium was followed by a return to a normal activity. The histochemical method for estimation of fibrinolytic activity used (Todd 1959, modified according to Risberg 1975), may therefore also be used for detection of areas of damage to the endothelium. Detection of fibrinolytic activity at the cellular level is still not possible, however.

In conclusion, several different methods may be used for the detection of endothelial injury. They all give different information and the choice of a suitable method must depend on the purpose for the investigation that is to be made.

## 4:4 ENDOTHELIAL ALTERATIONS FOLLOWING CHOLESTEROL FEEDING

Several years ago, it was discovered that rabbits fed a cholesterol enriched diet developed intimal thickenings (Anitschkoff 1912). Deposits of cholesterol were found in these plaques (Anitschkoff 1913). Many experiments concerning the connection between cholesterol feeding and the development of atherosclerosis-like lesions have since then been done. In several of these, however, high concentrations of cholesterol have been added to the diet of the animals in order to rapidly produce lesions. The serum cholesterol levels have often ranged between 1000 and 2000 mg% (25 to 50 mmol/l) despite the fact that these levels are far beyond those seen in unmanipulated animals and in man.

Constantinides (1965) suggested that the results of such experiments often merely reflected a generalized cholesterol overload. Further evidence for this was given by Bondjers et al. (1976) who showed that endothelium remote from branching points could retain its normal barrier function only if the serum cholesterol levels were kept below 400 mg % (10,0 mmol/l).

In order to induce atherosclerosis-like lesions with the lower levels, it was necessary to feed the animals the diet described above for long periods of time (I and II). The lesions obtained by this method showed a more pronounced preferential localization to areas of hemodynamic trauma, as compared to when high serum cholesterol levels were used.

On the dietary induced lesions (paper I and II) the endothelial cells had a quadrangular shape. Many of them also appeared swollen in SEM. This sign, together with the discontinuities seen in the luminal cell membrane,

was particularly frequent in the periphery of the lesions. These areas had
also the highest number of Evans blue-positive cells and an increased IgG-
like immunoreactivity (II).

It was proposed by Fry (1968) that augmented hemodynamic trauma may
result in endothelial injury. Papahadjoupoulos (1974) considered it possible
that high cholesterol levels make plasma membranes less fluid and the cells
more prone to injury. It may be speculated, that these mechanisms may act
in an additive way leading to the preferential localization of lesions to
areas of turbulent blood flow (I and II).

It may also be suggested, that the increasing size of the dietary induced
lesions was due to the formation of the zone of severely injured cells
seen along the borders of the lesions. Turbulence following from protrusion
of the lesions into the arterial lumen may be significant for this. Varying
mechanical properties of the lesions and the surrounding, intact, tissue
may also be important.

4:5 MECHANICAL INJURY

As earlier mentioned, endothelial injury has been proposed to be an im-
portant link in the development of atherosclerosis (reviews: French 1966,
Björkerud and Bondjers 1976, Ross and Glomset 1976). The endpoint for en-
dothelial cell injury is lysis and desquamation (Tsão 1970). Even if it
is still unknown for how long the resulting denudation of subendothelial
tissue persists, repeated injury may lead to an increased leakage of plasma
constituents into the vessel wall. It has been shown, that de-endotheliali-
zation produces atherosclerosis-like lesions (review: Björkerud and Bond-
jers 1976, Ross et al. 1977) and it has therefore been suggested that the
endothelial barrier is crucial in preventing atherosclerosis also in hu-
mans. Further studies on mechanically induced experimental atherosclerosis
were considered necessary, particularly on the ultrastructural level.

Endothelial cell injury and desquamation was induced through rotation
of a microsurgical instrument inside the aorta of rabbits (paper V and VI)
Ruptures in the subendothelial tissue were also induced at certain sites.

Large numbers of platelets and leukocytes were seen to have adhered al-
ready within the first hour. After about 3 days, the lesions were demarca-
ted, surrounded at most sites by endothelial cells with an intact appea-
rance. At seven days after injury, cytoplasmic extensions from underlying
cells were seen at the lumen. These formed a surface which had some of

the characteristics of endothelium: flattened cytoplasms lining the inti-
ma. A clearly defined border towards the normal endothelium was still no-
ted, however.

IN TEM, the cytoplasms of these new intimal cells contained densely
packed thin filaments (diameter between 4 and 7 nm) immediately below the
surface. Below these, large amounts of rough endoplasmic reticulum was found.
The nucleus of these cells was located at varying distances from the lumen.
The deeper portion of the cytoplasm had some of the characteristics de-
scribed for the smooth muscle cell while it is in its so-called "synthetic
state" (review: Campbell et al. 1981). The cells had few, scattered bund-
les of thin filaments seen among large numbers of organelles known to be in-
volved in the synthetic activity of a cell. This cell type was called "the
pseudoendothelial cell", a name originally derived from studies by Pugatch
(1964) (see also separate chapter). Some areas remained covered by such
cells even one year after injury. Adhesion of leukocytes and platelets, al-
so spherical, was then still seen. Signs of dilatation of the vessels were
also noted after one year.

Zones of endothelial injury were seen at certain sites along the borders
to the areas covered by pseudoendothelial cells, at all time intervals.
Fibrinous strands with an apparent banding of approximately 23 nm were al-
so occasionally noted adhering to the surface following mechanical injury.
These strands were predominantly found in large aggregates and thrombi.

The response to application of a Diffenbach clamp over the femoral arte-
ry of rats was also studied (paper IV). This clamp is used also clinically
during surgery. The morphological findings were in this study correlated to
changes in fibrinolytic activity of the vessel wall. The fibrinolytic sys-
tem is important for the regulation of fibrin deposition and the resolu-
tion of thrombi. Fibrinolysis is stimulated plaminogen activitors (PA) de-
rived from endothelial cells (Todd 1959).

The vessels were, in the present study (paper IV), subjected to clamping
for 30 sec. They were excised and prepared for the studies after different
time intervals: from 1 hour to 7-8 weeks. At time intervals within one day
after application, denuded areas, endothelial cells which were almost de-
tached as well as large numbers of platelets, were seen. Some leukocytes
could also be noted as well as deposition of small amounts of fibrinous
strands and erythrocytes. After 24 hours, all remaining endothelial cells had
an intact appearance. Re-endothelialization was almost completed after one
week and after three weeks the surface showed full restitution of endothe-

lial integrity. A marked thickening of the wall was seen at the injured
sites two months after application of the clamp. On these thickenings, some
endothelial cells had an uneven surface with microvillous blebs and dis-
continuities. The fibrinolytic activity was increased when the surface was
re - endothelialized and reached pre-injury levels after three weeks, i.e.
when full restitution had occurred.

Ashford and Freiman (1968) showed that, if an inhibitor of the fibrino-
lytic activity, EACA, was given, the amount of fibrin deposited on an in-
jured vessel wall increased. Despite the low fibrinolytic activity demonst-
rated in de-endothelialized areas in these and other studies (IV, Silver
1969), relatively small amounts of fibrin were found in the studies included
in this thesis (IV, V and VI). Theoretically, this may be due to the re-
maining fibrinolytic activity of the blood but artefactual loss of fibrin
strands during preparation may,although not very likely, also be an expla-
nation to this.


Some differences between the lesions induced in the rats and in the rab-
bits deserve particular mention. Following clampling, the femoral arteries
of the rats showed complete re-endothelialization after three weeks. No
pseudoendothelial cells could be detected, while such cells covered large
parts of the initially injured lesions in the de-endothelialized aortae of
the rabbits. A thickening of all parts of the vessel wall was seen in the
rats while only the intimal compartment showed thickening in the rabbits.
Up to 50 leukocytes/mm$^2$ could be found on the injured areas in the rats
while between 500 and 5000/mm$^2$ were seen in the aortae of the rabbits.

Both species differences and methodological differences may account for
these diverging results. A comparison with experiments performed by other
groups suggest, however, that the different methodology used for the induc-
tion of the lesions was the most important factor. The presence of pseudo-
endothelial cells has, for example, been described also in the rat after
drying the endothelium (Clowes et al. 1978).


The effects of mechanical injury to the artery has long been an object
of study. Several methods have been used: Prior and Hartmann (1956) stu-
died the long-term structural effects of the insertion of a needle into
the aorta of rabbits. Poole et al. (1958), who used a brass rod, described
a rapid phase of restitution of the intimal lining. This was then followed
by a slow phase, leading to a return to a normal morphology of the lining
cells. In the light of later studies, the first phase seems to correlate

to the development of the pseudoendothelium, but a discrimination between endothelial and pseudoendothelial cells was not done by Poole and co-workers. Friedman and Byers (1963) studied the response to the insertion of an intra-aortic coil. Baumgartner (1963) developed the balloon-catheter model: at present the dominating system for induction of mechanical injury (for references, see: Ross et al. 1977 and 4:8).In 1965, Friedman and Byers described persisting lesions following the insertion of a wire-stiffened vinyl tubing into the aorta of rabbits. Björkerud (1969) presented the microsurgical instrument used in paper V and VI. The different responses following from rotating or pulling the instrument and using light or heavy pressure were studied (for references, see: Björkerud and Bondjers 1976). Vascular lesions have also been described after the insertion of a roughened metal probe into arteries (Sheppard and French 1971) and a vibrating catheter filled with mercury was used by Hirsch and Robertson (1977). The rapid sequence of healing following a light and narrow scratch to the endothelial layer has recently been studied (Reidy and Schwartz 1981).

Other methods to induce injury have also been utilized. Partial ligation was used to Tsáo (1970) in a paper where he described different grades of intimal injury. Gertz et al. (1981) have recently also been using this method. The reaction following the placement of a suture through the aortic wall of the rat was studied by Poole et al. (1971) and in the rabbit this was studied by Webster et al. (1974 a and b). Fallon et al. (1973) found signs of injury within the vessel wall after exposing the artery to ultrasound. The effects of electrocautery were studied by Webster et al. (1974 a and b) using the rabbit aorta. Vascular clamps were used by Slayback et al. (1976) and by Mansfield et al. (1978). Application of a cold probe to the outside of the aorta of rats was also shown to result in endothelial injury and desquamation (Malczak and Buck 1977) and the effects of drying the endothelium were studied by Fishman et al. (1975). In the latter experiments, using the rat common carotid artery, endothelial desquamation was found to occur without accompanying leukocyte adhesion.

Mechanical injury accentuated by cholesterol feeding has also been an object of several studies. Prior and Hartmann (1956) considered the resulting lesions to be very similar to early human atherosclerotic plaques. The lesions produced in their experiments were also considered to have many characteristics in common with thickenings resulting from hypercholesterolemia alone. Friedman and Byers also described an additive effect of the two components and the resulting lesions were in their models found to consist of neatly arranged SMCs and extracellular lipids which were deposited

in the centre of the plaque (Friedman and Byers 1965). In their experiments,
however, thickenings induced by hypercholesterolemia alone consisted main-
ly of cells containing lipids. Björkerud and Bondjers (1977) found that
the repair of mechanically induced lesions was delayed if the animals
were rendered hypercholesterolemic. Also in their experiments, the resul-
ting lesions were considered to have many properties in common with early
human atherosclerotic plaques. In accordance with these results it was
suggested that high levels of serum cholesterol either interfered with re-
endothelialization or caused an overloading of the tissue beyond its
lipid eliminating capacity (Björkerud and Bondjers 1977).

4:6  SOME ASPECTS ON THE ROLE OF THE PLATELET IN ATHEROGENESIS

Endothelial desquamation has been shown to lead to platelet adhesion
(Baumgartner 1972, IV, V and VI). The platelets adhere to collagen while
elastic tissue is considered to be virtually non-thrombogenic (Hugues
1960, Baumgartner 1972). According to Groves et al. (1979) the majority
of the platelets adhere within the first 10 min after de-endothelialization.
The event of degranulation following adhesion to the wall has been shown
to be associated with a release of the contents of platelets granules.
One substance,the platelet derived growth factor, PDGF, deserves particular
mention. Ross et al. (1974) found that it seemed to stimulate mitosis in
SMC cultures. The release of this substance into the arterial wall was
suggested to provide an explanation for the high number of SMC´s seen in
some atherosclerotic lesions (Ross et al. 1977). Recently, a modified ver-
sion of this hypothesis has been proposed since it was found that PDGF al-
so  seems to be chemotactic for SMCs in culture (Grotendorst et al. 1981).
These authors suggested that a migration of SMC´s from the media to the
intima could result in the formation of plaques and that this was a response
to PDGF released by platelets adhering to areas of endothelial injury.

Evidence for the relevance of platelet adherance and degranulation for
atherogenesis had, in any case, already been given by Moore et al. (1976)
and by Friedman et al. (1977). They showed that animals rendered thrombo-
cytopenic after infusion of antiplatelet serum failed to respond with in-
timal hyperplasia after de-endothelialization. An inhibitor of platelet
aggregation, dipyridamole, has also been shown to have a beneficial effect
in baboons subjected to experimental homocysteinemia where the extent of
the lesions was reduced after distribution of this drug (Harker et al. 1976).
The platelet may thus play an important role in atherogenesis if the endo-

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thelium is significantly injured.

## 4:7 ADHESION OF LEUKOCYTES TO THE ARTERIAL WALL IN EXPERIMENTAL ATHERO-
### SCLEROSIS

Leukocyte adhesion to the vessel wall has been shown to be related to endothelial injury. In this thesis, it was found both after induction of hypercholesterolemia (III) and after mechanical trama (IV, V and VI).

It was not possible to determine the type of the leukocytes involved in the reaction to hypercholesterolemia (III, see also Bondjers et al. 1978). According to similar experiments done by other groups, however, a large proportion of the cells described may be monocytes (Haust 1974, Fowlet et al. 1979, Schaffner et al. 1980, Gerrity and Naito 1980 a and b). If large numbers of leukocytes are involved also in human atherosclerosis, one explanation for the increased cell numbers of intimal thickenings, may be provided.

In the clamped femoral arteries of the rats (paper IV), 0 to 50 leukocytes were found per square mm. Leukocytes were seen both on the injured areas and on areas covered by normal endothelial cells in the vicinity of the injured parts.

Following mechanical trauma induced from the inside of the aorta of rabbits, higher number of leukocytes were often found (V and VI). On the deendothelialized areas, 500 to 5000 leukocytes were counted per square mm. The majority of the leukocytes involved were at all time intervals polymorphonuclear granulocytes, PMNs. Also monocytes and, in some cases, lymphocytes, could be found. The leukocytes involved successively showed a more well defined and characteristic pattern of distribution. They were seen in ring-like formations around large thrombi and rows of leukocytes were found along intercellular borders of pseudoendothelial cells. Large numbers of leukocytes were also particularly frequent along borders of lesions. A connection between leukocytes and platelets was also noted. These results are consistent with the findings of Ratcliff et al. (1979), who described a process of interaction between platelets and PMNs in both balloon-catheter-injured arteries of dogs and arteries of rabbits and rats injured by infusion of arachidonic acid. In their study, it was suggested that a mechanism of platelet-PMN interaction ("cupping") exists, and that this may be an integrated part of the arterial response to injury.

In a series of studies by Hansson and co-workers it has been shown that

the immunological system may be highly significant for the adhesion of mo-
nocytes to injured areas (Hansson 1980). Monocytes bear Fc-receptors on
their surface (Berken and Benacerraf 1966) and these are capable of forming
tight complexes with IgG. IgG has been shown to accumulate within injured
endothelial cells, within the intimal compartment under such cells (II,
Hansson et al. 1979, 1980) as well as under pseudoendothelial cells (VI).
Blocking of the Fc-receptors of the monocytes by means of the addition of
staphylococcal protein A did, in an in vitro-system, cause a 50% reduction
of the number of monocytes adhering to injured areas of rabbit aortae
(Hansson et al. 1981). These data thus suggest, that the adhesion of mono-
cytes may, to a large part, be mediated through an interaction between IgG,
adsorbed to injured parts of the intima,and the Fc-receptor of the mono-
cytes.

More specific data on the role of the leukocytes in human atherogenesis
are still lacking but, since a pseudoendothelium and adhering leukocytes
could be seen even one year after mechanical injury, it is suggested that
a chronic inflammatory process may be involved, at least in experimental
atherosclerosis.

4:8 THE PSEUDOENDOTHELIAL CELL

In areas where mechanical injury had been induced, a surface cell was
seen, lacking several of the characteristics common to endothelial cells
(paper V and VI). As seen from the lumen, the first signs of this cell type,
the so-called pseudoendothelial cell, were noted 7 days after injury. We
first observed large processes which extended from underlying cells up to
the luminal surface. The main part of the cytoplasm as well as the nucle-
us was often found 20 to 40 µm below the surface. The long axis of the
surface of the luminal process was oriented at a right angle to the blood
flow. After about 2 weeks, the large processes had formed a relatively ho-
mogenous new lining of the affected areas. By SEM, the size of the area
which was covered by each process differed remarcably, extending up to
70 x 70 µm. The processes showed an arrangement resembling that of roofing-
tiles, i.e. the distal part of each process covered the proximal part of
its downstream neighbour. Microvillous projections, occasionally bud-like,
could be found in large numbers on some of these cells. Along the luminal
borders, small interdigitating processes were seen. Among these, platelets
and leukocytes were also found. Even one year after injury, many of the

platelets were spherical, a sign suggesting that degranulation had not oc-
curred(review: Baumgartner and Muggli 1976). Pseudoendothelial cells at the
border to normal endothelium did not have a different structure from those
in other non-endothelialized areas. In the normal endothelium, however, the
border regions were characterized either by a zone of necrotic endothelium,
thrombi or a demarcation line formed by a lip-like swelling connecting the
normal endothelial cells. A fusion between endothelial and pseudoendothe-
lial cells was never observed.

By TEM, both the interdigitating, finger-like, processes along the cell
borders and the smaller, microvillous or bud-like, protrusions seen by SEM,
could be confirmed. A condensation of thin filaments which had a diameter
of about 4 to 7 nm, was seen at the luminal part of the cytoplasm. Below
this zone, large quantities of rough endoplasmic reticulum were present.
Vesicles were common along abluminal membranes, but no signs suggesting
the presence of transcellular pathways, were seen. No Weibel-Palade bodies
could be found and luminal junctional complexes were absent. Due to the
deep localization of the nucleus, the luminal surface covered by pseudoen-
dothelial cells was flat, in contrast to the bulging appearance seen when
ordinary endothelial cells constituted the intimal lining.

With the dye-exclusion test, access of Evans blue was gained to compart-
ments underlying pseudoendothelial cells. Likewise, a material showing a
positive reaction to antibodies against IgG could be found under these cells.
Björkerud and Bondjers (1971) reported that also when injecting Evans blue
in vivo binding of the dye to these areas occurs. An increased transfer of
[3]H-cholesterol into these blue parts was also found (Bondjers and Björke-
rud 1973b),and all these data, taken together, indicate a higher permeabi-
lity to plasma constituents.


The term"pseudoendothelium" was first used to describe the sheets of
cells that appeared before normal vascular endothelial cells in healing
rabbit ear chambers (Pugatch 1964). In that study, macrophages interspersed
with fibroblasts and other mesenchymal cells constituted the surface. Baum-
gartner (1971) used this description for the layer of platelets which ad-
hered to a de-endothelialized blood vessel,but more often it has been app-
lied to describe a single layer of cells, other than platelets,lining the
intima .

At present,the most common opinion about the origin of the pseudoendo-
thelial cell, as defined in this study,seems to be that it is derived from
SMC´s (Stchelkounoff 1936, Friedman and Byers 1965, Stemerman and Ross

1972, Ross and Glomset 1973, Björkerud and Bondjers 1973, Spaet et al. 1975, Stemerman et al. 1977, Clowes et al. 1978, Richardson and Moore 1980, Eldor et al. 1981),but also other opinions about its origin have prevailed. Poole et al. (1958) considered these cells to be of endothelial origin, oriented in a bizarre pattern, while French (1966) and Haudenshild and Studer (1971) thought that flattened monocytes constituted the surface of lesions induced by mechanical injury. Collatz-Christensen and Garbasch (1973) considered it possible that these cells could be derived either from the blood, from the underlying tissue or from both sources. Since implanted non-viable aortic homografts became covered solely from the sites of junction in an experiment by Spaet et al. (1975), the possibility of a hematogenous origin of these cells was, by these authors, considered unlikely.

The smooth muscle cell is the main cellular component of the vessel wall. It is capable of producing all extracellular connective tissue components present in arteries (review: Ross and Kariya 1980). The SMC of the artery is spindle-shaped or multipolar and has an elongated nucleus which is oriented along the long axis of the cell. The main components of the contractile SMC are thin (diameter 4-7 nm) and thick (diameter 12-18 nm) filaments consisting of actin and myosin (Gerrity and Cliff 1975, review: Campbell et al. 1981) and numerous mitochondria. Occasionally, gap junctions or less well-defined contacts can be seen between neighbouring cells in the normal vessel (review: Ross and Kariya 1980). Peripheral dense bodies, peripheral vesicles and a basal lamina are also characteristic of the contractile SMC. Despite these criteria, however,the identification of this cell type may occasionally prove to be difficult since SMC´s adapt to their enviromental conditions (comments and references: Campbell et al. 1981). It has even been shown, that the oestrus cykle may affect the morphology of SMC´s (Poole et al. 1971).
Arterial cells in lesions induced by mechanical injury have proved to be difficult to identify. Schwartz et al. (1975) described for example three types of cells in healing lesions: SMC´s, endothelial cells and, as a third type, "probably dedifferentiated SMC´s".
It has also been suggested that endothelial cells (Crawford 1956, Haust et al. 1960, Eiken and Norden 1961) or blood-borne cells (Jörgensen et al. 1967) should be able to alter their differentiation pattern sufficiently enough to resemble SMC´s in character. To date, no direct evidence has been reported for this rather unlikely hypothesis. However, the border of non-fusion between endothelial and pseudoendothelial cells, which then

would be considered an intermediate cell type (paper VI),may suggest the opposite. Evidence further suggesting this possibility was provided by the lack of reactivity to antibodies towards factor VIII seen in the pseudo-endothelium of rats (Clowes et al. 1978) and rabbits (paper VI).

As has been mentioned earlier, the SMC is multipotent and capable of responding to various stimuli. This ability may be the factor leading to the formation of a pseudoendothelium since endothelial desquamation probably leads to gross alterations in the growth conditions within the vessel wall. Another example of this may be the storing of lipid droplets seen in SMC´s of animals subjected to experimental hypercholesterolemia (references and comments: Fowler et al. 1979). Other gross alterations have been seen in SMC´s subjected to in vitro conditions (Fowler et al. 1979, review with references: Campbell et al. 1981). Not only morphological but also chemical properties of SMC´s may change extensively along with altered conditions . While present within the media, the SMC produces only minor amounts of the anti-thrombogenic substance $PGI_2$, prostacyklin (Moncada et al. 1977). After the surface of an artery has been subjected to mechanical injury, the pseudoendothelial cell continuously increases its ability to produce this substance until the produced amounts reach levels seen in normal endothelial cells (Eldor et al. 1981).

The concept of modulation of the SMC phenotype has recently been emphasized (Campbell et al. 1981). According to this concept, SMC´s may change their phenotype as a response to alterations in their environments. In culture, SMC´s remain in a contractile state for about one week. Being in this state, they have an abundance of thick and thin filaments with associated dense bodies (Campbell and Chamley 1975) and they are, due to this, easily identified in TEM. Modulation of the phenotype to a synthetic state has been shown to occur after prolonged culture (Chamley et al. 1974) and this was accompanied by a loss of thick filaments and an increase in the number of organelles involved in synthesis, mainly ribosomes. This modulation may also facilitate proliferation since most SMC´s seen undergoing mitosis have been in the synthetic state (references: Campbell et al. 1981).

In cell culture, modulation back to the contractile state may take place if either a confluent layer of SMC´s has developed or if another layer of confluent cells, for example endothelial cells, is present in the culture. It has been suggested that this effect may be chemically mediated through a release of some form of substance produced in cells which are contact inhibited (Campbell et al. 1981).

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The characteristics of the pseudoendothelial cells shown     in this
thesis (paper VI) do not allow classification in any of the two states de-
scribed. The luminal part of these cells was, in most cases, filled with
densely packed thin filaments. The remaining part of the cytoplasm con-
tained a large number of organelles, thus resembling the synthetic state
of the SMC. It is postulated, that also phenotypic states other than the
pure contractile and the synthetic, may exist. Whether this "pseudoendo-
thelial state" represents an intermediate between the two earlier mentioned
states or it should be considered as a separate one, remains to be clari-
fied. A lack of endothelial cover  as well as of specialized intercellular
junctions may possibly be important due to the fact that no SMC´s in the
contractile state were seen close to the lumen (paper VI). According to
the earlier mentioned findings that SMC´s preferentially seem to go into
mitosis in a non-contractile state, a connection between the lack of endo-
thelial cover and the high degree of proliferation,noted in de-endothelia-
lized areas, may be seen. PDGF released from platelets adhering may possib-
ly also affect the phenotypic state of the SMC´s.

In cell cultures it has been shown, that SMC´s lose their ability of re-
turning to a contractile state after more than 3 weeks of culture (review:
Campbell et al. 1981). If such an inability of SMC´s in the synthetic state
to return to the contractile should prevail also in the in vivo situation,
it may provide an explanation to why some lesions became  stationary after
mechanical injury to the intima  (paper IV).

# 5. SUMMARY

Endothelial injury has, for a number of years, been considered to be an
important factor for the development of atherosclerotic lesions in large
blood vessels and for the formation of thrombi on the vessel wall. The ob-
ject of these investigations was to study the surface ultrastructure of ar-
teries subjected to mechanical trauma and to study the integrity of the en-
dothelial lining in experimental hypercholesterolemia in rabbits.

Scanning electron microscopy served as the basic method for the studies
and a preparational scheme, particularly suitable for arteries, was develop-
ed  in order to provide optimal reproducibility and resolution. Transmission
electron microscopy was used in some studies where a characterization of
different cell types and a gradation of cellular injury was found necessary.
A new method for staining of specimens for transmission electron microscopy
was also developed, providing an enhanced contrast and membrane delineation.
Light microscopy, interference contrast microscopy, and fluorescence micro-
scopy were other methods that were used in order to detect endothelial in-
jury. A dye-exclusion test was performed in several experiments, allowing
a macroscopic evaluation of the endothelial integrity as well as localiza-
tion of endothelial injury on the cellular level. Injured endothelial cells
showed swelling, desintegration of their cellular membranes, uptake of
Evans blue and a binding of antibodies  against immunoglobulin G.

The effects of a direct mechanical trauma to the intimal lining of the
aorta of rabbits was studied. Endothelial cells became desquamated and ad-
hering platelets, leukocytes, erythrocytes and fibrinous strands were seen
on injured areas. 7 days after injury, a characteristic cell type, the
"pseudoendothelial cell", was observed at de-endothelialized parts of the
vessels. At some sites, it could be seen lining the intima even one year
after injury. A binding of Evans blue and a positive reaction to antibo-
dies against immunoglobulin G was seen in the intimal compartment under-
lying such cells. Since no specialized junctions between the pseudoendothe-
lial cells could be seen, it was suggested that this kind of surface al-
lowed an increased leakage of substances of the blood into the vessel wall.
The pseudoendothelial cells did not contain detectable amounts of factor
VIII and no Weibel-Palade bodies were seen, two signs making and endothe-
lial origin unlikely. The zone of non-fusion between endothelial and pseu-
doendothelial cells was also considered to speak against a common origin.
It is suggested that the pseudoendothelial cells were smooth muscle cells
that had been modified even though they did not resemble either of the

two defined phenotypic states of this kind of cell: the contractile or the synthetic state.

The effects of the application of a surgical clamp to the femoral artery were studied in rats. The endothelial surface showed signs of injury and desquamation of cells and an adhesion of platelets, erythrocytes, leukocytes and fibrinous strands, was noted. The fibrinolytic activity of the vessel was measured after injury and a reduction to levels not detectable with the methods was found. A return to pre-injury levels was noted when re-endothelialized was completed, 3 weeks after clamping.

In rabbits, moderate hypercholesterolemia was induced by the addition of cholesterol to their diet. Intimal thickenings were found to occur in their aorta. A predominance for areas close to ostia of branches was seen. On these lesions, particularly along their borders, a discontinuous endothelial lining with swollen and immunoglobulin G-containing cells could be noted. Leukocytes were also occasionally seen adhering to the intimal surface of these hypercholesterolemic rabbits.

The possible connection between endothelial injury and the formation and growth of atherosclerotic lesions has been discussed in the light of the present studies and observations made by other groups.

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