Experimental Arterial Thrombosis

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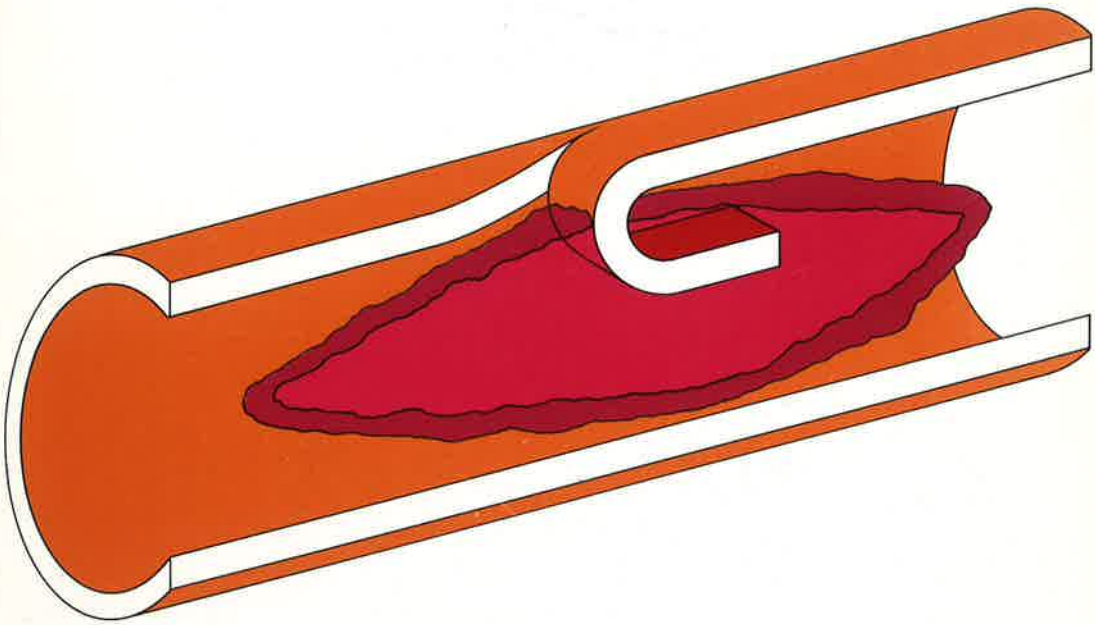

P. J. van Aken

EXPERIMENTAL ARTERIAL THROMBOSIS

Investigations based on a new microsurgical method for arterial thrombosis in rat aorta.

Experimental arterial thrombosis

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by

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Aan mijn ouders

Lieke

Berend

Tijmen

CHAPTER I

INTRODUCTION

I.1

The importance of arterial thrombosis

Cardiac and vascular disorders contribute to the total mortality in the Dutch population. Accounting for 12% of total mortality in the beginning of the century, the number of deaths attributable to such disorders had increased to 47% in 1976 (data from the "Commissie Coördinatie Onderzoek Hart- en Vaatziekten").

The magnitude of this problem still stimulates the investigation of the underlying causes. Despite many clinical and experimental investigations, no real causal therapy has been developed for this important disease. Symptomatic therapy is the only weapon against vascular occlusions and their sequelae. Arterial thrombosis seems to play an important part in arterial and cardiac diseases, as already described by Duguid in 1946.

Arterial thrombosis is also important concerning the patency rate in vascular surgery, thus forming a direct problem to deal with.

The question remains whether arterial thrombosis is also an underlying factor in the development of longterm vascular disease, by causing repeated arterial thrombus formation until occlusion of the vessel has occurred. The possible relation between arterial thrombosis and longterm vascular disease, is worth an investigation.

For a better understanding of the development and organization of arterial thrombosis, a detailed description of the latter has to be available. In human material it proves difficult to find fresh arterial thrombus formation because one usually deals with older thrombus material. Experimental thrombus formation has to be produced in an animal model, without using exogenous material. Because of this, a new experimental model is developed in which reproducable, mural arterial thrombosis is evoked.

The main causes for mural thrombus formation are described in Virchows' triad; changes in the vessel wall, changes in blood flow and changes in the

composition of the blood. These thrombogenic factors will be elaborated in detail in the following sections of this chapter.
Because of the growing evidence of the importance of platelets in the development and organization of arterial thrombosis, special attention will be given to platelet physiology.

I.2

The role of platelets in thrombogenesis

The role of platelets in the development of both an atherosclerotic lesion and an arterial thrombus is getting more and more attention. The role of the platelets has a number of aspects. Adhesion to the vessel wall as a reaction to endothelial lesions seems to be important. This carpet-like covering could possibly stimulate arterial smooth muscle cells to grow into the intima. Another aspect is the formation of platelet aggregates and incorporation of aggregates in the vessel wall. The formation of platelet-fibrin thrombi could be considered as the last step (Geer and Haust - 1972, Ross and Glomset - 1973/1976, Haust and More - 1974, Friedman - 1977/1978, White and Heptinstall - 1978).
Moreover, in the last few years, a number of new aspects of the platelet has been discovered. The importance of the cohesion and adhesion of platelets, influenced by a shear stress of the passing blood, has been clearly demonstrated (Turrito - 1979, Stevens - 1980). The interaction between the thromboxane production by platelets and the prostacyclin production by endothelial cells, also seems to be of importance in order to maintain the balance between aggregation and non-aggregation of platelets (Moncada, Gryglyewski - 1980).

The role of platelets in arteriosclerosis is put forward very clearly in the theory of Ross and Glomset (1976). They indicate that, if the endothelium is injured by for instance a balloon catheter, a number of events will follow. When the lesion has been produced, the platelets attach within minutes to the subendothelial tissue and degranulate. In 1976 Harker showed that, in the presence of an arterial lesion, platelet survival was diminished, corresponding to the consumption of platelets on the endothelial lesion.

Later on, in clinical situations too, a diminished survival time for the platelets was affirmed in patients with arteriosclerosis. Moreover, Ross and Harker found that an induced hypercholesterolaemia in monkeys resulted in a significantly decreased platelet survival time; from 8.0 days to 6.2 days. This finding suggests that hypercholesterolaemia causes an increased platelet consumption because of vessel wall trauma. Apart from hypercholesterolaemia Harker (1976) could also evoke endothelial lesions with the aid of a homocystein infusion. These findings prove that endothelial lesions can be caused by an abnormal chemical metabolite as well.
Five to seven days after an endothelial lesion has been produced, migration through the fenestrae of the lamina elastica interna, and proliferation of smooth muscle cells can be observed. These smooth muscle cells migrate into the injured intimal area. Within a few weeks, a large number of layers of these cells is present, surrounded by collagen fibrils and elastin lamellae; all of them newly formed.

If the noxe is permanent, aggregation of the platelets will take place again locally and thus, possibly, a laminar construction of the lesion is formed, which is typical for the formation of arterial thrombosis.
Aggregation of platelets can take place by activation from the platelets themselves. Aggregation can also be induced by factors coming from the vessel wall. If the platelets attach themselves to the collagen of the intima, they will degranulate. During this process a number of factors are released from the platelets, i.e. ADP. Under the influence of ADP, the shape of the platelets, passing the lesion, changes, due to which they can turn to aggregation (with the aid of fibrin), (French - 1971, Chandler and Pope - 1972). Subsequently, further organization can take place if these microthrombi are not washed away. The lesion thus initiated, will be able to expand because of proliferation of smooth muscle cells. In this way the mechanism becomes clear which, on account of a recurrent noxe, may cause an artherosclerotic lesion from a small arterial thrombosis. This lesion becomes ever bigger and might cause obstruction of the vessel concerned. All these findings are confirmed by Friedman and Stemerman (1977), who effected lesions in the aortas of rabbits, which were treated with anti-platelet serum, resulting in thrombocytopenia. The proliferation of the smooth muscle cells was clearly suppressed. Burns

and Friedman (1978) emphasized, once again, the importance of proliferation of smooth muscle cells in the development of atherosclerosis; they especially underlined the importance of the platelet actions.

What are these actions, what is their influence on the vessel wall and notably on the proliferation of smooth muscle cells? Is it a hormone or purely a chemotactical substance, coming from the granulae of the platelets. Are there any restrictive factors in this process?
With the aid of the important model of Ross and Glomset (1976), a correlation could be demonstrated between lesion of the arterial wall, adhesion of platelets, release of granulae from these platelets, and also the subsequent proliferation of smooth muscle cells. Ross and Glomset reinforced their theory by culturing arterial smooth muscle cells. They showed that a quiescent culture of smooth muscle cells could be induced to grow by adding serum prepared from blood in the presence of platelets, but not by adding serum prepared from platelet-free plasma. An extract of platelets showed a similar activity. Consequently, they concluded that the platelets contained a substance which incited smooth muscle cells to proliferate. This factor released at the degranulation of the platelets has been the subject of many experimental investigations. Tiell (1978) alleges that the hypophysis seems to play an important role. During experiments with hypophysectomized rats, he observed a clearly decreased myointimal thickening and proliferation. A hormonal influence from the hypophysis, via platelet factors, is thus made probable. In the same year, however, Tiell also alleged that the suppression of myointimal proliferation by hypophysectomy can be removed by a non-specific inflammation. If turpentine is injected subcutaneously, a revival of the myointimal proliferation of smooth muscle cells is seen in hypophysectomized rats. The mechanism responsible has not been explained. The possible importance of a stimulating role of leucocytes and macrophages is being investigated.

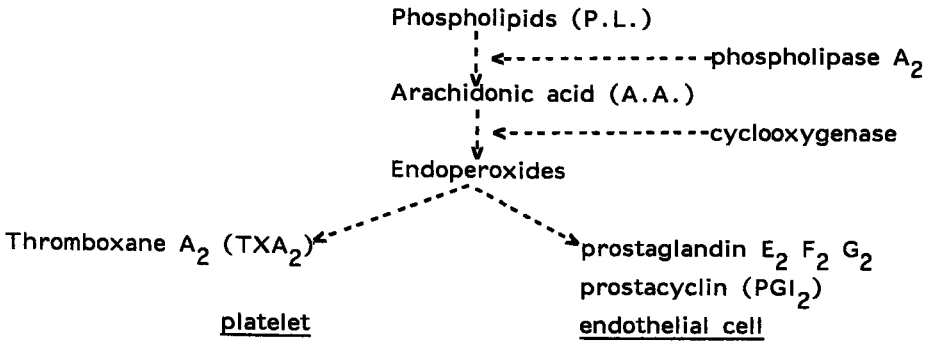
Clowes (1978) determined that, in case of an endothelial lesion, the proliferation of smooth muscle cells was suppressed in the presence of heparin. He presumed that the inhibiting effect of heparin, via antithrombin III, on thrombin may play a role. Recent investigations in vitro and in vivo have shown, however, that heparin directly effects the smooth muscle cells, irrespective of its anticoagulating effect (Guyton - 1980, Castellot - 1980).

On the other hand it is suggested that the coagulation system plays a role in smooth muscle cell proliferation. Goldberg and Stemerman (1980) indicate, by means of immunofluorescence methods, that platelet factor 4, after being released at a degranulation on an endothelial lesion, penetrates the vessel wall. After about four hours this factor has disappeared from the vessel wall again. Anyway, this shows that platelet factors can penetrate the vessel wall; this is, however, a self-limiting process that does not last long.
Moreover, Ross and Glomset determined, by culturing smooth muscle cells in vitro, that these cells can form non-cellular connective tissue components; in the first place, the formation of collagen, in the second place, elastin, and in the third place, glycosaminoglycans, consisting of dermatan sulphate (60-80%), of chondroitin sulphate (10-20%) and of hyaluronic acid (less than 5%). At a physiological pH, dermatan sulphate strongly binds low density lipoproteins. This is possibly a factor in the accumulation of these lipoproteins in the later developed lesions.
Moore (1979) reviews the relationship between endothelial lesions and development of atherosclerosis. He thinks that there is currently enough evidence to believe that repeated arterial trauma can lead to lipid-containing, atherosclerotic lesions in animals that have low concentrations of lipids in their blood. The causes of repeated endothelial damage can be various (see above). Regarding the accumulation of lipids, Moore strongly underlines further investigations into the role of glycosaminoglycans; these substances, notably dermatan sulphate, might play a central role in lipid accumulation.
Apart from that, lipid deposits can also be explained otherwise.

Stchelkounoff (1936) probably was one of the first to propose, that the smooth muscle cells from the media are responsible for the formation of a neointima. His hypothesis, as described, has obtained a sound basis; also owing to the work of Hassler (1970, 1976), Webster (1974) and Burns (1976), who traced the origin of these cells by means of autoradiographical techniques.
Spaet et al. (1975) found that the neointimal cells were indeed smooth muscle cells derived from the media which, penetrating the lamina elastica interna through fenestrae, covered the vessel wall again. They stated that, after a trauma, the intima surely does not seem to be regenerated by the adjoining endothelial cells from the wall, but by the smooth muscle cells from the media.

Tyson (1976) and Moore (1979) observed atheromatous lesions in man after an endothelial damage. In coronary surgery Moore saw atherosclerotic lipid-rich lesions in interposed vein grafts. The lipid was mainly situated in the intima and, to a lesser extent, in the media of the vessel wall of the graft. Tyson alleged that at autopsies of neonates, in whom an intra-aortic catheter has been introduced to measure blood gasses, lipid-rich atheromatous lesions in the aorta could be observed because of repeated endothelial trauma.
It is of note, that blood cholesterol levels in neonates are very low during the first thirty days of life. Many experiments have been performed to find substances which slow down the aggregation of platelets. Intensive research has been going on for a long time on this subject by authors to mention.
Baumgartner (1979) concluded, in an experimental study with rabbits, that acetylsalicylic acid (ASA), sulfinpyrazone and dipyramidole had the following effects on the adhesion of platelets on endothelial lesions in normal and citrated blood. In citrated blood, the above mentioned substances had a decelerating effect on the growth of the thrombus. The adhesion of platelets to the lesion, however, was not affected.
Rossi (1979) confirmed this for ASA and dipyramidole. The aggregation of platelets, and, subsequently the organizing arterial thrombus can possibly be influenced by ASA. Jaffe and Weksler (1979) alleged that low doses of ASA suppressed prostacyclin production in the endothelial cells. The production of prostacyclin by the endothelial cells recovered very quickly, whereas cyclo-oxygenase, responsible for thromboxane A_2 production in the platelets, is probably eliminated permanently; this might explain the findings of Baumgartner.
Clopath (1980) found a clear decrease in balloon catheter induced atherosclerotic lesions with pigs by giving them 30 mg/kg ASA twice a day. According to this author, ASA has an inhibiting influence on atherosclerosis, induced by endothelial trauma. Many investigators have studied the effect of ASA on arterial thrombosis; it appeared that this substance does indeed influence the formation and the size of an arterial thrombus (Danese et al. - 1971, Barth - 1975 and Meng - 1974, 1977). Sometimes, the results of these experiments were slightly contradictory. With the present knowledge of the functions of platelets and endothelial cells, regarding the production of prostacyclin and thromboxane, the effect of ASA in different doses can apparently be explained well.

By virtue of elaborate investigations done by Moncada and Grygliewski, from the end of 1970 to early in 1980, much value is now set on the presence of prostacyclin and thromboxane.
The synthesis of these substances proceeds as follows:



TXA_2 is, with ADP, thrombin and platelet aggregating factor (P.A.F.) a potent aggregator of platelets. Apart from this, TXA_2 causes a strong vasoconstriction. On the other hand, prostacyclin is a potent inhibitor of platelet aggregation and a strong vasodilatator.
The next step of thought would be to specifically slow down TXA_2 production and to promote the production of prostacyclin, in order to prevent platelet aggregation. Jaffe and Weksler (1979) described the effect of a low dose of ASA, preventing production of TXA_2 in the platelet, as a result of the effect of ASA on cyclooxygenase. This function of the platelet is then eliminated permanently, i.e. for the lifetime of a platelet. In cultures of endothelial cells, the above-mentioned authors showed a fast recuperation of PGI_2 production after the administration of ASA (within 36 hours).
PGI_2 can also be produced by blood cells; Matejka (1980) demonstrated this by means of a dacron prosthesis into which mesenchymal cells could not penetrate. Despite the absence of cells of mesenchymal origin, production of PGI_2 could yet be proven in the cellular ingrowth in this prosthesis (see also Sinzinger - 1978).
The nature of these blood-born cells remains uncertain, but there are indications that monocytes play a role in the production of PGI_2.
Usually, there is an adjusted balance between the production of TXA_2 and PGI_2. If this balance is disturbed (by, for instance, a lesion of the vessel

wall) the production of TXA_2 or PGI_2 will be increased. In the case of a vessel wall lesion, the balance will dip into an increased production of TXA_2. Besides, in case of a haemorrhage, the vasoconstrictor activity of TXA_2 may be useful. If, however, frequent trauma to vessel walls occur, the recuperative character of TXA_2, by high platelet aggregation and vasoconstriction, may be the trigger for arterial thrombosis. As stated above, ASA and dipyramidole diminish the synthesis of TXA_2 and PGI_2. Consequently, they prevent platelet aggregation, as has been described in many experimental studies (Ross - 1976, Meng - 1977, Baumgartner - 1979). Butyl-Imidazol specifically slows down the production of TXA_2, whereas the remaining metabolites are converted into prostacyclin. This substance, however, is not yet applicable to man.

In 1980 Coughlin and co-workers found that β-thromboglobulin, which is secreted by the platelets during degranulation, brings on an increase in the PGI_2 production in the endothelial cell. This could indicate a part of the feedback on the aggregation of platelets. Hope et al. (1979), however, found that human β-thromboglobulin binds to a specific site of bovine aortic endothelial cells and inhibits PGI_2 production.

Eastcott (1980) and Szczeklik et al. (1980) performed clinical studies on PGI_2 with a 40% succes rate in peripheral arterial pathology. Szczeklik also found a salutary effect of PGI_2 with spontaneous angina pectoris.

It must be clearly stated that these studies were not double-blind, so clinical conclusions may not yet be drawn from this result.

I.3 HAEMODYNAMICS

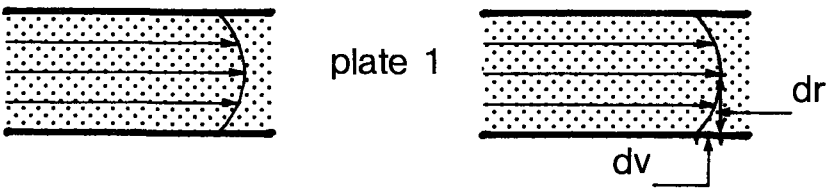
A possible cause of vessel wall lesions.

In recent years much research has been done on the importance of haemodynamics, see review by Nerem and Cornhill (1980). Moreover, the theory of Duguid (1976) about the expansion of the vessel wall is based on haemodynamical changes. To get a better insight into haemodynamical changes, a concise survey of the physics and pathophysiology of haemodynamics will be given, as haemodynamical factors play a role in the development of endothelial lesions on predilection sites (De Boer - 1978).

Physics

Shear stress

While considering a laminar current through a tube, the fluid can be seen to move in layers, parallel to the tube. The layers of fluid closest to the wall have the most friction with the wall, and the fluid front will consequently slow down. The layers in the axis of the tube will move the fastest. The outer layer exercises a decelerating influence on the inner layers, whereas the more axial layers exert an accelerating influence on the outer layers. Thus, a different velocity develops in the various layers. This phenomenon depends on the difference in velocity, the difference in distance between the layer and the vessel wall and the blood viscosity, and is called "shear stress".



Newton developed the following formula:

$$T = \eta \frac{dv}{dr}$$

T = shear stress
η = dynamic viscosity T in dyn/cm^2

shear rate : dv/dr times/sec.

dv - difference in velocity of the layers

dr - difference in distance of the layers to the wall

The greater the difference dv/dr, the greater the shear stress. The dynamic viscosity is mainly dependent on the temperature of a fluid, and not on the pressure. In a Newtonian fluid, there is a linear relationship between the shear stress and dv/dr. As velocity increases, the shear stress will increase proportionately. A fluid in which this correlation does not exist, for instance blood, is called non-Newtonian. A non-Newtonian fluid will have a non-linear correlation between shear stress and dv/dr, as is shown in plate 2.

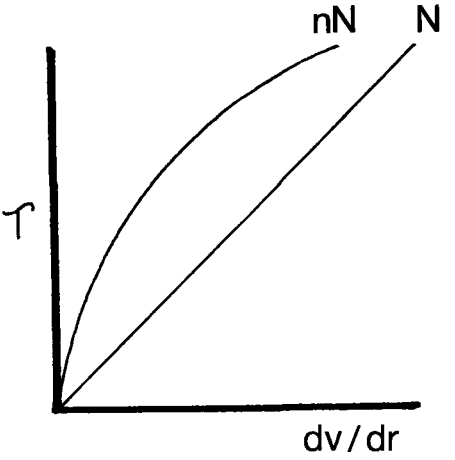


plate 2

With small values of dv/dr in a non-Newtonian fluid, there will be a greater increase in shear stress than in a Newtonian fluid.

The law of Bernoulli

This law refers to the maintenance of kinetic and potential energy of a moving particle in fluid.

$$P_1 + \tfrac{1}{2}\rho v^2_1 + \rho g h_1 = P_2 + \tfrac{1}{2}\rho v^2_2 + \rho g h_2$$

P = pressure energy of a fluid particle
$\tfrac{1}{2}\ v^2$ = kinetic energy of a fluid particle
ρgh = potential energy of a fluid particle
g = gravity
h = height
ρ = density g and h have constant values

To summarize, the law of Bernoulli:
The sum of pressure energy, kinetic energy and potential energy per volume is constant. Hence, the pressure along the streamline decreases as the speed increases. This law is only applicable to a fluid with a viscosity of zero. In the greater part of a streaming field for a Newtonian fluid, the areas of friction are so small that this friction force is of practically no importance. The layer which adjoins the wall, however, should no longer be regarded according Bernoulli's law, because of the great friction forces.

The law of Poiseuille

This law refers to the shape of the fluid front, moving through a smooth tube. Via a number of complicated calculations, which is more the domain of a physicist, the following formula is derived.

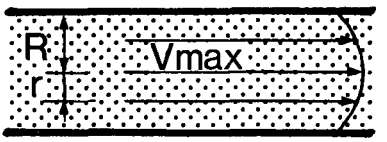
$$V = \frac{(P_1 - P_2)\ (R - r)}{4\ \eta L}$$

V - velocity
P - pressure on two different places L_1 and L_2
R - radius of the tube
r - distance to the axis of the tube
η - viscosity
L - distance between the two places L_1 and L_2

From this formula it follows, that the distribution of velocity over the diameter of the tube is parabolic.
V is maximal in the axis of the tube where r=0.
For r=0, the formula will be as follows:

$$V_{max} = \frac{(P_1 - P_2)\ R}{4\ \eta L}$$ when the velocity in the outermost layer is zero.

With this formula, it can also be calculated that the average velocity is equal to half the maximum velocity in the axis of the tube (plate 3).



parabolic form of the fluid front

$V_{av} = 1/2\ V_{max}$

plate 3

The character of the stream is dependent on the average speed of the stream. If the velocity becomes too high, the individual fluid particles will leave their original layer and will thus disturb the laminar flow; this will cause turbulence.
The laws of Bernoulli and Poiseuille are only applicable to a laminar continuous flow in rigid tubes.

The number of Reynolds

In 1883 Reynolds found a dimensionless number, derived from the average velocity (V), kinematic viscosity (v) and diameter of the tube (d).

$$Re = \frac{V_{av}\ d}{v}$$

Fluids with a Reynolds number below 2300 behave in a laminar way. Fluids with a Reynolds number over 2300 behave in a turbulent way.
Towards the Reynolds number (2300) there is an increase in laminar flow stabilization; above this number turbulence will develop. Blood is a non-Newtonian fluid; the Reynolds number is ± 1800. Blood can be considered as a suspension of rather large particles in a colloidal solution (plasma). Plasma itselfs behaves like a Newtonian fluid. Blood shows an increased viscosity at a lower speed in large vessels. In smaller vessels, however, the viscosity decreases at a lower speed.

The velocity of a stream causing turbulence, is the critical speed for this fluid.

$$V_{crit} = \frac{2300\ v}{d}$$

Tnus, the larger the diameter of the tube, the lower the critical velocity will be. In a laminar flow, molecular energy exchange will take place between the different layers, which expresses itself in a difference in speed and a decline in pressure. In a turbulent flow there will be a greater exchange of molecular energy, involving large volumes of fluid. To maintain a turbulent flow, a greater difference in pressure will be needed than during laminar flow. A pulsatile flow can be both laminar and turbulent. A pulsatile flow, however, is more inclined to turbulence than a non-pulsatile flow with the same number of Reynolds. As a flow in a pulsatile system is dependent on the frequency of the pulsations, the Reynolds number does not apply. Womersley (1957) derived a new formula from Poiseuille's, to obtain a special number for pulsatile flows. It is unnecessary in this context to expatiate on this very complicated formula. To be brief, Womersley's formula states that by increasing the number of pulsations or by increasing the diameter of the tube, the total flow will decrease.

A fluid front will be retarded on entering a tube, according to the law of Poiseuille. The layers closest to the wall will become denser. The central layers of the parabolic fluid front will gain speed in order to displace an equal amount of fluid through the tube. This phenomenon will take place over a certain length of the tube, until stabilization of a parabolic fluid front has been achieved. These changes in speed between the layers at the entrance of a tube are known as "entrance phenomena" (De Boer - 1978).

Convergent and divergent flows

Elevations in the surface of a vessel wall cause turbulence in the flowing blood. In proportion to the ruggedness, and the position of the elevations of the vessel wall, the turbulences will rapidly fade away or will develop into large turbulences.

In the event of a slight convergence, the average speed of the fluid front will increase at the cost of a decline of pressure energy (Law of Bernoulli). At the same time, there will be an increase in pressure gradient. By raising Reynolds number there will be a shift to stabilization of the laminar flow.

In the case of a divergence, there will be a decrease in speed, with a tendency towards turbulence. At the same time there will be an increase in pressure energy and a decrease in pressure gradient.
To make this clear, the law of Bernoulli is used.

$$P_1 + \tfrac{1}{2} \rho v^2_1 + \rho g h_1 = P_2 + \tfrac{1}{2} \rho v^2_2 + \rho g h_2$$

$$P_1 - P_2 + \tfrac{1}{2} \rho v^2_1 - \tfrac{1}{2} \rho v^2_2 + \rho g h_1 - \rho g h_2 = 0$$

$$P_1 - P_2 + \tfrac{1}{2} \rho (v^2_1 - v^2_2) = 0, \text{ when } h_1 = h_2$$

$$P_1 - P_2 = - \tfrac{1}{2} \rho (v^2_1 - v^2_2)$$

$$P_1 - P_2 = \tfrac{1}{2} \rho (v^2_2 - v^2_1)$$

In a convergent area, the difference in velocity ($V_2 - V_1$) will cause an increase in pressure gradient ($P_1 - P_2$). In a divergent flow ($V_2 - V_1$) the pressure gradient will consequently decrease.
In a diverting flow the boundary layers will decrease markedly in speed, so that even a complete stop of the fluid particles or backflow may occur, resulting in local turbulences and a decrease in pressure gradient.

Changes of the flow in case of a local stricture

During a flow through a local stricture, three changes of the flow, with changes of the pressure gradients will take place.

1. changes in pressure at the transition from the wide part to the narrow part
2. changes in pressure in the narrow part itself
3. changes in pressure at the transition from the narrow part to the wide part

Distally of an abrupt stricture, the fluid front will not instantly follow the vessel wall. The front will first follow a smaller lumen than the diameter of the tube and will only return to the wall after a certain length. A gradual narrowing will not cause this phenomenon.
From this, it can be deduced that, in the case of a abrupt narrowing, the velocity of the fluid front increases even more (as expected). When the fluid

front enters a wide part of the tube from a part with a small diameter, the velocity will be too high for the diameter of the tube.
At the transition from a narrow to a wide part, the boundary layers become subject to the pressure gradients of a dead corner (plate 4).

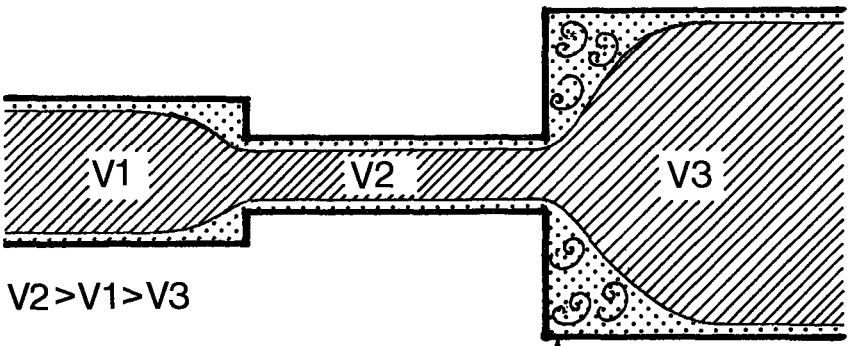


plate 4

When a laminar flow suddenly enters a tube with a wide diameter, coming from one with a narrow diameter, it takes a part of the surrounding fluid particles with it. This causes the pressure between the wall and the entering laminar flow to decrease. At the very entrance of the tube, this effect will be the strongest. A backflow may occur between the entering laminar flow and the wall. When this laminar flow symmetrically enters the wider part, an unstable situation will develop because of the backflow on both sides of the entering stream. When the stream enters the wider part asymmetrically, the laminar flow curves away to the wall, where the pressure is the lowest. This will cause a stable situation; this stabilizing effect is known as "the Coanda effect".

Physiological conclusions based on these physics

The vessel wall consists of non-homogenous visco-elastic material, while the composition of the central part differs from the peripheral parts of the vascular system.
The arteries are connected to the surrounding tissue by the adventitia. Longitudinal and radial movements are small, but visible. The arterial vascular system can be considered as quite a rigid system of tubes. In this sys-

tem, a laminar pulsatile flow of a non-Newtonian fluid is present. The vessel wall is subject to aging, hormonal and neural influences. During the aging process, the wall becomes more rigid. From a haemodynamical point of view, the behaviour of this rigid system is difficult to predict. On top of every systole, however, there is a tendency towards turbulence in the ascending aorta. Due to the high speed of the blood the effective number of Reynolds sometimes reaches more than 10.000. The effective number of Reynolds differs from the basic number, by the pulsating character of the flow. This turbulence does not last long enough to accomplish a total turbulence throughout the entire aorta. Consequently, it is generally assumed, that there is a pulsatile laminar flow in the human arterial system. The perfusion is determined by the pressure gradient and the resistance; not by the pressure itself. The resistance in the vessel is determined by the diameter and the viscosity of the blood. Because of the pulsating character of the bloodflow, the total perfusion is also dependent on the frequency of the pulsations.
Womersley's formula indicates a smaller perfusion proximally in the aorta than would be expected in view of the law of Poiseuille. In the a. femoralis, the flow approximates to the flow according to Poiseuille. Because of the bigger diameter of the aorta, mass and compliance are more important effects than viscosity. The beating of the heart produces a pulse wave in the arterial system, which is subject to a number of factors. For instance, damping and reflection of the pulse waves are important factors. At the site of the bifurcation of the aorta the amplitude of the pressure oscillation is increased by reflection in peripheral direction; peripherally these pressure oscillations are fully damped out.

Pathology

It seems possible to apply haemodynamical laws to physiological and pathological situations in this system, in order to acquire a number of indications which explain the origin of vascular pathology. Segmental narrowings or dilatations and ramifications could serve as examples.
The stenosis in an artery is usually the result of a segmental atherosclerotic process; it is known that the total flow of a vessel will only decrease when about 2/3 of the diameter of the vessel is obliterated, dependent on the

length of this stenosis (plate 6). Whenever the stenosis is less than 2/3 of the diameter of the vessel, flow alterations will occur, causing pathological changes. A small segmental narrowing will divert the flow around this obstacle. Consequently, the velocity of the flow will increase while passing the stenosis. Another effect is the movement of the border layer away from the wall, giving rise to a so-called "dead water zone" distal to the stenosis (plate 5). In this dead water zone, stasis of blood and, consequently, sedimentation can occur. This zone is also prone to develop turbulences because of the interaction between the flowing blood particles and the motionless particles in the dead water zone.

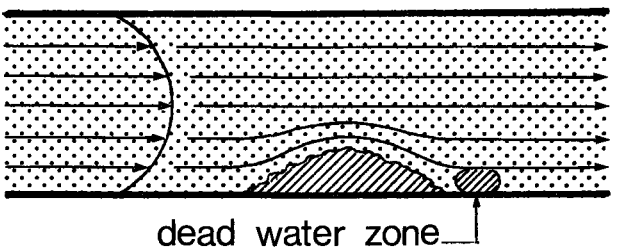


plate 5

As stated earlier, a stenosis not only causes changes in velocity but also a fall in pressure. Once, at an increasing stenosis, the maximum fall in pressure has been reached (determined by the arteriovenous difference in pressure), no further increase in velocity can occur.
At every point in the arterial system, the maximum fall in pressure, appertaining to that place, will require a minimal luminal diameter, which is called "the critical arterial stenosis". If the diameter becomes ever smaller, the flow will suffer seriously, and ischaemic phenomena will develop (plate 6).
Besides, the effect of the morphology of a stenosis in a vessel should be pointed out. If the stenosis is gradual, the laminar flow will follow it; the border layers stay close to the wall. An increase in flow velocity is likely to take place as well as an increase in fall of pressure. If the stenosis is abrupt, a contraction of the laminar flow will occur, the border layers are led away from the wall, thus causing dead water zones directly behind the stenosis (plate 7).

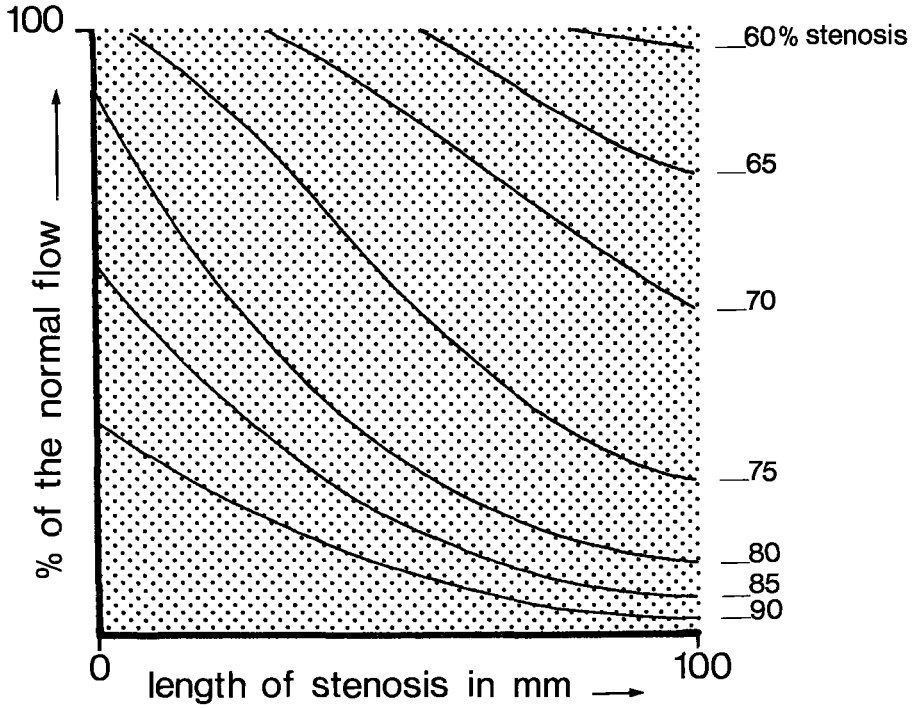
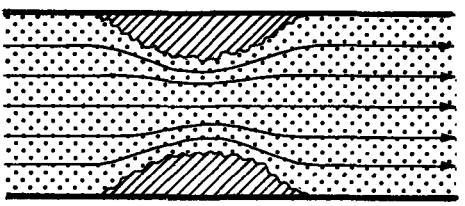


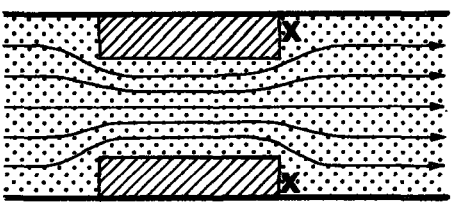
plate 6

If such a stenosis is long enough, the border layers will return to the wall again. This phenomenon is called "contraction" of the laminar flow.
The Coanda effect, reviewed above, can even completely deprive a blood vessel of its supply in the region of bifurcations or trifurcations. These situations are seen in the heart, Circulus Willisii and in the celiac trunk.



gradual stricture

plate 7



abrupt stricture

x = dead water zone

From the literature it is known that changes in shear stress can cause changes in speed of laminar flows, turbulences and dead water zones, changes in the vessel wall and sedimentation (Wesolowski - 1965, Fry - 1968, 1969, Stevens - 1980, Padmanabhan - 1980).
Nerem and Cornhill (1980) described the present state of affairs regarding the importance of haemodynamics in atherogenesis. They especially emphasize the varying shear stress and the frequent appearence of zones with stasis due to contraction of the fluid front, which zones are, in their opinion, predilection sites for vessel wall lesions. Local endothelial lesions are experimentally found at these zones. Platelets will attach to these lesions (Nerem and Cornhill - 1980). Whether platelet aggregates will also be incorporated into the wall, depends (according to French - 1971) on the local fibrinolytic activity and on the amount of change in flow. A prolonged change in flow, indicating a constant stimulus, will have a more serious effect and, therefore, an organizing platelet thrombus may develop. Apposition of a platelet-fibrin mass can be found in the arterial vessel trunk, where a haemodynamical change has taken place (Nerem and Cornhill - 1980).
Fry (1968) described a model with a varying stricture in the thoracic aorta of dogs. By varying the stenosis, he found a crucial shear stress, above which acute endothelial changes took place. These endothelial cell changes consisted of swelling of cytoplasm, deformation and disintegration of cells, and finally degeneration and erosion of the endothelial surface. Fry emphatically stated that these changes are seen in an experimental model. Whether these high shear stresses can develop in human pathophysiology is unknown, but it is likely, considering the many causes that may result in an increased shear stress. In what way the haemodynamical changes influence the vessel wall can not be determined with certainty. Is, apart from the physical trauma, the transport through the vessel wall affected, or are biochemical processes in the vessel wall also influenced? Can tissue anoxia at the site of the haemodynamical change play a role? All these questions still have to be answered.

The dilatation

In the case of a dilatation, the velocity will decrease, resulting in a fall in pressure. Thus, a change in the Reynolds number will occur, dependent on changes in speed and diameter. An abrupt dilatation will have more serious flow dynamical consequences than a gradual dilatation.

In the case of an abrupt dilatation, a zone with stasis may easily develop with sedimentation of erythrocytes, platelets and with formation of fibrin. (A mural thrombosis is often seen in saccular aneurysms.) In the event of a gradual dilatation, however, the dead angle does not develop and consequently, there is little chance of stasis.

Fry (1968) described that the development of a pathological dilatation is due to a change in the elastic qualities of the vessel wall. Once a dilatation is present, it will not easily disappear, not even after elimination of the cause. A stenosis, proximal to a weak spot in the vessel wall, would facilitate a pathological dilatation. Hence, the often observed poststenotic dilatation develops (Holman - 1954, Roach - 1972). The dilatation can be seen as a decompensation of the vessel wall by the pulse reflections, the changes in blood pressure and the so-called "jetstreams" due to stenotic changes. On account of these pathophysiological influences on the vessel wall, permanent changes in the shape of a dilatation can develop. The rise in lateral pressure in the wide part will only enhance this development.

Moreover, other phenomena play a role in the pulsatile laminar flow of human and animal arteries; among other things, the phenomena of pulse reflection.

Two pulses can be in phase with each other. When the reflected flow is in phase with the normal flow, maximum oscillation develops locally. When the reversed flow is out of phase, a minimum oscillation develops.

Kot and Rose (1978) paid much attention to this; especially at the bifurcation of the aorta into the two iliac arteries and at places where strong changes in diameter and the possibility of distension occurs: this could be very significant. Whether these pulse reflections are of importance to the endothelial lesions is not yet clear. There is also the phenomenon of the reversed flow in the aorta and in the femoral arteries. This arrest of flow, or backflow, develops late in the systolic fase.

Goldsmith (1974) states that in the arterial system there is a greater chance of platelet thrombi forming at strictures and bifurcations, due to varying shear stress of the laminar flow pattern and turbulence. He showed experimentally that, in a suspension of albumin and C^{14} serotonin-labelled platelets in vitro, the platelets released more serotonin at shear rates above 2000/sec. This caused an increased tendency towards platelet aggregation. The same effects could be reached when adrenalin, or Ca^{++} in high concentration was added to the suspension.

Once again, this in vitro study points out the importance of physical factors in the development of arterial platelet thrombi, notably the presence of varying shear stresses and turbulences. Moreover, the influence of shear stress on the platelets themselves should not be overlooked. Stevens (1980) showed that an increased shear stress resulted in degranulation of the platelets and an increased synthesis of TXA_2. He notes, however, that the TXA_2 synthesis alone does not seem to play an important role in the aggregation of platelets. The cause of this increased TXA_2 production, is unknown. It is generally assumed that shear stresses over 100-150 dyn/cm^2 have an activating influence on platelets (Born - 1977 and Stevens - 1980).

With the aid of a mathematical model, Padmanabhan (1980) showed, that stenotic arteries with large diameters, produce a greater flow separation, thus producing more atherosclerotic plaques than smaller arteries (Caro - 1971). These calculations were made for pulsatile flows. He also stated, that the presence of branches and the elasticity of the vessel are of great importance in atherosclerotic processes.

It is generally assumed, that high shear stresses cause intimal damage to the arterial wall. By investigating vein interposition grafts (Rittgers - 1978), it appeared, however, that the proliferation of the intima is less in high shear stress areas than in low shear stress areas. These experiments were performed with veins placed at different angles to the artery. No significant correlation could be found between the graft positioning and the degree of intimal proliferation. There was, however, a weak inverse correlation between fluid shear rates and the corresponding degree of intimal proliferation; the greatest proliferation occurred in the region, experiencing the lowest shear stresses. Therefore, proliferation of the intima in an arterialized vein is the crucial point at stake. From a flow technical point of view the shear stress along the wall of a vein must be high.

Matsuda et al. (1978) described the development of a thickening of the arterial wall, as a result of changing haemodynamical factors in rats. They, too, indicated that bifurcations, branches and strictures are predilection sites for the development of atherosclerotic plaques in man and in animal. The pressure-related hypothesis (Texon - 1957, Fox - 1966), the shear stress hypothesis (Fry - 1969, Caro - 1971), the turbulence hypothesis (Wesolowski - 1965) and the wall stress hypothesis are specifically emphasized.

Matsuda et al. (1978) made a kind of semi-circular bypass model in the carotid artery of the rat. They found wall thickening in the regions of bifurcations and in the concave parts of the bypass; these parts corresponded with the low shear stresses in the flow pattern. They saw intima and media thickening, notably in the poststenotic areas, where much turbulence was observed. The arrangement of elastic fibres differed pre and poststenotically. According to the authors, the poststenotic thickening of the wall possibly had another cause than a thickening at bifurcations or ramifications. The number of Reynolds or Womersley is much lower in the rat, so that extrapolation to man can not automatically be made. Here, too, an indication is found for the possibly important role of haemodynamical factors in the development of arterial thrombosis or atherosclerosis.

Turrito and Baumgartner (1979) investigated platelet interaction with subendothelium, dependent on the shear rate of the flowing blood. They concluded, that with shear rates below 200/sec., no thrombi (platelet-fibrin) were formed on the subendothelium. Between shear rates of 200 and 1000/sec. transient thrombi were found. Above 1000/sec., the formation of thrombi increased with the shear rate up to 10.000/sec., and the thrombi may become irreversible in some experimental animals. According to these authors, different experimental animals have a different sensitivity to thrombus formation or degradation, which is only apparent at high shear rates.

Rieger (1980) investigated platelet aggregations in vitro with and without ADP stimulation; he found an increased and spontaneous aggregation up to shear rates of 35/sec., and a decreasing aggregation towards 460/sec. With low chemical stimulation (ADP 10^{-7} M), increased platelet aggregation with the same shear rates could be seen. With high stimulation (ADP 10^{-3} M), however, platelet aggregation not only increased, but remained at a high level. Rieger concluded that platelet aggregation is not only dependent on chemical stimulation but also on local shear rates.

Texon (1976) describes why a bifurcation is a predilection site for atherosclerosis. By applying the haemodynamical laws, he indicates that areas with low pressures, on account of a change in flow, are predilection sites for the development of atherosclerotic lesions. When a fluid front enters a bifurcation, the pressure is divided into the two legs. In spite of the higher velocity, the developing pressure on the median side is lower than on the lateral side of the vessel. These pressure proportions are dependent on the

bifurcation angle. If the bifurcation becomes more acute-angled, the pressure on the median wall will become less, compared to the lateral wall. Owing to the low wall pressure, the possibility of atherosclerosis becomes greater. If the angle becomes less acute, the pressure on the median wall will increase, followed by a decreased tendency towards formation of atherosclerotic lesions.

Summary

From this survey of the literature, two main points arise about the theories of arterial thrombosis development and their relation with arteriosclerosis.

Haemodynamics, modestly used by Duguid, are of logical importance.

In recent years it has also become clear that platelets also have an important, if not crucial, role in the healing of vessel wall damage and, consequently, also in the development of arteriosclerotic aberrations, which might follow the repeated trauma of the vessel wall. It is known that any endothelial lesion is followed by adhesion of platelets or microthrombus development, dependent on the depth of the lesion (Bondjers - 1970, Jørgensen - 1972, Ross and Glomset - 1976, Friedman - 1977).

Subsequently, migration and proliferation of arterial smooth muscle cells occur, leading to repair. This activity of smooth muscle cells is started by still partly unknown stimuli, about which many speculations are being made.

On the other hand, an uncontrolled healing process of the vessel wall takes place in the case of repeated surface damage, or of a very deep and extended lesion. As for the mechanical trauma, haemodynamics are important.

Undeniably it has been shown (Texon - 1957, 1976) that varying shear stresses, turbulences and dead water zones not only have a noxious impact on the vessel wall, but also promote the aggregation and the tendency of platelets towards adhesion (Born - 1977, Baumgartner - 1979, Stevens - 1980). Platelets and haemodynamics, therefore, seem to occupy a central place in the development of arteriosclerosis.

1.4

Objectives of investigation

- Is it possible to produce arterial thrombosis in rat aorta by changing the arterial wall, the blood flow and the composition of the blood?
- Is it possible to give a summarized, detailed description of the development and organization of this arterial thrombosis?
- Is such an organizing arterial thrombus comparable to arteriosclerotic lesions found in man?

In order to be able to answer these questions, a model of arterial thrombosis in the aorta of rats was developed. Comparative investigations on human atherosclerotic lesions were performed as well.

CHAPTER II

ANIMAL MODELS OF ARTERIAL THROMBOSIS

Based on the theoretical considerations in chapter I and the literature cited, a pilot study to induce arterial thrombosis was started. The purpose of this study was to develop an experimental model for arterial thrombosis by inducing a stable platelet thrombus. This thrombus had to be induced in a perfused artery, without the use of any exogenous material, under disturbed haemodynamical conditions.

II.1
Choice of the experimental animal

The rat was chosen as the experimental animal. The reason for this choice was the resemblance of the thromboplastic and fibrinolytic characteristics of the rat to those of man, as described by for instance Astrup and Coccheri (1962). In addition, rat platelets behave similarly to human platelets, although their number is about three times as high. Other advantages are the easy way of handling these animals and their low maintenance costs. The fact that there is no need to work under strictly sterile conditions is another time and cost-saving factor. Microsurgical manipulations of the vascular system on this level could be regarded as a potential disadvantage of using the rat as an experimental animal; this did, however, not offer many problems. Another disadvantage concerning the rat is the small size of the vessels, necessitating meticulous techniques in preparing the histological slides.

II.2
History of experimental models for arterial thrombosis

As mentioned in chapter I, the interest in thrombosis goes back to the seventeenth century. In 1676 Wisemann published one of the first descriptions of thrombogenesis.

Hanson (1772) applied two ligatures to the vena jugularis of a dog and observed the blood remaining fluid for three hours; this was the first known attempt to cause thrombosis in vivo.
In the first half of the twentieth century, the experimental studies on vascular disease in a model system, were mainly focussed on lipid infiltration; Anitschkow (1913), Aschoff (1914) and Leary (1934) represent this tendency.
After the second world war, a variety of methods were developed to induce arterial thrombosis in vivo. Most investigators, however, used exogenous material to damage the vessel wall or introduced foreign surfaces, in order to initiate thrombogenesis. It is difficult to judge whether these thrombi represent human thrombi, and can thus be used for experimental studies. The genesis, and subsequent development of these arterial thrombi, will presumably be influenced by these exogenous factors, in such a way that no thrombi, comparable to human thrombi, will be induced. Many methods have been developed and subsequently refined in order to obtain the best possible resemblance to human athero- and arteriosclerosis and arterial thrombosis.
Excellent reviews of methods to induce arterial thrombosis have been published by Henry (1962), Johnson (1965), Didesheim (1972) and Wessler and Yin (1973).
The following methods have been widely used:

- Freezing of the vessel (Meng - 1977).
- Application of an electrical current (Reber - 1966, Duval - 1970, Hladovec - 1971, Potvliege - 1976, Bourgain - 1974).
- The injection into the vessel of damaging etching compounds, such as formaldehyde (Hassler - 1976) and sulfuric acid (Deaton - 1960).
- The injection of thrombin into the vessel, combined with clamping of the vessel (Deaton - 1960).
- The introduction of metal wires and plastic surfaces (Friedman and Byers - 1961).
- The injection of fibrin into a clamped vessel segment (Chakravarti - 1975, Olson - 1975).
- The construction of an extracorporeal loop (Hornstra - 1973, Umetsu - 1978).
- Arteriovenous shunts (Hovig - 1970, Benis et al. - 1975).
- Air drying of the intima (Fishman - 1975, Clowes - 1977).

- Mechanical trauma and clamping (Williams - 1955, Johnson - 1966, De Palma - 1977, Gertz - 1979).
- Diet-induced thrombi (Thomas and Hartcroft - 1959, Wexler - 1967).
- Balloon catheterization or mechanically induced superficial intimal damage (Baumgartner - 1966, Woolf - 1968, Danese - 1971, Ross -1971).
- Ligation of the vessel (Jørgensen - 1964).
- Vascular incisions and sutures, anastomoses or prostheses (Huggins - 1960, Engler - 1961, Inokuchi - 1961, Wilkinson - 1979).
- Obstructive changes in the vessel wall (Constantine - 1972, Rosenbaum - 1977).

All these methods have proved to produce arterial thrombosis, and have provided valuable data on thrombus development. These methods should not be discarded as irrelevant. Arterial thrombosis, however, should be produced resembling the natural human formation as closely as possible, which makes the use of exogenous material undesirable. In 1972 Constantine described a method which appeared to be a very natural one. In dogs, he inverted a side branch of the carotid artery into the vessel itself. This method met the conditions described above. A platelet-fibrin thrombus with a high reproducability is formed on this intra-luminal inverted branch. To perform large series of various time intervals, however, the use of dogs is unsuitable for obvious reasons. A pilot study was therefore started, using the rat as experimental animal. Because of its anatomy, it proved impossible to perform this method on the carotid artery of the rat. The inversion of a side branch of the a. iliaca communis proved to be possible, but technically difficult. Besides, the small size of the vessel offered too many problems in histotechnology and the results were difficult to interpret.
Another possibility would be to invert a side branch of the aorta between the a. renalis dextra and the bifurcation, although the anatomy of the aorta and its side branches is variable. This approach is microsurgically possible, but it is much more difficult than the procedure described below.
The changes introduced by inverting a side branch can be summarized as follows. The side branch, protruding into the lumen, changes the laminar bloodflow and exposes tissue thromboplastins from the media and the adventitia to the blood.

Taking this as a starting-point, it should be possible to produce these changes in a more simpler way. In pilot experiments, a small opening was made in the aorta, between the a. renalis and the bifurcation, and subsequently closed by a purse-string suture. By this manoeuvre, the perforated part of the vessel wall was brought into the lumen. This, however, proved to be an insufficient stimulus for producing arterial thrombosis. Better results, up to 100% thrombosis, could be obtained by inserting a small strip of the vessel wall into the vascular lumen, consisting of intima, media and part of the adventitia. This inversion was made in the above-mentioned aortic area. An attempt to perform this method in the carotid artery failed. For the final series, the inversion of a strip of the aortic wall, a so called "aortic flap", was chosen.

This microsurgical procedure resulted in:

1. a slight stenosis of the aorta
2. the introduction of a non-occluding obstruction in a perfused vessel
3. the exposure of tissue thromboplastins without the use of exogenous material, except for the presence of non-reactive suture material

Point 1. and 2. are important because of their haemodynamical effects.

This surgical procedure might also serve as a model for badly constructed vascular anastomoses. For that reason, everting and inverting aortic anastomosis were performed in separate series of experiments in order to compare the results of these procedures with those of the aortic flap procedures.

CHAPTER III

MATERIAL AND METHODS

III.1

Microsurgical method for inducing an arterial thrombus in rat aorta

Male Wistar rats (Centraal Proefdierbedrijf T.N.O., Zeist), weighing between 250-290 gram, were used. They were kept in an artificial twelve hour day-night rythm, food pellets and tap water were provided ad lib.

All surgical procedures were performed under general anaesthesia, induced by an intraperitoneal injection of Nembutal^{R} (Abbott), at a dose of 60 mg/kg bodyweight. The animals were breathing spontaneously. During the surgical procedure, no additional medication was given.

After induction of anaesthesia, the ventral skin of the animals was shaven from the os pubis to the ribcage, and the animals were fixed on the operating table. After moisturing the skin with saline, a midline laparotomy was made. The skin, the musculature and the peritoneum of the abdominal wall were cut from the processus xyphoideus to the os pubis. Stomach and small intestines, together with the colon ascendens and transversum were taken out of the abdominal cavity, and kept in a moist environment. The sigmoid and the distal part of the colon descendens were left in situ. After applying two spatulas to the split parts of the abdominal wall, a good access was obtained to the retroperitoneal area, which was opened by blunt dissection. With this manoeuvre, the parietal peritoneum is moved to two sides, together with the retroperitoneal fat. The large vessels (aorta and vena cava) were now clearly in view, from the renal arteries to the aortic bifurcation; this area was freed from surrounding tissue.

During the following steps of the procedure, an operation microscope (Zeiss Opmi I), at a tenfold magnification was used. Normal microsurgical instruments were used, for example a needle holder, dissecting scissors and simple watchmaker forceps, adapted by the surgeon. As suture material 10/0 Dermalon with a C4 needle (Davis and Geck) was applied.

The vessel was dissected from the vena cava and from surrounding tissue. Using two fine sharp forceps, it proved possible to perform this dissection

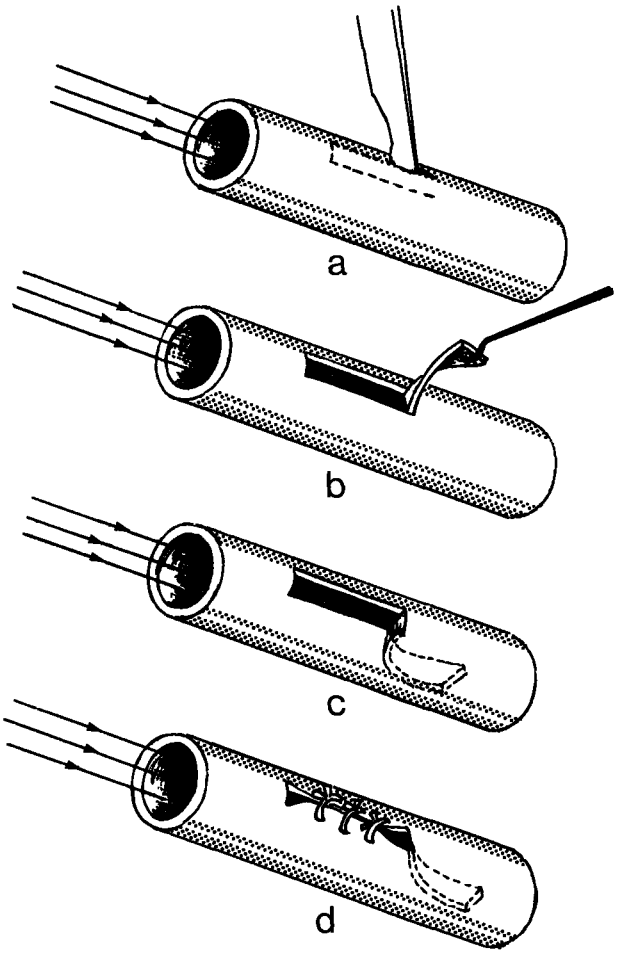


plate 8

DIAGRAM OF THE AORTIC FLAP PROCEDURE

very accurately, without loosing any blood. The aortic wall should not be touched directly, but only handled by grasping the adventitia. The aorta was freed from the level of the aa. renales to its bifurcation. This can be dangerous because, in the bifurcation area, the vena cava runs very close to the aorta; particularly in this area, which is difficult to survey, bleeding can occur. Sometimes, when there was not enough space to perform the subsequent steps, it proved necessary to ligate a side branch. Once the aorta had been separated, a large part of the adventitia was also removed by dissection. Two atraumatic vascular clamps (Aesculap microclips), at a pressure of 20-30 grams/mm^2, were placed on the aorta at a spacing of 5 mm. After the flow in the aorta had stopped, the vessel was opened by an arteriotomy between two clamps, from proximal to distal with dissecting scissors (plate 8a). The arteriotomy should be about 2 mm long. In order to remove all blood, the isolated part of the aorta was flushed with saline.

Then a second incision was made, parallel to the first one, at a distance of about 1 mm. The vessel wall was cut between the two incisions at the proximal side, thus creating a free piece of the aortic wall, measuring 1 x 2 mm (plate 8b). This part, distally still continuous with the wall, consisted of intima, media and a little adventitial tissue.

This free section of the wall -the so-called "aortic flap"- was brought into the lumen of the aorta (plate 8c). After flushing, the arteriotomy was closed in an everting way with 5-6 stitches, without causing too much narrowing of the vessel diameter. The flap is then floating freely in the lumen and will be pushed distally by the blood flow (plate 8d).

At first, the distal clamp was removed; the backflow from the distal aorta should be sufficient to cause some backbleeding. Subsequently, the proximal clamp was removed and the circulation restored. A surgical bleeding must be closed with another stitch; the arteriotomy should be tight in 20-30 seconds. By observing the colour of the hind legs and toes, it is possible to judge the patency of the aorta before closing the wound. A complete obstruction of the aorta is considered a failure. This, however, was never observed. A slight narrowing of the aorta was always present. After repositioning the gut, the abdominal wall (musculature, peritoneum and skin) was closed.

The sham operation

In this operation the surgical procedure is the same, except for the fact that no arteriotomy is made. The microvascular atraumatic clamps were left in place for about 5-10 minutes, removed and the abdominal wall closed.

To perform this microsurgical "flap" procedure, some practice and experience in microsurgery is advisable. In experienced hands, however, the whole procedure takes about 20-25 minutes, including the induction of anaesthesia.

III.2
Microsurgical procedure for producing everting and inverting aortic anastomoses in rat

After performing the same steps as in the aortic "flap" procedure up to the dissection of the aorta, a special frame with two atraumatic movable vessel clamps is placed on the aorta. This frame is necessary to approximate the two ends of the severed aorta. When the aorta has been cut, both ends tend to retract because of the elasticity of the aortic wall. These cut ends of the aorta were rinsed with saline.
Two groups of animals have been operated upon to produce aortic anastomoses: one group with an everting anastomosis (the proper way) and one group with an inverting anastomosis (the wrong method for vascular anastomosis).

The everting anastomosis (Acland - 1980)
An everting anastomosis was made after the method of Carrel (1902). First, two stay sutures at 120° are placed. These two sutures determine the quality of the anastomosis. By pulling the two stay sutures and applying a slight longitudinal traction, the cut ends of the aorta tend to evert. At first, the front part between the two stay sutures was closed. The frame with the aorta was then turned upside down, and the back part of the aorta was closed. Care must be taken not to pick up the back wall in suturing the front wall, and vice versa. A running suture is not advisable because of the danger of narrowing the anastomotic line.

In making an everting anastomosis, the intima on both cut ends should be put together in such a way that no narrowing is present after completion of the anastomosis. For that purpose, the suture should be placed from the outside to the inside, and from the inside to the outside again. The suture is fixed with three non-slip knots. In this way, only the intima is in contact with the blood. Contact of the adventitia with the blood should be avoided.

The inverting anastomosis

In an inverting anastomosis, the suture was placed from the inside to the outside and from the outside to the inside, whereby a firm narrowing cannot be avoided; all vessel wall elements (intima, media and adventitia) are in contact with the flowing blood. After completion of the anastomosis and obtaining haemostasis, the abdomen was closed in the same way as in the "flap" procedure.

Patency control was done by observing the colour of the hind legs of the animal. To perform an aortic anastomosis in a rat takes, after some practice, about 45 minutes. Postoperative control of the rats was performed by observing their clinical status. Whenever there is a major vascular complication, this will result in paralysis of the hind legs. After one week, the skin agraves of the animals were removed.

III.3

Protocolling and protocol sheet

For every animal a protocol sheet (page 43) was used, containing all surgical data, weight, anaesthesia and the results after sacrificing the animals.

III.4

Method of sacrifice

All animals were sacrificed under general Nembutal^{R} anaesthesia (60 mg/kg bodyweight). The abdomen was re-opened, and the aorta exposed as described. After freeing the aorta from surrounding tissue, the left heart was punctured under direct vision after opening the thorax. The aorta was cut below its bifurcation, and perfused with saline at a pressure of 130 cm H_2O until the fluid, coming out of the cut aa. iliacae, became clear. The part of the aorta with the lesion, was taken out and processed as will be described in the next chapter.

PROTOCOL SHEET

Date: Time: Surgeon:

Rat: m/f Anaesthesia: cc Nembutal^{R}:

Kind of operation:

aorta
- flap
- everting anastomosis
- inverting anastomosis
- sham

Remarks:
- loss of blood
- respiration
- colour of the hind legs
- postoper. conscience
- suture material

Duration of surgical procedure:

Time interval between surgery and sacrifice:

Date of sacrifice:

Remarks at preparation:

Fixation: Method: Remarks:

Summary of results:

Staining:

Frozen sections:

III.5

Histological and histochemical methods

Fixation and embedding

Histology

After perfusion with saline at a pressure of 130 cm H_2O, the aortas were fixed by perfusion with 4% p-formaldehyde in 0.1 M phosphate buffer (pH= 7.4) at the same pressure. The fixative was always freshly prepared from polymerized p-formaldehyde (Merck, Darmstadt, Germany). After perfusion fixation, the tissues were post fixed for at least 24 hours in the same fixative, and embedded in paraplast.

Immunofluorescence

After perfusion with saline as described, the aortas were excised, frozen at -90^{o} C, and kept in air-tight containers until use.

Transmission electron microscopy

For ultrastructural studies, the aortas were fixed, after perfusion with saline, with 1.5% glutardialdehyde (Fluka, Buchs, Switserland) in 0.067 M cacodylate buffer (pH=7.4), containing 1% sucrose. Tissue was post fixed in 1% osmium-tetroxide in 0.1 M phosphate buffer (pH=7.4) and embedded in Epon.

Scanning electron microscopy

The specimens were perfused with glutardialdehyde and rinsed with a cacodylate buffer solution, followed by dehydration in ethanol series of 20-50-70-80-90-100%.

The specimens underwent critical point drying with CO_2, and were sputter coated with gold.

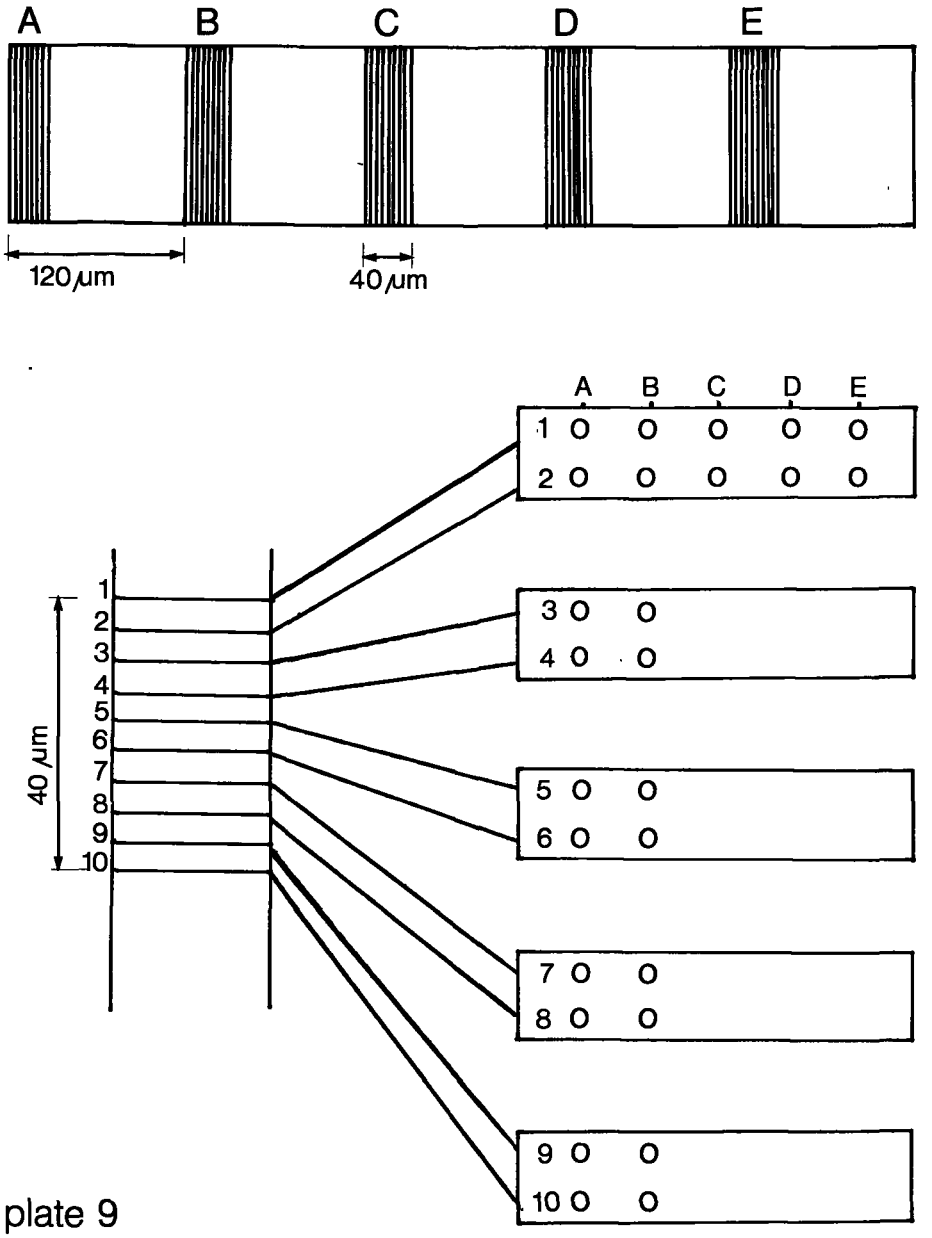


plate 9

diagram of the sectioning procedure

III.6

Sectioning

Paraplast-embedded tissue

The aortas were positioned in such a way that cross-sections of the vessel could be obtained, starting from the distal end of the vessel segment. The entire vessel segment was sampled in the following way.

Ten serial sections of 4 um thickness were obtained for histological or (immuno) histochemical analysis. These ten sections were mounted pairwise on five slides. Next, eight sections with a thickness of 10 um were cut and discarded, etc. After five cycles, the whole procedure was resumed with a different set of slides.

Using this procedure, on every slide, five sets of sections, obtained at intervals of 120 um, are available, while five slides are available for analysis with various staining techniques.

Frozen sections

Frozen tissue was sampled identically, except that the ten serial sections had a thickness of 7 um.

III.7

Staining procedures

- LMSB - Lawson-Martius-scarlet-blue staining:
  this staining procedure (Lindeman - 1976) is a combination of Lawson's elastica staining (1936) with the MSB-staining according to Lendrum (1962).
- Levanol Fast Cyanine-staining:
  performed according to Van Pelt-Verkuil (1982 - submitted).
- Collagen-staining:
  by the acid fuchsin-picrin procedure with Hansen's iron oxyhaemateine counterstaining (Romeis - 1968).

- Elastin-staining:
  the Weigert staining method was used.
- Frozen sections were stained with the Lawson MSB procedure.
- The Todd slide technique for the determination of fibrinolytic activity was performed after Noordhoek-Hegt (1976).

III.8
Antisera

Anti-rat fibrin monomer antiserum

Rat fibrinogen was purified from rat plasma as described by Van Ruyven-Vermeer and Nieuwenhuizen (1978). Rat fibrin monomer was prepared from this fibrinogen according to Haverkate and Timan (1976). Rabbits were immunized with fibrin monomer as described (Nieuwenhuizen et al., 1977). Specifity was tested by immuno-electrophoresis and double immunodiffusion against rat plasma, serum, fibrinogen and fibrin degradation products, and by using an enzyme-linked immunoabsorbent assay (Emeis et al. - 1981). The antiserum was specific for fibrin-related antigens, and did not react with rat serum proteins.

Anti-rat platelet antiserum

Rat platelets were obtained from EDTA-anticoagulated rat blood by differential centrifugation. The platelet-rich pellet (free of leucocytes) was washed ten times with phosphate-buffered saline, resuspended in distilled water, and repeatedly frozen and thawed. The resulting platelet lysate (protein content 1 mg/ml) was emulsified in Freund's complete adjuvans, and used for producing an antiserum in rabbits. The antiserum obtained, proved to be contaminated with antibodies against fibrinogen, and (weakly) against some serum proteins, as found by immuno-electrophoresis. These contaminating antibodies were absorbed by solid-phase absorbtion, using platelet-free rat plasma cross-linked with glutaraldehyde as immunoabsorbent, according to Avrameas and Ternynck (1969). After absorbtion, the antiserum did not show any reaction with rat plasma or serum when tested by immuno-electrophoresis or double immunodiffusion. The reactivity towards cellular blood components was tested with indirect immunofluorescence on smears of platelet-rich plasma (containing

leucocytes), and on frozen sections of rat lung with ADP-induced platelet aggregates.

Anti-smooth muscle cell antiserum

Human auto-immune serum against smooth muscle cells was kindly provided by Dr. J.L. Molenaar (Dept. of Clinical Immunology, S.S.D.Z., Delft).
Antiserum specifity for smooth muscle cells was tested by indirect immunofluorescence on frozen sections of rat stomach, liver and aorta.

Anti-factor-VIII antiserum

Rabbit anti-human factor VIII-related antigen was obtained from the Central Laboratory of the Netherlands Red Cross (Amsterdam).
The specifity of the antiserum was tested as described by Van Pelt-Verkuil and Emeis (1981). No antibodies against fibrinogen, fibronectin or other serum proteins were detected.

Rabbit anti-human lysozyme, rabbit-anti-human Ig (FITC-conjugated), and swine-anti-rabbit IgG (FITC-conjugated) were purchased from Dako (Copenhagen, Denmark).

Goat-anti-rabbit IgG (peroxidase-conjugated) was obtained from Nordic (Tilburg).

3,3'-diaminobenzidine. 4 HCl (DAB) and hydrogen-peroxide were obtained from Merck (Darmstadt, Germany).

III.9
Immunohistochemical procedures

Paraplast-embedded sections

In order to demonstrate the presence of fibrin, an indirect immunoperoxidase method was used on paraplast-embedded sections. Rabbit-anti-rat fibrin monomer antiserum (diluted 1:100 in 12% normal goat serum) was used in the first step of the procedure, followed by peroxidase-conjugated goat-anti-rabbit IgG antiserum (diluted 1:50 in PBS) in the second step. The tissue-

bound peroxidase was visualized with DAB and hydrogen peroxidase according to Graham and Karnovsky (1966). Incubated sections were counterstained with haematoxylin. For further details see Craane et al. (1978) and Emeis et al. (1981).

Frozen sections

Indirect immunofluorescence was performed on unfixed frozen sections, using a 1:40 diluted FITC-conjugated antiserum in the second step. Sections were studied using a Leitz Orthoplan microscope equipped with incident light fluorescence optics. Micrographs were made on 27 Din black and white films. The antisera used in the first step, are detailed in Table III.1.

III.10

Human material

Human vascular specimens were obtained during peripheral vascular surgery at the St. Hippolytus Hospital and the Oude en Nieuwe Gasthuis (Dr. J. Maier) in Delft.

These specimens were sent to the Department of Pathology (S.S.D.Z. Delft, Dr. J. Lindeman) for macroscopical investigation, and were subsequently divided into three parts. One part was frozen at -90° C, one part was fixed in 96% ethanol and one part fixed in 4% formaldehyde. These latter specimens were embedded in paraplast, stained with haemotoxylin-eosin and investigated by Dr. J. Lindeman.
Other sections of these specimens were stained as mentioned above for rat aorta.
Thirty specimens of coronary arteries were routinely investigated with three staining methods, i.e. LMSB, Levanol and immunoperoxidase staining for fibrin. These coronary arteries were obtained from routinely performed autopsies (Dept. of Pathology, S.S.D.Z. Delft).

TABLE III.1

ANTISERA USED IN IMMUNOFLUORESCENCE STUDIES

Antigen	Primary antiserum	Secondary antiserum
Fibrin	Rabbit-anti-rat fibrin monomer (1:100)	Goat-anti-rabbit IgG (FITC-conj.) (1:40)
F VIII:RAG	Rabbit-anti-human F VIII:RAG (1:60)	id
Platelets	Rabbit-anti-rat platelet (1:80)	id
Lysozyme	Rabbit-anti-human lysozyme (1:20)	id
Smooth muscle cells	Human-anti-smooth muscle cell (1:20)	Swine-anti-human Ig (FITC-conj.) (1:40)
Control	Pre-immune rabbit serum (1:20)	Goat-anti-rabbit IgG (FITC-conj.) (1:40)

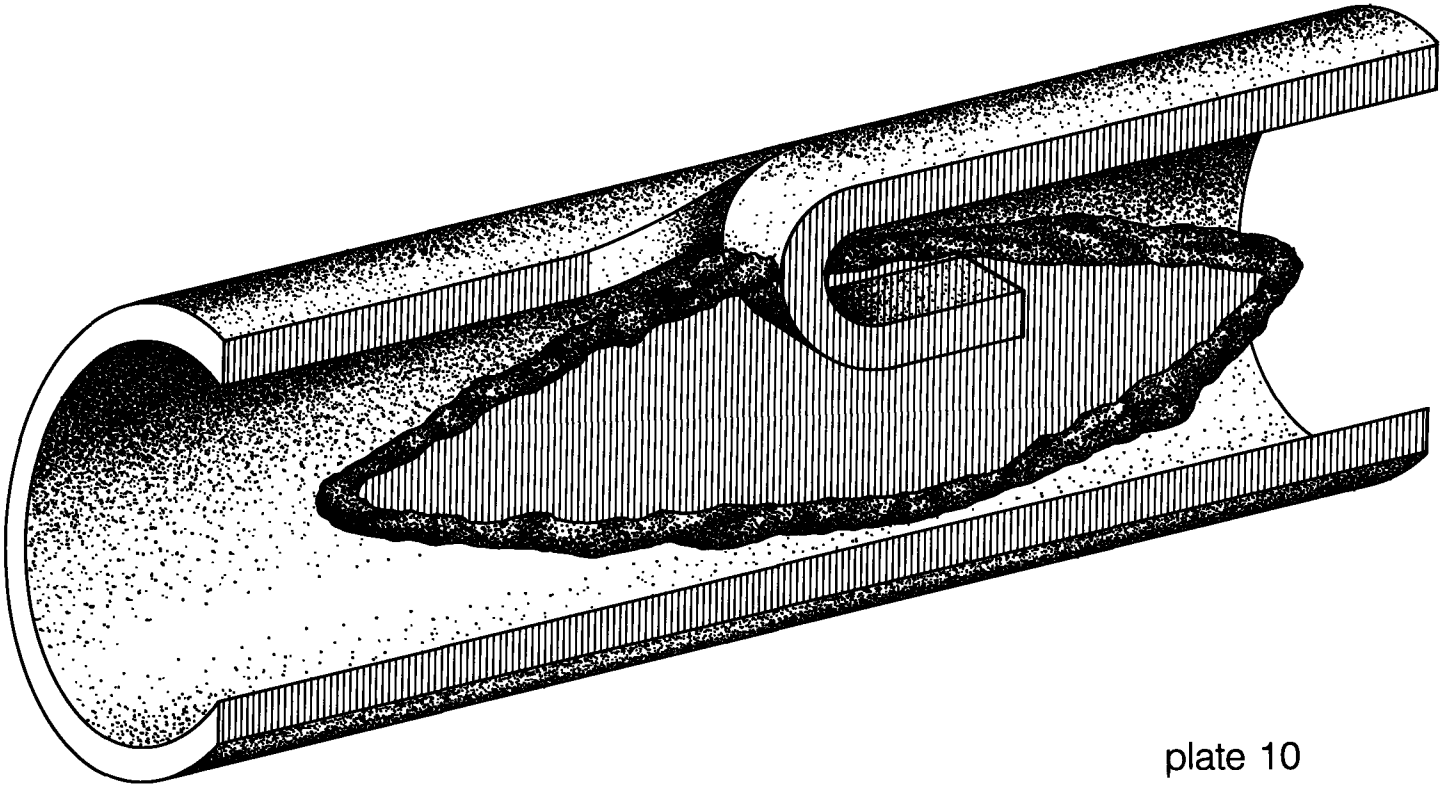


plate 10

diagram of a developing arterial thrombosis after aortic flap construction

CHAPTER IV

RESULTS

IV.1

Macroscopical results of flap procedures

A

Experimental animals

An aortic flap was inverted into the lumen of the aorta of one hundred rats. The number of rats sacrificed at the various time intervals, after operation, is shown in Table IV.1. This table also shows the number of rats used for the various morphological methods. Out of 100 rats, 66 were used for histological study, using paraplast sections, 16 for immunohistochemical purposes using frozen sections, 13 for transmission electron microscopy, and 4 for scanning electron microscopy. One animal died before the intended time of sacrifice.

B

Macroscopical observations

None of the animals died during surgery (Table IV.2). One animal, intended for sacrifice at 4 days, died on the day following surgery. At autopsy the aorta arteriotomy did not show any bleeding. No signs of infection or sepsis were seen; the cause of death could not be ascertained.

All the other animals behaved normally; no signs of aortic occlusion, such as paralysis of the hind legs, or necrosis of the tail were ever observed. At sacrifice, nearly all aortas appeared normal, or showed only a slight stenosis in the surgical area. In 5 rats (see Table IV.2), a stenosis of the aorta and/or extensive collateral formation was seen.

Rat no. 266 (1 hour) had a totally occluded aorta, but did not show any pathological effects.

Rat no. 268 (2 hours) had a poorly perfused aorta, without any signs of ischaemia of the legs or tail.

Rat no. 180 (1 day) showed a patent aorta, but also extensive collateral formation.
Rat no. 194 (2 days) showed a blue colouring of the toes, but no clear-cut obstruction of the aorta. No collateral formation was observed.
Rat no. 129 (3 months) had an extensive stenosis without collateral formation. (At microscopy a recanalized occluding thrombus was found.)
A false aneurysm was found once, as well as one true aneurysm.
Macroscopically, no infections were seen, although the rats were not operated upon under strictly sterile conditions. Microscopically, one animal proved to have an infection around the aorta, near the surgical area.
Necrosis of the gut or kidneys did not occur. No thrombi were seen in the vena cava or renal vessels.
In the sham-operated animals no complications were encountered.

TABLE IV-I

PURPOSE OF INVESTIGATION

Time interval between surgery & sacrifice	Total number of rats	Histology	Immuno-histochem.	Transmission E.M.	Scanning E.M.
10 months	3	2	0	1	-
3 months	6	4	1	1	-
2 months	9	6	2	1	-
1 month	6	5	1	-	-
3 weeks	6	4	2	-	-
2 weeks	6	4	1	1	-
6 days	6	4	1	1	-
4 days	7	4	1	1	-
3 days	8	4	2	1	1
2 days	6	5	1	-	-
1 day	7	6	-	1	-
12 hours	6	4	1	1	-
6 hours	6	3	1	2	-
3 hours	4	3	-	-	1
2 hours	6	4	1	1	-
1 hour	7	4	1	1	-
0 hour	1	-	-	-	1

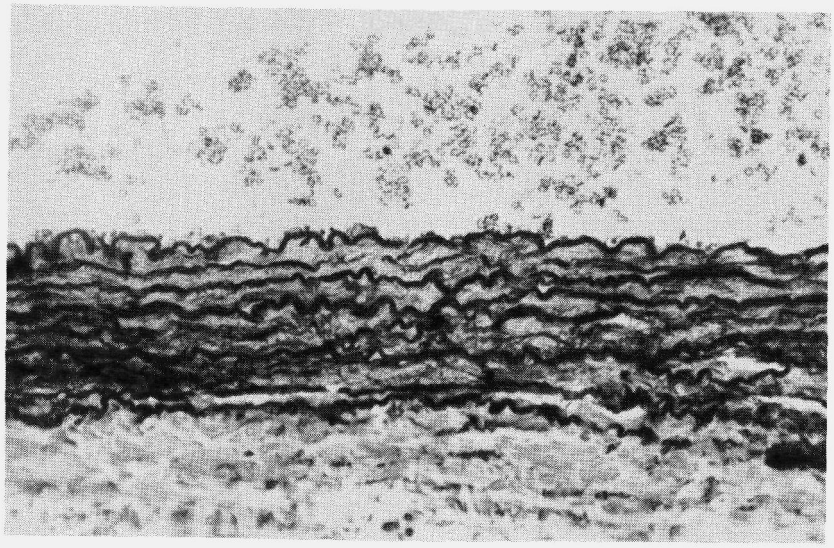
At every time interval two sham operations were performed.

TABLE IV.2

MACROSCOPICAL OBSERVATIONS

	Number of animals
Aortic flaps produced	100
Mortality during surgery	0
Mortality after sugery	1
Severe stenosis or occlusion	4
Formation of collaterals	1
False aneurysm	1
Aneurysm	1
No complications	92

Following the sham procedure, no complications were seen (34 animals).



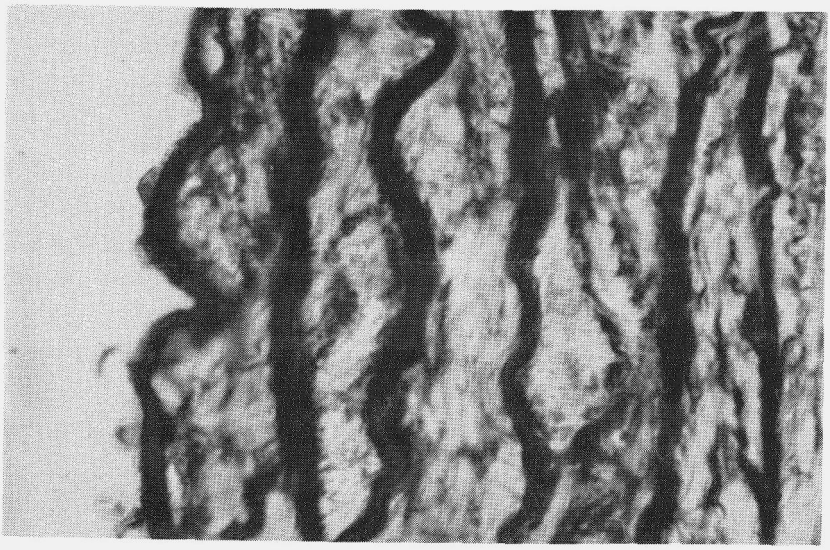
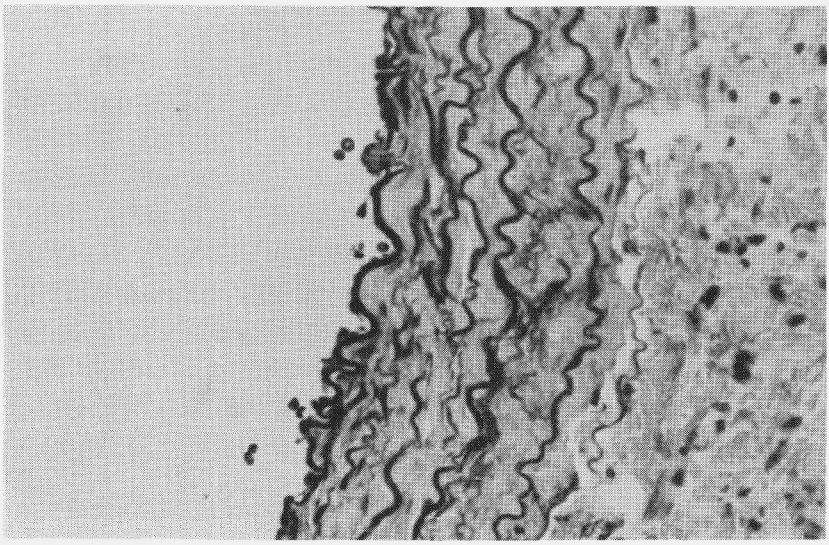


FIG. 1 Low power survey of normal rat aorta, stained by the Lawson MSB procedure. (magn. 300x)

FIG. 2 Detail of fig. 1, showing darkly stained elastic lamellae, with smooth muscle cells in between. The luminal side of the vessel wall is covered by endothelial cells. Lawson MSB. (magn. 1250x)



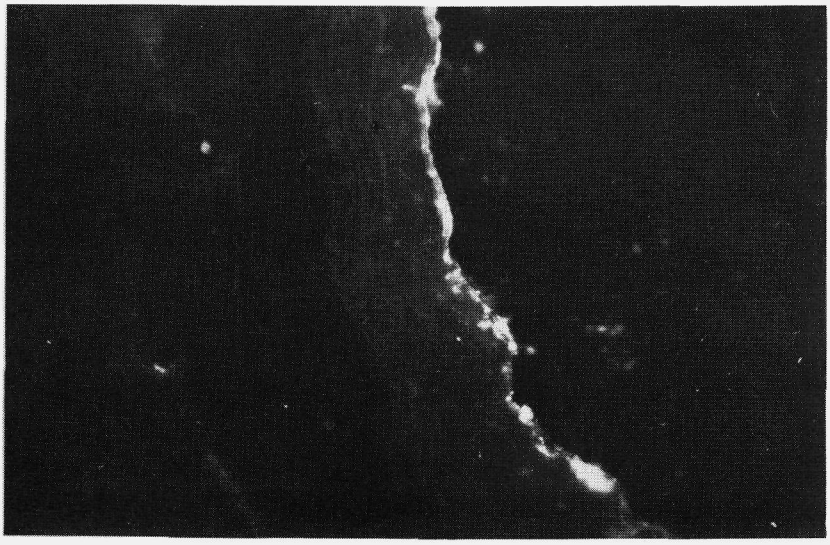


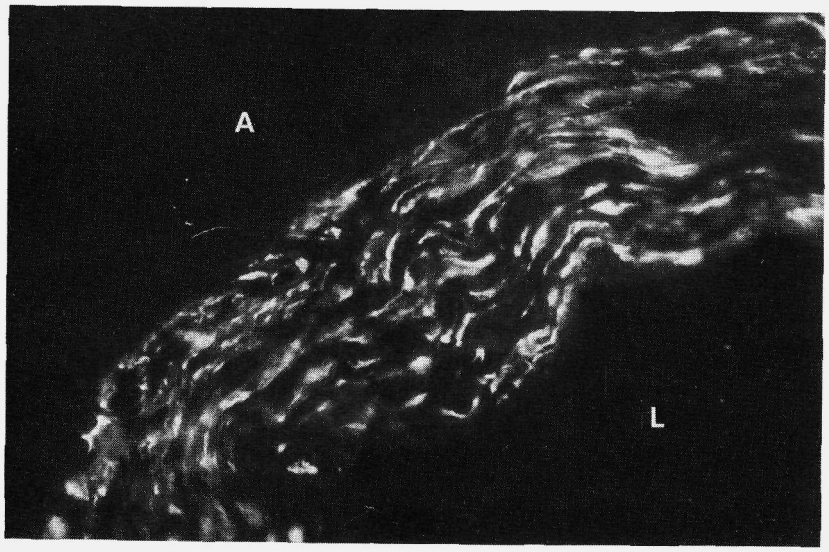
FIG. 3 Normal rat aorta, stained by the Levanol procedure. (magn. 500x)

FIG. 4 Immunofluorescent staining for coagulation factor VIII in a frozen section of normal rat aorta. (magn. 625x)

IV.2

Normal microscopical anatomy of the rat aorta

The wall of the normal rat aorta is composed of three layers: intima, media and adventitia (fig. 1, 2). The intima forms the inner lining of the vessel wall, and is composed of a thin layer of collagenous fibres, covered by a single layer of endothelial cells. These cells contain small spindle-shaped darkly staining nuclei, surrounded by a thin layer of cytoplasm, and form a continuous lining of the vessel lumen.
In surface view, these cells are closely apposed polygonal cells with a centrally located, single nucleus. Directly below the intima lies a continuous elastin layer, the lamina elastica interna, the first of 6-9 concentric elastic lamellae of the media. Between these elastin lamellae, smooth muscle cells, small amounts of collagen, and thin elastin fibres can be seen, especially near the surface of the elastin lamellae. The elastin lamellae often split and rejoin at random. Thin, elastin fibres can also be found surrounding the smooth muscle cells of the media. These cells are regularly distributed between the elastin lamellae, and are spirally arranged around the axis of the vessel. The nuclei have an irregular, ovoid shape and contain 2-3 nucleoli. In Lawson MSB (LMSB)-stained sections of paraformaldehyde-fixed tissue, these nuclei take a pink to light red colour and appear to be vacuolated. The cytoplasm has an ill-defined outline and contains, in Levanol-stained sections, dark blue fibrils (fig. 3).
The outer, or adventitial, layer of the aorta is predominantly composed of collagen fibres, interspersed with thin fragments of elastin. Fibroblasts are randomly distributed throughout this collagenous layer. The vasa vasorum are poorly developed and consist of a few capillaries. The adventitial layer gradually becomes more loosely arranged and finally merges with surrounding connective tissue. In LMSB-stained sections, the collagen in the adventitial layer stains bright blue. Before the construction of an aortic flap, a greater part of the adventitia, especially the more loosely arranged outer layer, is removed in order to get a better view of the vessel wall while suturing. This reduces the amount of adventitial tissue introduced into the vessel lumen during the inversion of the flap.



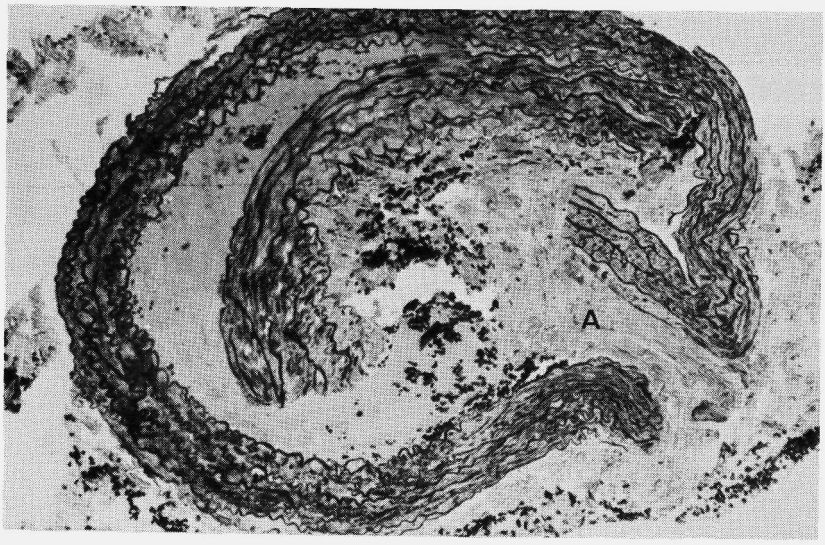


FIG. 5 Normal rat aorta, stained with anti-smooth muscle cell serum. Frozen section. (A: adventitia; L: lumen.) (magn. 500x)

FIG. 6 Aortic flap near its point of attachment to the vascular wall, surrounded by a virtually occluding thrombus mass after one hour. The end of the arteriotomy is occluded by adventitial tissue (A). Lawson MSB. (magn. 125x)

In immunofluorescence and immunoperoxidase histochemistry, no fibrin-related material can be found on the surface of the endothelial cells, or in the vessel wall. Coagulation factor VIII can be demonstrated in the endothelial cells as a granular fluorescence (fig. 4).
Antiserum against smooth muscle cells (SMCs) produces a strong fluorescence in the cells of the media, while endothelial cells and fibroblasts remain unstained (fig. 5). The pattern of fluorescence is identical to the staining pattern of the medial cells in LMSB or Levanol-stained sections. Sections stained with anti-lysozyme antiserum showed no specific fluorescence, nor did sections incubated with non-immune rabbit serum.
With anti-platelet serum, no specific fluorescence is detected in normal aortic tissue. (A background autofluorescence of elastin fibres is always present.)
In the normal aorta of the rat, no spontaneously formed thrombi are seen; no single platelets are attached to the endothelial lining.

IV.3
Microscopical results of the flap procedures

Microscopical findings after one and two hours

In all aortas, thrombi were found after construction of a flap in animals sacrificed after one and two hours. The thrombus mass was attached to and surrounded the flap, and extended in proximal and distal direction into the vessel (fig. 6). The results obtained at one and two hours were so much alike, that these results will be described together. In this section also a detailed description will be presented, to give a general survey of the reactivity of various tissue components to the stainings employed.

LMSB-staining

The major part of the thrombus stained greyish-blue, without any obvious structure. In this greyish-blue mass, clusters of erythrocytes were embedded which stain bright red. Strands of red stained material, sometimes with a fibrillar structure (fig. 11), were seen, mainly in the erythrocyte-containing areas of the thrombus and occasionally on its surface (fig. 7). Proximal to and surrounding the flap, the major part of the thrombus was composed of light-blue material, interspersed with small clusters of erythrocytes, while in

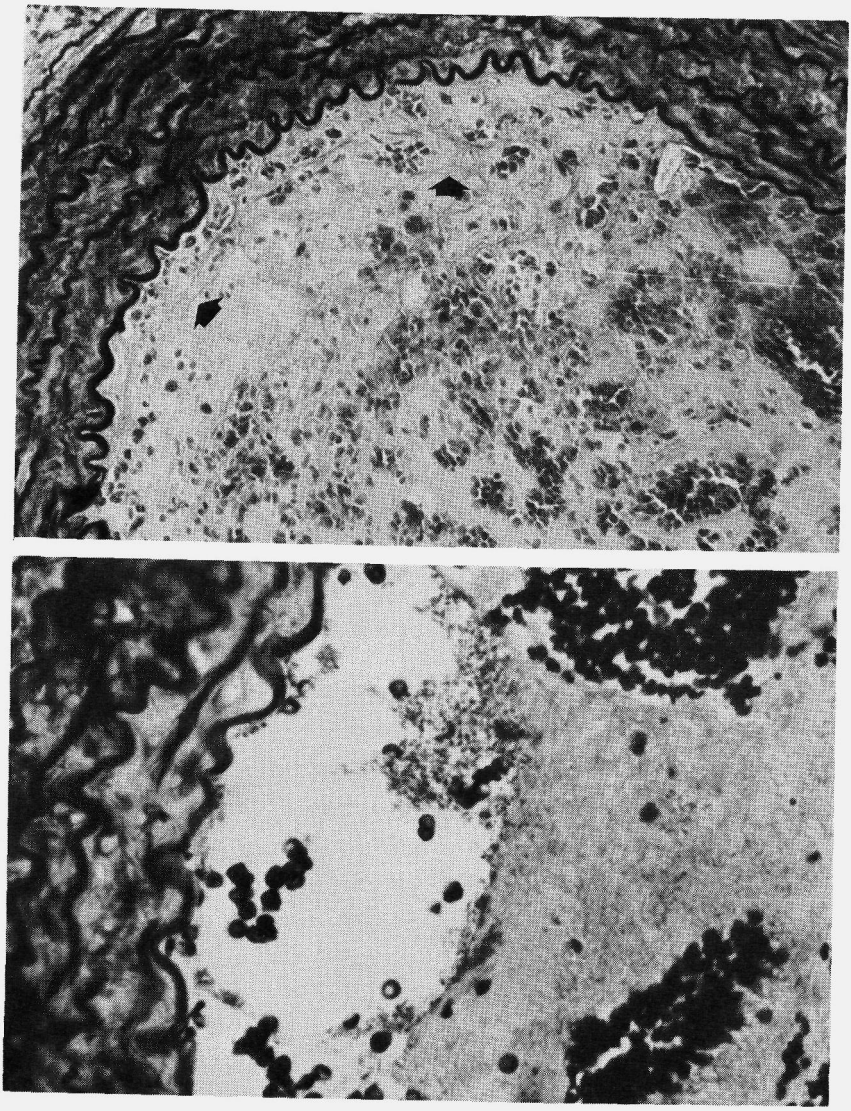


FIG. 7 Two-hour old thrombus, showing erythrocytes embedded in a platelet-rich mass. Strands of fibrillar material (e.g. at arrows; dark blue in original) are seen between the platelets. Lawson MSB. (magn. 350x)

FIG. 8 Freshly deposited platelets can be seen as distinct dark blue dots on the surface of a two-hour thrombus. Levanol staining. (magn. 500x)

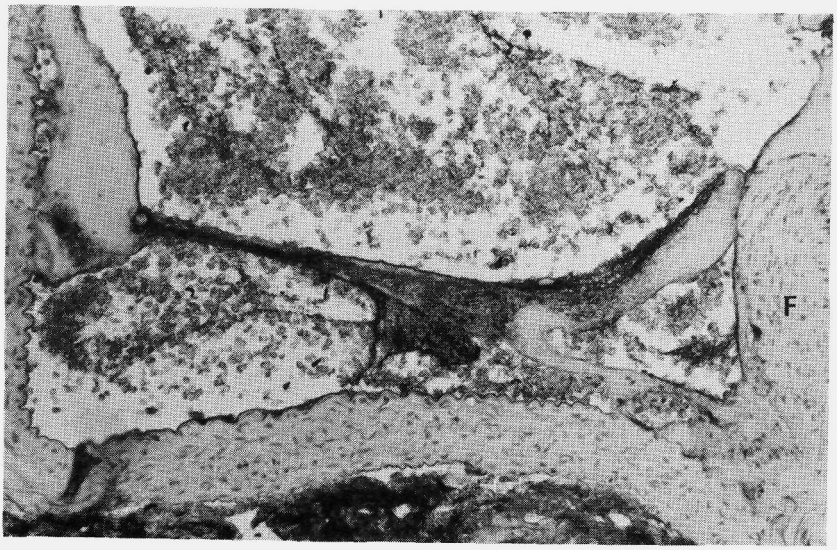
the distal part of the thrombus, the erythrocytes were often centrally located as a large mass.
In the proximal part of the thrombus, a laminar pattern could be seen, consisting of thin layers of erythrocytes between thicker layers of blue stained material. This laminar pattern was not observed in the distal part of the thrombus. The flap consisted of the same components as the vessel wall itself, and contained the same amount of smooth muscle cells. The adventitia on the flap could easily be distinguished from the greyish-blue stained thrombus mass by its bright blue colour. In the direct surroundings of the flap, smaller numbers of endothelial cells were found on the vessel wall than in the remainder of the vessel.

Levanol-staining
Following this staining procedure, the erythrocytes and strands of fibrillar material were dark blue. The major part of the thrombus stained yellow, with dispersed dark blue dots. On the surface of the thrombus, single blue particles of the size of platelets were found (fig. 8). The flap contained the same components as the vessel wall itself; the smooth muscle cell cytoplasm and the elastic fibres were dark blue, the collagen light yellow. The nuclei of the smooth muscle cells were pink.

Weigerts Elastic Tissue staining
This staining method only demonstrated the elastic lamellae in the vascular wall and in the flap. These lamellae had a black to dark blue colour.

Collagen staining
This method stained the collagen light red to pink. The thrombus remained unstained, while the adventitial part of the flap was clearly demonstrated. The more collagen was present, the more intense the red colour became; for instance, in the adventitia compared to the media.

Immunoperoxidase staining for fibrin
The strands between the erythrocytes of the thrombus were stained dark brown to black, whereas the surface of the thrombus was covered with a small rim of dark brown material. In those areas of the thrombus that did not contain erythrocytes, a diffuse light brown to yellow colouring was found (fig. 9).



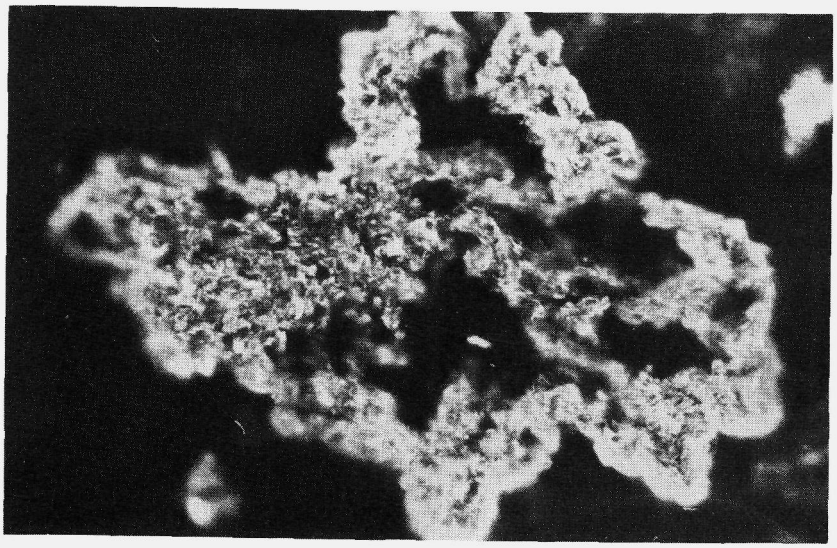


FIG. 9 Young thrombus of two hours, stained for fibrin by the immunoperoxidase procedure. The thrombus spans the lumen form flap (F) to original vessel wall. Note intense staining of thrombus surface. (magn. 150x)

FIG. 10 Two-hour old thrombus, stained for platelet antigens by anti-rat platelet serum. Frozen section, immunofluorescence procedure. (magn. 450x)

On the surface of the vessel wall, small wisps of darkly stained material were sometimes observed. Using this method, no staining was seen outside the thrombus, nor in the vessel wall. No staining was found in sections treated with non-immune rabbit serum. Due to haematoxylin counterstain, the nuclei were beautifully displayed in these sections; virtually no nucleated cells were seen in the thrombus.

- Immunofluorescence staining of fibrin-related antigen
  On frozen sections, the thrombus showed strong diffuse fluorescence. The surface of the thrombus showed a thin rim of strongly fluorescent material, while the surface of the aortic wall was lined with a thin, less fluorescent layer. The vessel wall and the flap showed no fluorescence.
- Immunofluorescence staining for Factor VIII-related antigen (Factor VIII:RAG)
  In the area directly adjacent to the flap, a thin lining of fluorescence could be observed on the vessel wall, although the intensity of fluorescence was less than in control aortas or in areas far from the flap.
  On the endothelial side of the flap, a thin fluorescent lining was found as well.

- Immunofluorescence staining for platelet antigen
  The thrombus demonstrated a strong fluorescence. No fluorescence was seen in the vessel wall (fig. 10).

- Immunofluorescence staining for smooth muscle cells
  In the media of the vessel wall and in the flap, a normal pattern of cellular staining was observed, identical to the pattern found in a normal vessel wall.

Considering the results obtained with anti-platelet serum, in combination with the staining pattern of LMSB or Levanol procedure, those areas of the thrombus staining greyish-blue in the LMSB procedure and yellow in the Levanol procedure, could be regarded as platelet masses. Therefore, these masses will be described in the following as platelets.

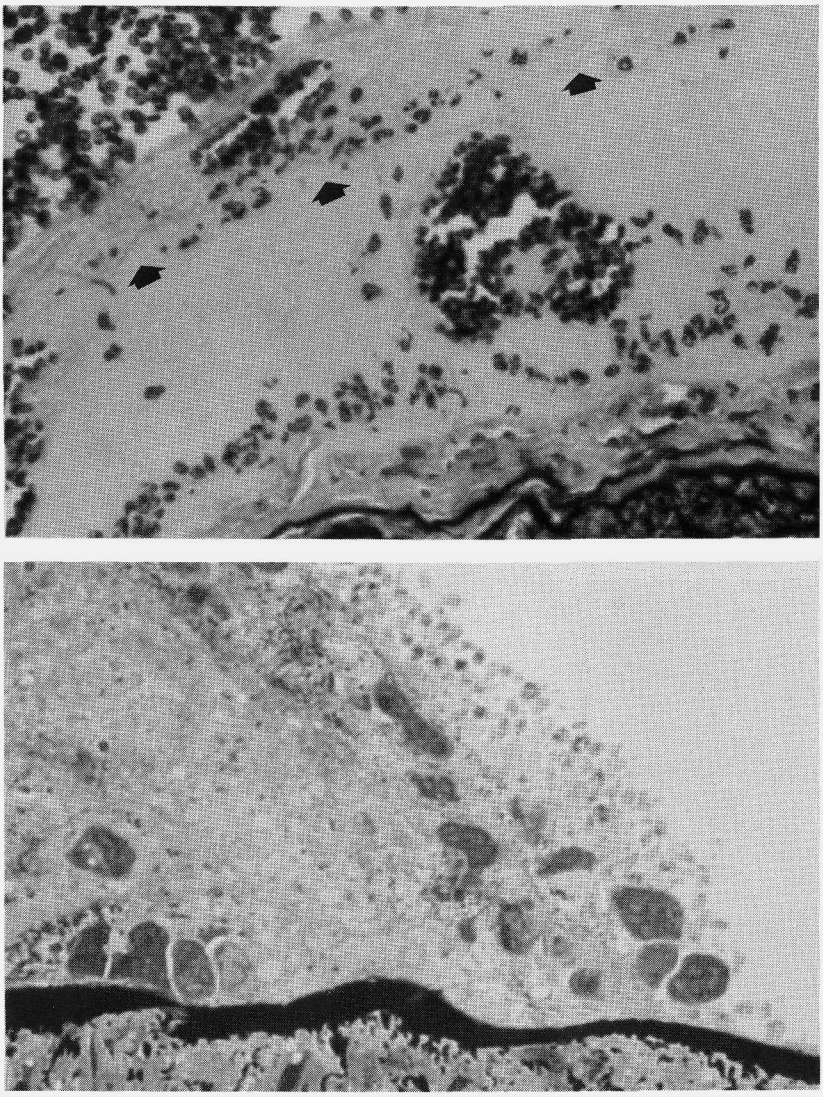


FIG. 11 Thrombus after two hours on the adventitial side of a flap, showing laminated structure with fibrin strands (arrows). Lawson MSB. (magn. 400x)

FIG. 12 Neutrophilic leucocytes invading a three-hour old thrombus. Note platelet aggregates on the thrombus surface. Semithin epon section, toliudine blue staining. (magn. 1375x)

In the immunoperoxidase procedure, areas of reactive material can be regarded to represent fibrin, corresponding with the red stained thrombus parts in the LMSB staining and with the blue stained thrombus parts in the Levanol staining; these structures will be described as fibrin.

Summary:

A fresh platelet-fibrin thrombus containing various amounts of erythrocytes was invariably found attached to the flap, extending mainly in a proximal direction.

The vessel wall did not show changes, except for a slight decrease in the number of endothelial cells in the vessel wall area around the flap.

Microscopical findings after three hours

After three hours, mixed platelet-fibrin thrombi were found in all vessels. The thrombi appeared slightly larger than at the previous time interval. The major part of the thrombus was composed of platelets with scattered areas of erythrocyte aggregates. Large parts of the thrombus had a laminated structure, recognizable by the presence of fibrin rims (fig. 11). Fibrin was also found on the surface of the thrombus; single platelets and platelet-aggregates were also present. In the Levanol staining these platelets stained dark blue. At this time, polymorphonuclear leucocytes (PMNs) were firstly seen in the thrombus (fig. 12). These PMNs, mainly neutrophils, were predominantly found on the surface of the thrombus, although scattered cells were also observed along fibrin rims inside the thrombus (fig. 13). At this time no fluorescence studies were performed. The vessel wall had a normal aspect.

Summary

At three hours a platelet-fibrin thrombus was found, resembling the thrombus found at one or two hours. The occurrence of platelet aggregates on the surface, together with fibrin, suggested a continuing growth of the thrombus. At this time, apposition of neutrophils on the thrombus was seen for the first time. The presence of neutrophils deep inside the thrombus could be explained by apposition followed by covering by new platelets and fibrin.

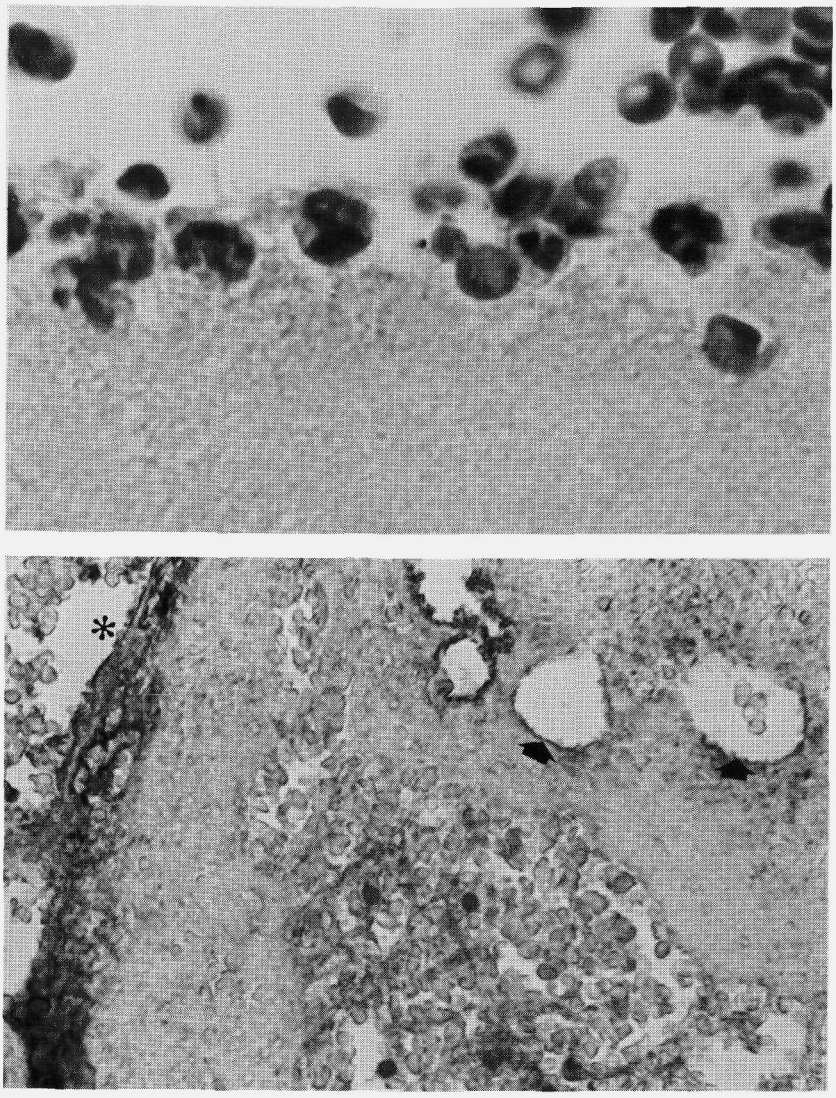


FIG. 13 Polymorphonuclear leucocytes and erythrocytes on the surface of a six-hour platelet thrombus. Lawson MSB. (magn. 1900x)

FIG. 14 Fibrin-rimmed canals (arrows) in the proximal part of a six-hour old thrombus. Strong staining for fibrin is also present on the thrombus surface (asterisk). Immunoperoxidase staining for fibrin. (magn. 575x)

Microscopical findings after six hours

The major difference in the composition of thrombi at six hours, compared to three-hour old thrombi, was an extensive infiltration of the thrombus by polymorphonuclear leucocytes (fig. 13). These leucocytes were found particularly in those areas of the thrombus, where the thrombus mass had a frayed appearance. Moreover, the localization of these cells was correlated with the presence of fibrin in the thrombus, as could be seen in immunoperoxidase stained sections, and confirmed in fluorescence staining. In all sections, the flap was completely covered by thrombus mass and did not show signs of degenerative changes. In one animal, the thrombus mass nearly occluded the aorta near the flap, with only a few channels near the vascular wall. Leucocyte apposition on the vessel wall opposite to the thrombus was seen. In the proximal part of one thrombus, recanalization was found (fig. 14); these channels contained large amounts of erythrocytes. In Levanol stained sections, rethrombosis was observed, as evidenced by the occurrence of dark blue stained single platelets and small platelet-aggregates on the surface of the thrombus.

The thrombus mass was strongly reactive to anti-platelet serum. Factor VIII reactivity was comparable with that seen at two hours.

In the thrombus fibrin was diffusely present. The vessel wall showed no changes.

Summary

After six hours a platelet-fibrin thrombus with an extensive infiltration of PMNs was observed, with rethrombosis on the surface.

At this time, as at previous time intervals, no fibrinolytic activity was detected in the thrombus or in the vessel wall with the Todd slide method. In the adventitial parts of the aortas, some fibrinolytic activity was seen.

Microscopical findings after twelve hours

At twelve hours, the thrombus was again characterized by an extensive infiltration of the entire thrombus mass by PMNs. These leucocytes were also still present on the surface of the thrombus. In immunoperoxidase staining the laminar structure of the thrombus could still clearly be seen, especially the rows of neutrophils along fibrin rims. The amount of fibrin appeared to have increased, most clearly on the surface of the thrombus and, to some extent,

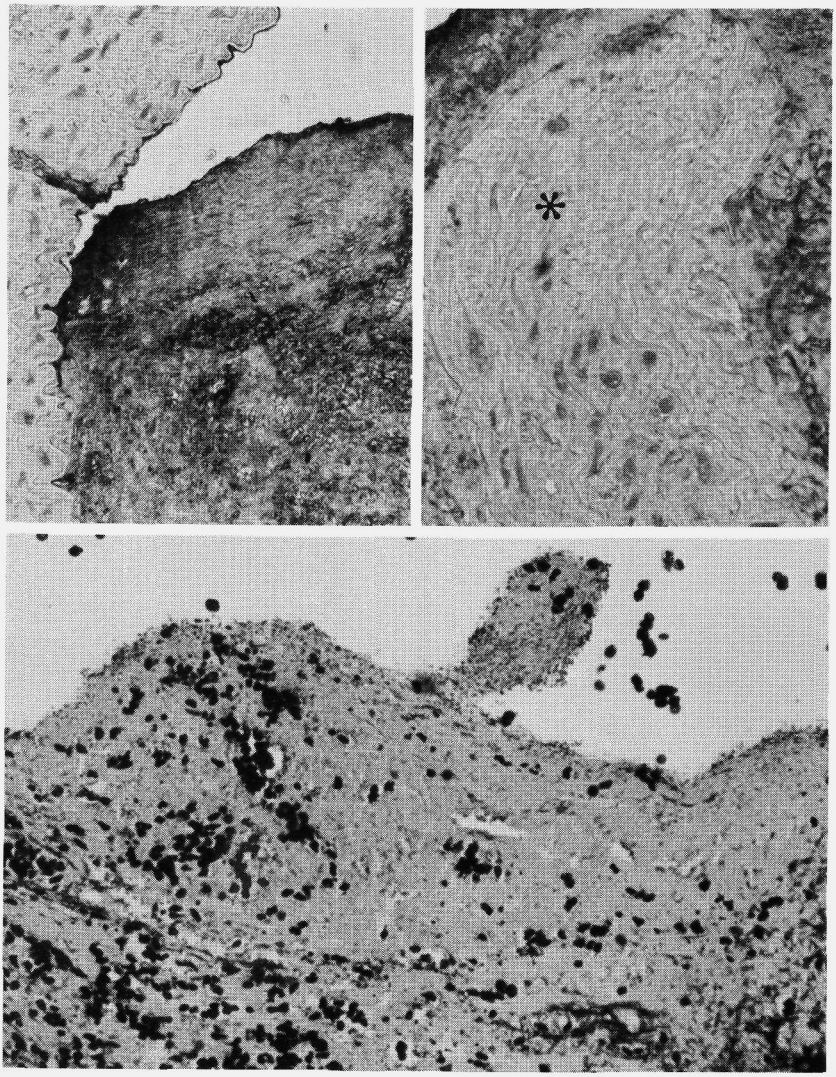


FIG. 15 Twelve-hour old thrombus, stained for fibrin. Increased amounts of fibrin are found inside, and especially on the surface of the thrombus. Immunoperoxidase-stained section. (magn. 500x)

FIG. 15a Decreased number of nuclei in the cut end of an aortic flap (asterisk), surrounded by a fibrin-rich twelve-hour old thrombus. *Immunoperoxidase-stained section; haematoxylin counterstaining.* (mag. 500x)

FIG. 16 Freshly-apposed platelets, covering the surface of a twelve hour old thrombus, are seen as stained dots in a Levanol-stained section. (magn. 350x)

diffusely in the thrombus (fig. 15). Fresh apposition of platelets on the surface of the thrombus was still present. These platelets seemed to adhere to the old platelet masses and to the fibrin deposited on the thrombus (fig. 16). The amount of SMCs, as judged in sections by Levanol and by immunofluorescence, using anti-SMC serum, was diminished in the flap, but not in the vascular wall.

Staining for fibrin by immunofluorescence was comparable to that seen with the immunoperoxidase method; although fluorescence staining also showed a thin lining on the inside of the vascular wall that was not visible in the peroxidase-stained sections.

Factor VIII:RAG was locally present in the vascular wall. On the luminal side of the flap no fluorescence was seen any longer.

The thrombus reacted positively to anti-platelet serum. After a ninety-minute incubation, the Todd slide method showed local, weak fibrinolytic activity on the surface of the vessel. No fibrinolytic activity was seen on or in the thrombus.

Summary

At this time interval, the thrombus was composed of platelets and fibrin, and was heavily infiltrated by leucocytes. Fresh platelet apposition was seen on the surface. The amount of SMCs in the flap had diminished (fig. 15a). On the inside of the vessel wall weak fibrinolytic activity was seen.

Microscopical findings after twenty-four hours

The general composition of the thrombus and the localization of fibrin were similar to the six and twelve-hour thrombi. Large amounts of PMNs were still present in the thrombus, and platelet apposition could still be seen on the surface (fig. 16). A smaller number of PMNs was seen on the surface of the thrombus. In one animal, a large thrombus mass, proximal to the flap, was not infiltrated by PMNs, suggesting this part of the thrombus to be less than six hours old. In twenty-four hour old thrombi, a few mononuclear cells were found for the first time. In another animal, mononuclear cells were found on the surface of a mural thrombus at its point of attachment to the vascular wall (fig. 17). These cells appeared to be continuous with cells lining the surface of the vascular wall.

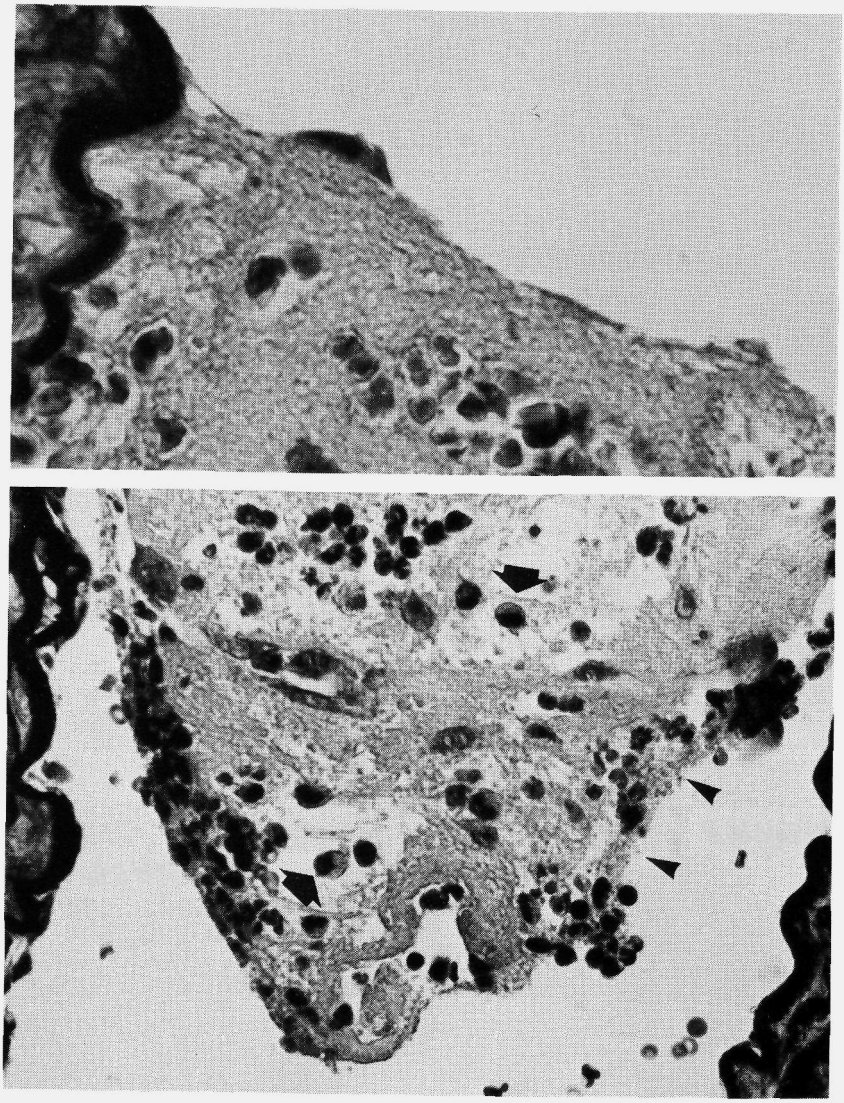


FIG. 17 Single mononuclear cell on a thrombus near the vascular wall. Lawson MSB-stained section of twenty four-hour old thrombus. (magn. 1600x)

FIG. 18 Mononuclear cells (arrows) surrounded by platelets inside a two-day old thrombus. Platelet aggregates (arrowheads) are still found on the thrombus surface. Lawson MSB. (magn. 550x)

Summary

No major changes were observed, apart from the presence of a few mononuclear cells and a smaller number of PMNs on the surface of the thrombus.

No immunofluorescence staining was performed.

Microscopical findings after two days

Thrombi found at two days, differed in many ways from thrombi of earlier time intervals, especially in respect to the cellular composition of the thrombus. The major thrombus mass still was composed of platelets and fibrin, and showed a laminar built-up with slit-like spaces filled with erythrocytes. As in the younger thrombi, fibrin was diffusely present throughout the thrombus, and focally in denser amounts on the surface of the thrombus. Scattered platelet aggregates were seen on non-invested parts of the surface, indicating that the thrombus still was thrombogenic (fig. 18). Moreover, PMNs were diffusely present in the thrombus. Some of these cells, however, showed pycnosis and karyorrhexis. A small number of eosinophilic leucocytes was seen.

Increased numbers of mononuclear cells were present in various forms; some, found throughout the thrombus, were small with a dark red-stained nucleus, while others had flattened, and formed a monocellular lining of the thrombus surface, lying directly on the platelet masses (fig. 19). This cellular lining was not continuous over the entire thrombus, but mainly covered discrete areas of the thrombus close to the vascular wall. The beginning of a similar form of investment has already been described above.

For the first time, another type of mononuclear cell was seen; these cells had large, lightly stained nuclei, and an extensive vacuolated cytoplasm, which gave these cells a foam-like appearance. This type of cell was mainly found in the superficial parts of the thrombus, surrounded by platelets (fig. 18).

These various cell types were also easily recognizable in the immunoperoxidase slides, because of the counterstain.

In the LMSB staining yet another cell type, found inside the thrombus, had large, pink to light red coloured nuclei and a vacuolated appearance.

In Levanol-stained sections, these latter cells had a dark blue stained nucleus while the cytoplasm stained light blue.

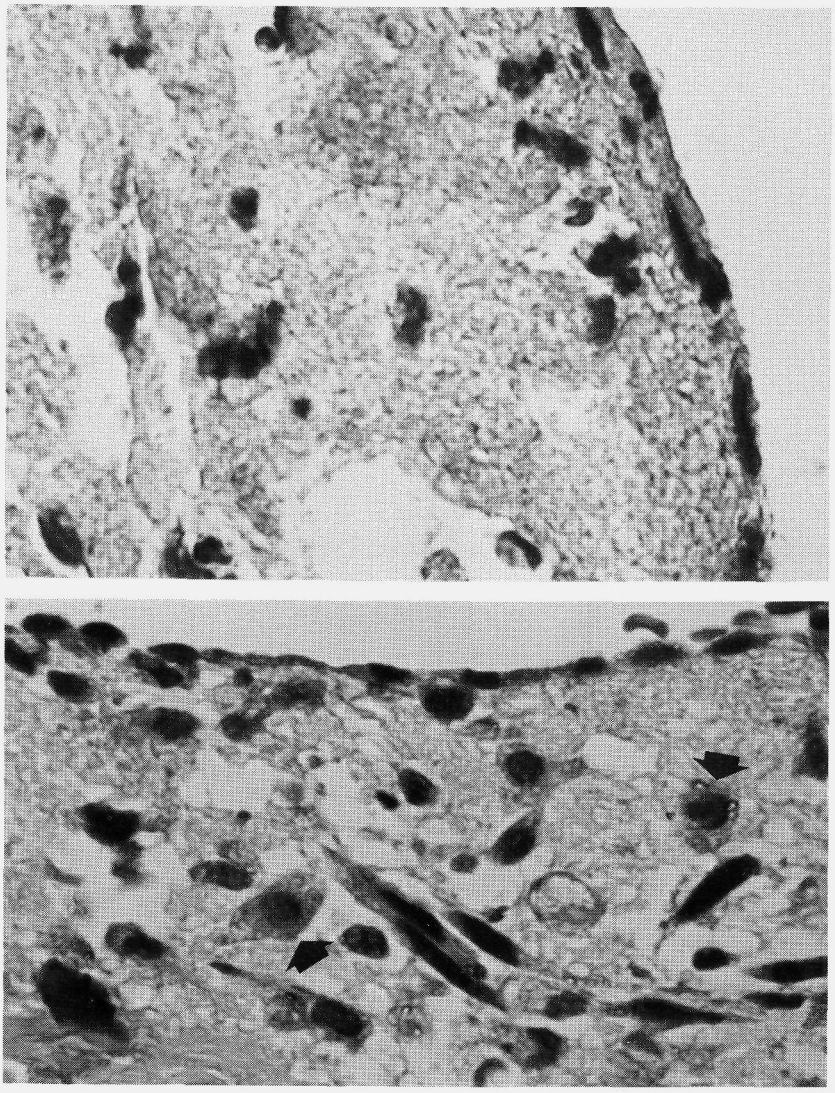


FIG. 19 Mononuclear cells lining the thrombus surface of a two-day old thrombus. Lawson MSB. (magn. 1600x)

FIG. 20 Three-day old thrombus, completely covered by mononuclear cells. Inside the thrombus a variety of cells can be seen, e.g. cells with a foamy cytoplasm (arrows). Lawson MSB. (magn. 1600x)

In both staining methods, these cells resembled the medial SMCs of the vessel wall. A few mononuclear cells with a polygonal nucleus and a large non-vacuolated cytoplasm were found, scattered throughout the thrombus; presumably these cells are macrophages. The vessel wall showed no changes.
No fluorescence staining was performed at this time.
Fibrinolytic activity was not determined.

Summary

The general impression of the findings after two days, was one of increased cellularity and increased cellular activity. Five new cell types were found, namely: eosinophilic granulocytes, macrophage-like cells, "foamy" cells, flattened mononuclear cells on the thrombus surface, and cells resembling medial SMCs. For the first time, extensive investment of the thrombus by a cellular lining was seen. Fresh platelet apposition still occurred on the non-invested areas of the thrombus.

Microscopical findings after three days

The general aspect of the findings after three days was similar to the picture one day earlier. Organization of the thrombus progressed by further infiltration of the thrombus by mononuclear cells. The amount of thrombus surface invested by a lining of mononuclear cells, as well as the number of vacuolated "foam"-like cells (fig. 20), had increased.
Peroxidase staining still showed fibrin lining the thrombus, while in the inner part of the thrombus, fibrin could still be demonstrated, although in smaller amounts.
An increased number of smooth muscle-like cells was seen; the origin of these cells could not be determined at this time.
Immunofluorescence staining for fibrin showed a positive reaction around the flap, and in and on the thrombus (fig. 21). Moreover, in the surgical area, a thin lining of fluorescence was seen on the intima. Some penetration of fibrin into the media was seen locally, probably due to surgical damage to the intima.
Factor VIII fluorescence showed weak endothelial activity in the vessel wall, while the luminal side of the flap also was slightly positive.

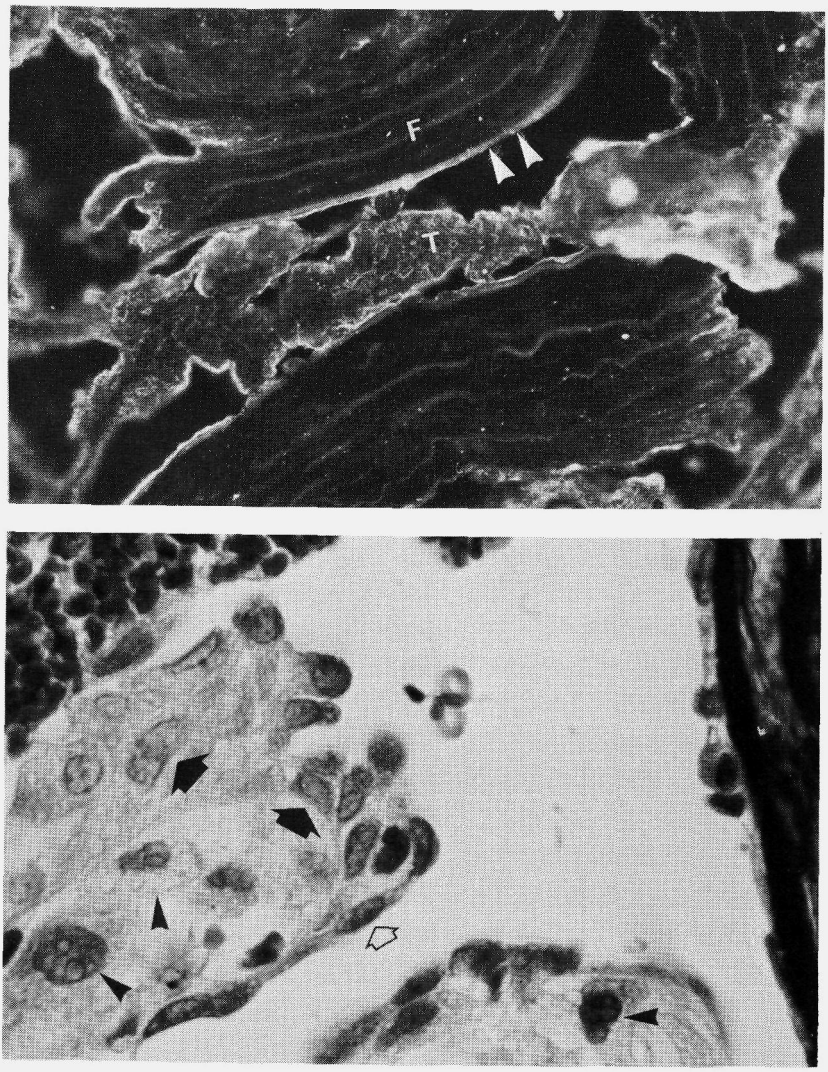


FIG. 21 Immunofluorescent staining for fibrin of a three-day old thrombus (T). Fibrin is seen on the thrombus surface, inside the thrombus mass, and on the luminal side (arrowheads) of the flap (F). (magn. 350x)

FIG. 22 Thrombus after four days, with various mononuclear cell types: smooth muscle-like cells (arrow), foamy cells with darkly stained nucleus (arrowheads), mononuclear lining cells (open arrow). Lawson MSB. (magn. 1600x)

Compared to the vessel wall, the flap showed a diminished staining with anti-SMC serum.
After 45 to 90 minutes of incubation, fibrinolytic activity was found focally in the endothelium, and in some parts of the adventitia. In one animal, a fibrinolytically active thrombus was seen. The vessel wall showed no abnormalities.

Summary
At this time, a continuing organization of the thrombus was observed, with increased numbers of all types of mononuclear cells. An increased investment of the thrombus by lining cells was observed. The number of SMCs in the flap had decreased compared to the number of cells in the vessel wall.

Microscopical findings after four days
A complete investment of the thrombi by flattened mononuclear cells was seen after four days. The thrombus itself was heavily infiltrated by mononuclear cells. At this time practically no PMNs remained inside the thrombus. The number of eosinophilic cells had decreased.
Four types of mononuclear cells could be differentiated in the thrombus. On the surface, flattened mononuclear cells with a small dark-red staining nucleus were seen, while large macrophage-like cells with a voluminous cytoplasm and a large, darkly stained nucleus were distributed throughout the entire thrombus. Other, similar cells showed a vacuolated cytoplasm with a lighter stained nucleus, giving them a "foamy" appearance (fig. 22).
Smooth muscle-like cells with large, lightly stained vacuolated nuclei were abundantly present. Migration of these cells, from the media into the thrombus, was suggested by the following observation. These cells were seen in a stream-like pattern between the surgically cut end of the media and the adherent thrombus, while a similar pattern of distribution of these cells was observed near the cut end of the aortic flap, surrounded by thrombus (fig. 23). By immunofluorescence, however, no cells staining with anti-SMC serum could be found inside the thrombus. The flap was nearly devoid of smooth muscle cells in this staining, while the vessel wall contained the same number of cells as normal vessels. In the surgically cut ends of the vessel wall, adjacent to the thrombus, however, less SMCs were present.

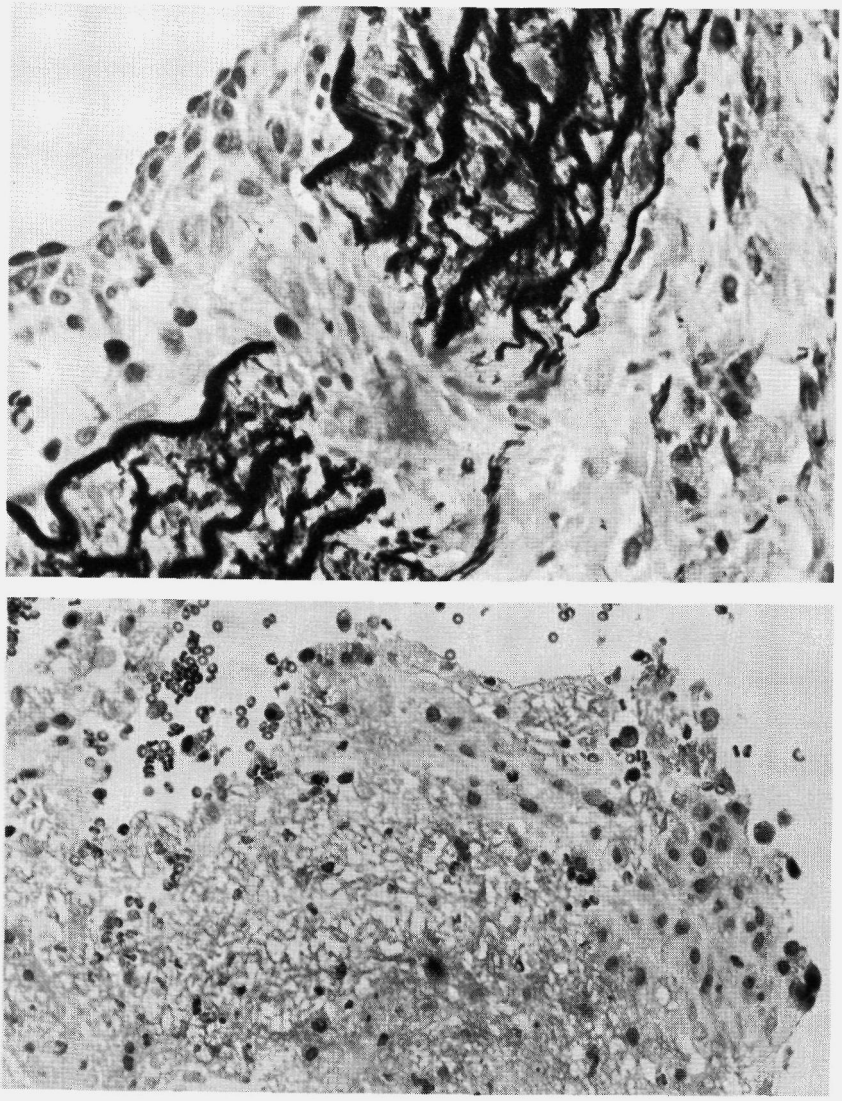


FIG. 23 *Smooth muscle-like* cells migrating into an organized six-day old thrombus. Note the lightly stained nuclei of these cells. Lawson MSB. (magn. 600x)

FIG. 24 Mixed thrombus at six days, composed of a well organized old part and a much younger part, devoid of mononuclear cells. Lawson MSB. (magn. 650x)

The amount of fibrin in the thrombus appeared to have decreased. Directly underneath the thrombus surface, more fibrin was present than deeper in the thrombus.
A normal distribution of Factor VIII fluorescence was found in the endothelium, even in the area near the thrombus. The luminal side of the flap also reacted positively, although weaker than the vessel wall. After sixty minutes of incubation, fibrinolytic activity was seen around the thrombus and in the adventitia. Fresh, platelet-rich thrombi were found attached to, or in the vicinity of the organized thrombus. Anti-platelet fluorescence was diffusely positive in the lesion.

Summary

At four days, the thrombi showed complete investment by mononuclear cells, and large numbers of macrophage-like cells, often with a foamy appearance. The number of smooth muscle-like cells inside the thrombus had increased, while the number of SMCs in the flap had decreased. Freshly deposited thrombi were seen as well.

Microscopical findings after six days

Completely organized and invested thrombi were found at six days, often in close contact with much younger thrombi; these two types could be easily differentiated. The older part of the thrombus was covered by flattened mononuclear cells, and was heavily infiltrated by various other types of mononuclear cells. Granulocytes were not seen in the older part. The younger thrombi, on the other hand, were not invested by cells, contained no mononuclear cells, and were rich in PMNs. The boundary between these two types was often beautifully displayed (fig. 24). The younger parts were found at the level of the flap and extended in a distal direction; they were not found proximal to the flap. The number of smooth muscle-like cells had increased when compared to four-day old thrombi, as determined in both LMSB and Levanol-stained sections. The greater number was found in the vicinity of the vessel wall and the flap. These cells often showed a parallel orientation, extending from the cut surfaces towards the thrombus (fig. 23, 25).
The stream-like patterns of these cells from the cut end of the flap and of the vessel wall, were confluent in the thrombus. Using anti-SMC serum, positively reacting cells could be seen inside the thrombus mass, in the vicinity

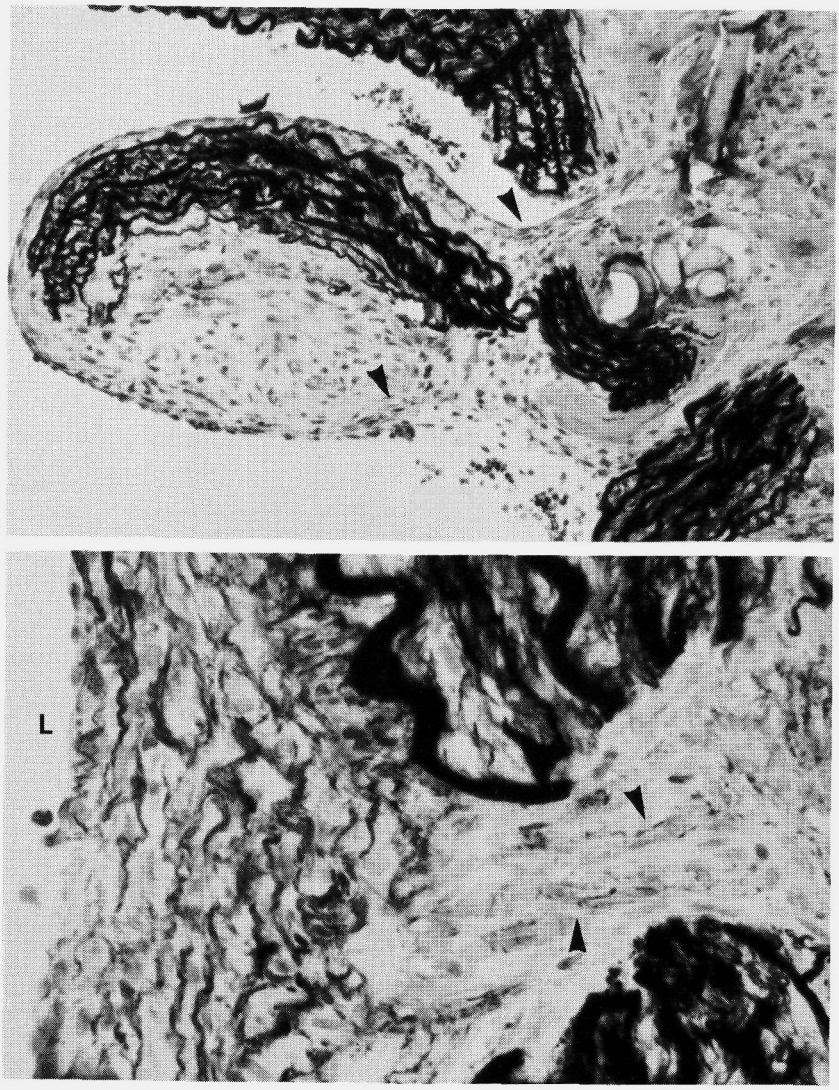


FIG. 25 Survey of a fully organized and invested thrombus around an aortic flap. Note parallel orientation of cells growing in and into the thrombus (arrowheads). Six days. Lawson MSB. (magn. 200x)

FIG. 26 Newly formed elastin strands parallel to the luminal surface. In the deeper parts smooth muscle cells are surrounded by granular elastin deposits (arrowheads). Two weeks. Lawson MSB. (magn. 650x)

of the aortic flap, and near the vessel wall. The flap itself did not contain positively reacting cells any longer. The cut ends of the media of the vessel wall showed a diminished number of positively reacting cells. These immunohistochemical observations showed a good correlation with the histological findings. The number of mononuclear cells with a vacuolated cytoplasm was variable, and appeared to be dependent on the size of the thrombus. The smaller the thrombus mass, the larger the number of foamy cells.

Freshly formed platelet aggregates were observed on the younger parts of the thrombus. A positive reaction to anti-platelet serum was seen focally in the thrombus.

In immunofluorescence, the thrombus was still reactive to anti-fibrin monomer antiserum. In immunoperoxidase-stained sections, less fibrin in an inhomogenous pattern was found. On the surface of the younger parts of the thrombus, however, the fibrin staining was still intense. In the deeper parts of the thrombus, weak staining for fibrin was seen, incorporated into the vessel wall.

Anti-Factor VIII:RAG showed a normal distribution in the vessel wall, whereas the surface of the thrombus was not reactive. As at previous time intervals, staining for collagen and elastin was confined to the vessel wall and to the flap, and did not show newly formed collagen or elastin in the thrombus.

With the Todd slide method, focal fibrinolytic activity was found on the normal vessel wall surface. In some specimens, the thrombus mass itself was fibrinolytically active.

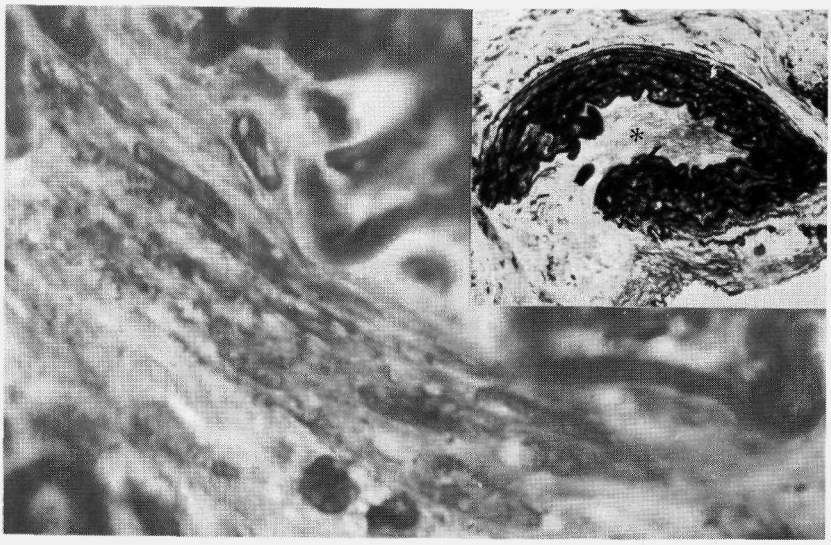
Summary

At six days, fully organized and younger thrombi were found in close relationship to each other. Smooth muscle-like cells were seen throughout the older thrombi, extending from the vessel wall and the flap in streamlike patterns.

Weak fibrin staining was still seen in the older thrombus parts, while fresh fibrin was present in the outer rim of the younger thrombi. Rethrombosis was a common finding.

Microscopical findings after two weeks

The most impressive change, seen at this time, was the presence of elastin and elastin lamellae. The morphology of the organized thrombus had changed



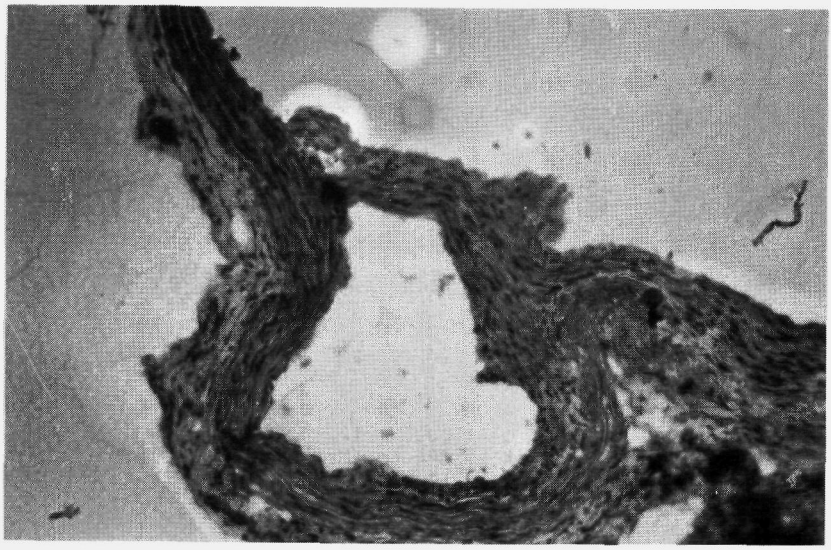


FIG. 27 Differences in elastin morphology. The inset shows the strand-like elastin at the luminal surface can be seen (Lawson MSB). At higher magnification the area at the asterisk shows granular elastin formation (Weigert's elastin staining). Two weeks. (magn. 1500x) (inset: magn. 100x)

FIG. 28 Focal fibrinolytical activity after two weeks at the junction of a bridge-like organized thrombus with the vessel wall. Todd slide method. (magn. 150x)

completely, in many areas no longer resembling the organized thrombus seen at six days. The organized cell mass, including the flap, was moved outwardly, causing the cell-rich, organized thrombus to become a part of the vessel wall. Part of the organized thrombus still protruded into the lumen of the vessel, forming an irregularity on the inner surface of the vessel wall.
In the deeper parts, elastin was present in an irregular, granular pattern around large smooth muscle-like cells. In the more superficial parts of the thrombus, close to the lumen, the elastin was more organized, running in strands parallel to the surface (fig. 26).
This elastin stained less intensely than the elastin lamellae of the normal vessel wall. No collagen was found in between the newly formed elastin (fig. 27). The superficially located cells differed from the cells in the deeper parts, by having smaller, oval, darkly stained nuclei.
Few "foamy" cells were seen in the deeper areas near the media of the original vessel wall. The surface of the newly formed vessel wall was covered by endothelial-like, flattened cells, except for the central area, where thicker and less regularly formed cells were exposed to the blood; these latter cells stained very dark blue in the Levanol staining, and platelets were seen attached to them. Close to these areas, granulocytes were found intraluminally. Small amounts of fibrin were found in both immunohistochemical staining techniques. This fibrin was located in the deeper parts of the organized cell mass, near the media of the original vessel wall.
The surface of the vessel wall showed Factor VIII immunoreactivity throughout. In the organized thrombus mass, no platelet antigens were observed by immunofluorescence any longer. Antiserum to SMCs, on the other hand, showed a diffuse positive reaction in all areas of the organized cell mass; the flap did not react to this antiserum.
In the Todd slide method, both the original vessel wall and the newly formed structure focally showed fibrinolytic activity (fig. 28).
The original vessel wall showed no abnormalities.

Summary

At this time, the thrombus had become a fully organized cell mass. Smooth muscle-like cells were abundantly present, and surrounded by newly formed elastin. Near the surface, strands of elastin could be seen. In between these strands, smooth muscle-like cells with a small darkly

stained nucleus were found. The thrombus was partly covered by endothelial-like cells; the central part of the thrombus, however, still appeared to be thrombogenic. Fibrin was found in the deeper parts of this cell mass.

Microscopical findings after three weeks

With all staining methods, the amount of elastin in the lesion had strongly increased after three weeks, and was now mainly present as strands parallel to the luminal surface. The cells between these lamellae had small darkly stained nuclei, while the differences in nuclear structure between the cells in the superficial and deeper parts of the lesion were less pronounced. Virtually no collagen was found in the elastin-containing areas. The peripheral parts of the lesion were covered by endothelial cells. In the central part of the lesion, however, no endothelial cells were seen, and platelets were attached to the surface of the lesion at this site. No foam cells were found in the lesions. In the deepest part of the lesion, next to the adventitial layer, collagen was found, which stained less intensely than collagen in the adventitia. By immunoperoxidase staining, remnants of fibrin were found in this area. Staining with anti-SMC serum showed positively reacting cells throughout the organized lesion. While the surface of the lesion reacted positively to anti-Factor VIII: RAG, no fluorescence was found in the lesion with anti-platelet serum or anti-fibrin monomer serum.

A peculiar finding in one of the aortas prepared for immunofluorescence studies was the presence of an organized lesion, extending across the vessel lumen. This "bridge" contained SMCs, while its surface reacted positively to anti-platelet and anti-fibrin monomer serum. The lesion focally showed Factor VIII immunoreactivity as well as fibrinolytic activity.

Summary

At three weeks, the lesions contained more elastin, predominantly present in strands along the luminal side of the lesion. Many SMCs were present between these lamellae. The central part of the lesion was not covered by endothelial cells, and platelet aggregates remained present on these areas. Small amounts of fibrin were still present in the deeper parts of the lesions.

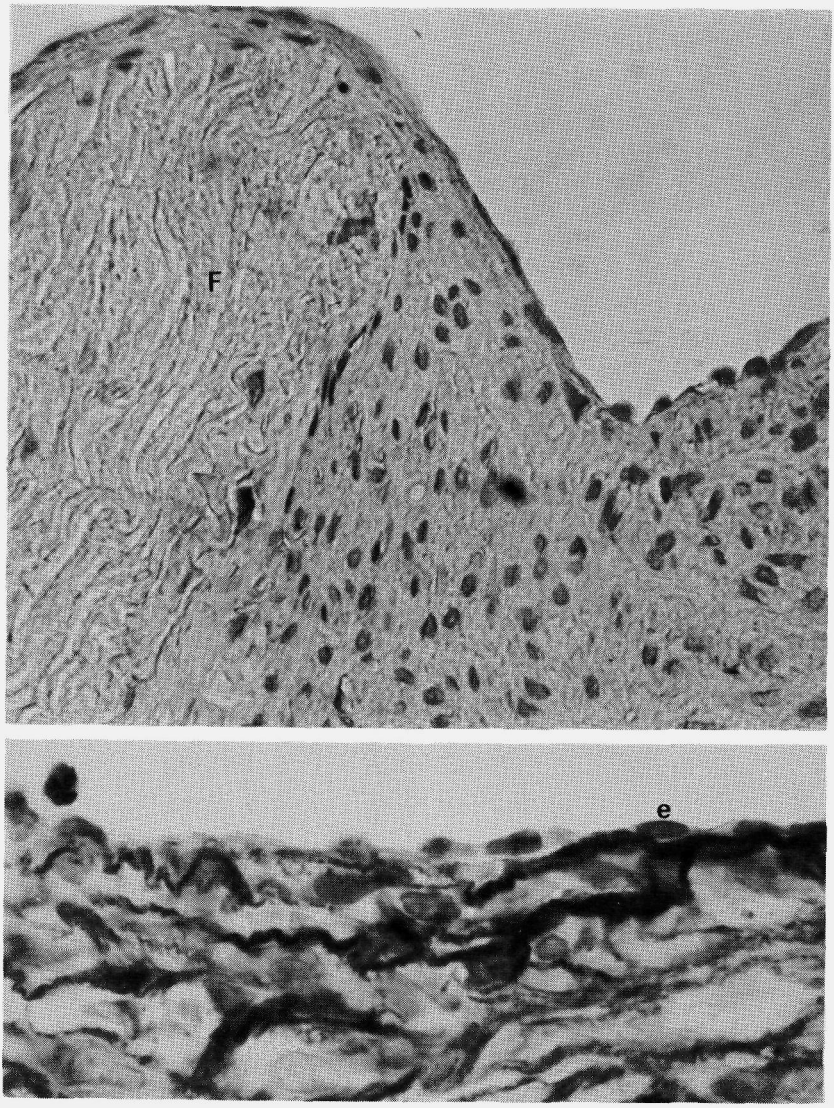


FIG. 29 "Empty" aortic flap (F) next to a fully organized thrombus, free of fibrin. One month. Immunoperoxidase staining for fibrin; haematoxylin counterstaining. (mag. 650x)

FIG. 30 Fully organized lesion after one month, partly covered by endothelial cells (e), partly denuded with an attached granulocyte. Lawson MSB. (magn. 1250x)

Macroscopical findings after one month

After one month, the lesion appeared more organized. The amount of elastin had increased further. The staining of the elastin strands was more intense, especially near the surface of the lesion. Many smooth muscle-like cells were present. Most of the surface of the lesion was now covered by endothelial cells, but in nearly every specimen areas could be seen where another cell type covered the luminal site. Whereas the endothelial cells had darkly stained, fusiform nuclei and a long, flattened cell body, these other cells had round, polygonal nuclei which stained not as darkly by LMSB, and had a smaller cell body. These latter cells were more densily packed than the endothelial cells, which were far apart as usual (fig. 30). On some of the sites, covered by non-endothelial-like cells, freshly deposited platelets were observed. In Levanol staining, the cells near the luminal side of the lesion contained more muscle protein than the cells in the deeper part of the lesion. Some collagen was found between the newly formed elastin lamellae. In peroxidase staining for fibrin, none was found in the lesion.

The cells in the deeper parts of the lesion, towards the adventitia, could be recognized as smooth muscle cells. This was affirmed with anti-smooth muscle cell serum. These cells were observed by immunofluorescence throughout the organized lesions. The "flap" and the cut ends of the original vessel wall contained virtually no smooth muscle cells (fig. 29). In Factor VIII immunofluorescence, a thin line of endothelial fluorescence could be seen on the luminal side of the vessel, and also on the lesion itself. But in some areas of the lesion, no luminal fluorescence was seen. Anti-platelet serum showed no positive reaction. In some areas of one specimen, a positive reaction to fibrin antiserum was observed. In LMSB and immunoperoxidase staining for fibrin, fresh and older rethrombosis, in various stages of organization, was detected, generally distal to the flap area.

In many areas, signs of rethrombosis were found. The organized lesion sometimes showed different stages of thrombus organization.

In the Todd slide method, fibrinolytic activity was found.

Summary

After one month, a new lamina elastica interna had formed. The luminal side of the lesions was covered with true endothelium, interspersed with areas covered with another cell type. Rethrombosis in different stages of

organization was a common finding (fig. 30). The lesions were fibrinolytically active. The original vessel wall showed no changes.

Microscopical findings after two months

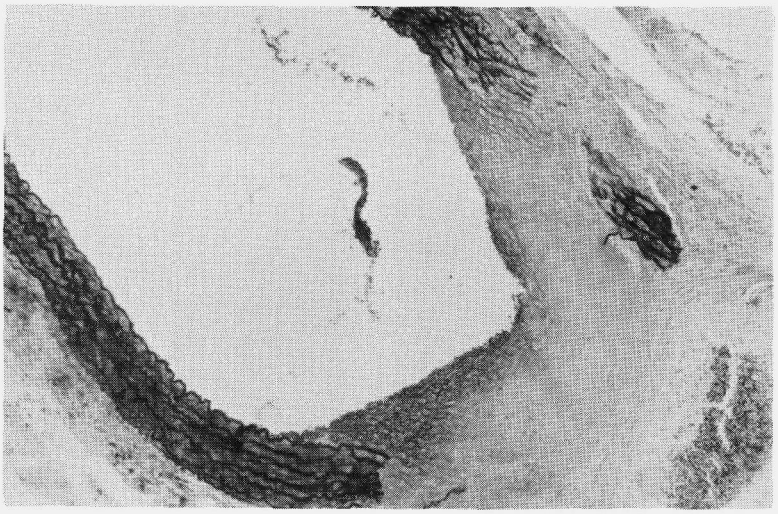
After two months, large parts of the lesion showed intense staining for elastin, while the newly formed lamina elastica interna had become more pronounced: the strands of elastin were now positioned parallel to each other. Also in the deeper parts of the thrombus, virtually no differences remained between the more deeply and the more superficially located SMCs. Their nuclei were larger and more lightly stained than at earlier times, and resembled the original smooth muscle cells nuclei in the media of the vessel wall. Between these cells and the elastin strands, newly formed collagen was found. The surface of the lesions was, for the greater part, covered by long, flattened endothelial cells, as could also be confirmed in immunofluorescence studies using anti-Factor VIII:RAG. The immunofluorescence, however, was interrupted at some places. This focal absence of endothelial cells was also observed in LMSB-stained and in Levanol-stained sections. On these denuded areas fresh fibrin was sometimes found with immunoperoxidase staining for fibrin. In the Levanol staining single and clustered platelets could be seen at these sites; thus, rethrombosis was a common finding. In one specimen, three different stages of thrombus organization could be observed; even an area with foamy cells was present.

No fibrin was located in the fully organized parts of the lesion. With anti-SMC serum a positive immunofluorescence was seen throughout the lesions, while the remnants of the flap were completely devoid of cells.

The organized lesion showed fibrinolytically active areas. No changes of the vessel wall were seen.

Summary

The most striking observation was the common finding of rethrombosis in different stages of organization. Furthermore, a pronounced staining of elastin in the organized areas, with diminished differences in form and staining intensity of the SMC nuclei in the various lesion areas, was found.



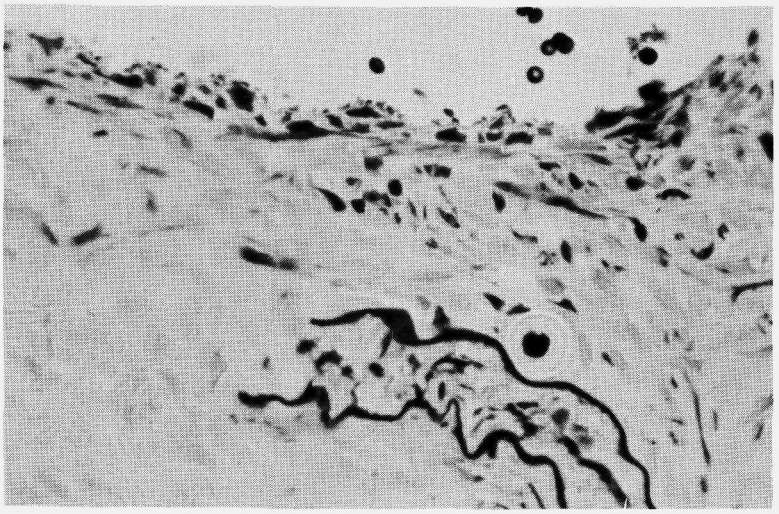


FIG. 31 At three months, the now cell-free flap, has moved to the adventitial side of the vessel wall, while the lesion shows fully developed, well-staining elastic lamellae. Lawson MSB. (magn. 125x)

FIG. 32 Platelets adhere to the surface of a two-week old, organized thrombus. Levanol staining. (magn. 1650x)

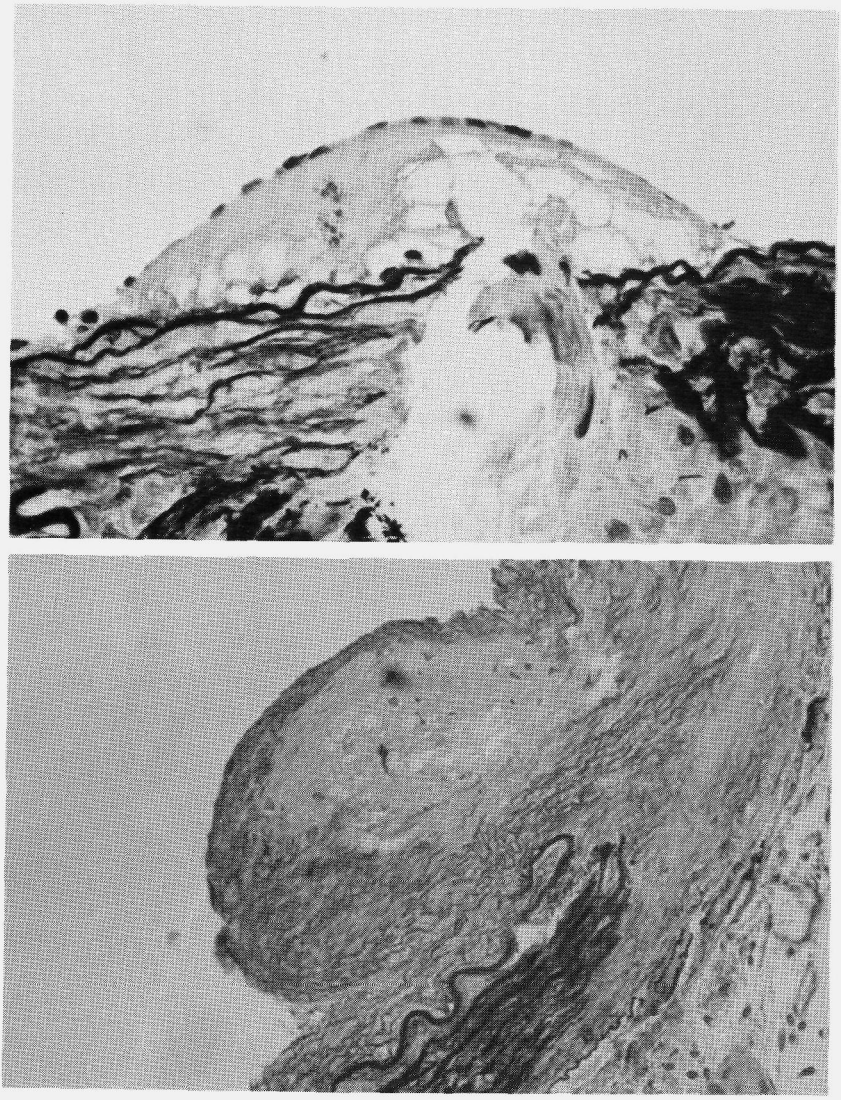
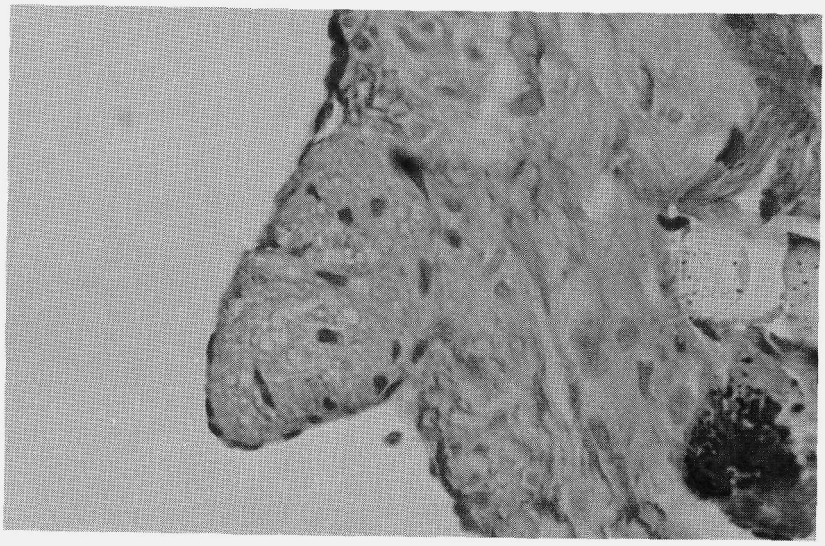


FIG. 33 Rethrombosis of four to six days is seen on a gap in the elastic lamellae of a three-month old organized lesion. Lawson MSB. (magn. 650x)

FIG. 34 Area of rethrombosis, of approximately three weeks of age, as judged by the pattern of elastin deposition, in a three-month old organized lesion. Lawson MSB. (magn. 425x)



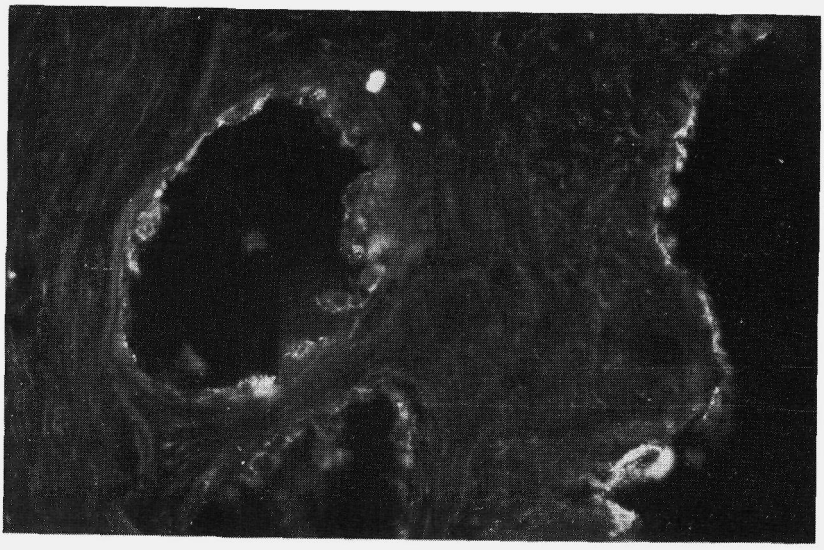


FIG. 35 Completely invested area of rethrombosis on a two-week old lesion. Lawson MSB. (magn. 650x)

FIG. 36 Punctate factor VIII immunofluorescence in the cellular lining of a completely organized, recanalized bridge-lesion of three months. Frozen section. (magn. 650x)

Microscopical findings after three months

The strands of elastic tissue, formed in the old thrombus mass, were sometimes difficult to distinguish from the original medial elastic fibres. Remnants of the aortic flap were invariably found outside the vessel wall in the adventitial layer. These remnants were devoid of cellular components. Compared with the results after two months, the elastin stained more darkly in LMSB and in elastin staining (fig. 31). The nuclei of the SMCs were still slightly smaller near the surface of the lesions, than deeper in the lesions. In the deeper parts, the nuclei became larger, stained more lightly in the LMSB staining, and resembled the SMCs of the original media.

In comparison with the two-month time interval, the collagen staining showed more collagen. The collagen was diffusely present between the elastin strands, but was more pronounced in the deeper parts of the lesion, thus forming a new adventitial layer. Most of the surface of the lesion was covered by endothelial cells; gaps in this lining, however, could regularly be observed. Rethrombosis always occurred at these locations. Thrombus masses of various ages, estimated from 1-3 hours up to 6 days could be seen (fig. 32, 33, 34, 35).

Fibrin was absent in peroxidase-stained sections neither in fluorescence studies on the surface, nor in the deeper parts of the lesion.

With anti-Factor VIII immunofluorescence, a thin lining inside the vessel wall could be observed, also on the surface of the healed lesion (fig. 36).

Anti-platelet serum showed no fluorescence.

Anti-SMC serum showed a normal composition of the vascular wall; virtually no differences between the normal wall in the healed lesions were detectable (fig. 37).

In the Todd slide method, the new vascular lining still showed little fibrinolytic activity. Intraluminally, young thrombi were repeatedly observed.

The shape of the vessel lumen after healing was ovoid instead of round, the lesion forming a crest in the lumen of the vessel.

Summary

After three months, a completely healed lesion could be observed. The elastin was more darkly stained, and more collagen had been formed throughout the entire lesion area. Rethrombosis was a common observation. Small thrombi of different ages were repeatedly observed.

Microscopical findings after ten months

The healed site of the vessel wall was still recognizable; no major changes had taken place, compared to three months. With different staining procedures, darkly staining, well-organized elastin could be seen, as well as normal amounts of collagen, forming a new adventitial layer.

No differences in SMC structure could be observed between the cells in the lesion and those in the original vessel wall. The endothelial lining still was not continuous and gaps, upon which rethrombosis areas of various ages were present. Some small free thrombi were seen as well.

In the surgical area, the vessel lumen did not have a round shape, and still showed a crest in the inner lining of the vessel wall.

At this time, no immunohistochemical studies were performed.

Summary

After ten months, a completely healed lesion with rethrombosis is observed, giving rise to an irregularity in the vessel wall surface.

IV.4

Thrombus volume and position

Method

The volume of thrombi was quantitated microscopically on LMSB stained sections, obtained at a distance of 120 um. Point-counting grids, with 438 points, were superimposed on micrographs, and the number of points lying on the thrombus (including the flap) was counted.
One point represents a volume of 19.10^{-5} mm^3 (uncorrected for tissue-shrinkage during processing).
The position of the thrombus, relative to the flap, was estimated by determining in which sections (obtained at 120 um) thrombus, or flap, or both were present.

Results

Thrombus volume and position were determined for 5 thrombi obtained at 4 hours and for 6 thrombi obtained at 24 hours.
At 4 hours, the thrombus volume (± s.d.) was 22 ± 8 $mm^3 10^{-3}$. The thrombus mass extended from 260 um proximal to the flap, up to 240 um distally. At 24 hours thrombus volume had not changed significantly (21 ± 3 mm^3 10^{-3}), but the thrombus had moved in distal direction, beginning 280 um proximal to the flap and extending to 560 um distally. The volume distribution, relative to the flap, however, did not show a change.

	proximal	%thrombus volume around the flap	distal
at 4 hours	23%	40%	37%
at 24 hours	17%	41%	42%

Effect of Ticlopidine on thrombus volume:

In animals which had been pretreated with Ticlopidine for 4 days (100 mg/kg body weight daily per os) thrombus volume at 24 hours had decreased, compared to control animals treated with vehicle only.

Thrombus volume	(mm^3 10^{-3})	
Controls	13 ± 2 mm^3 10^{-3}	(n=4)
Ticlopidine	5 ± 3 mm^3 10^{-3}	(n=5)

The difference in thrombus volume between control and Ticlopidine-treated animals, was statistically significant (Main-Whitney test; $p = 0.005$).
The difference in mean thrombus volume between the first investigated group (22 ± 8 mm^3 10^{-3}) and the control group in the Ticlopidine study (13 ± 2 mm^3 10^{-3}) could not be accounted for.

Summary of morphological data seen in primary thrombosis, including rethrombosis

	Fibrin	Platel.	PMN	Eos	Macroph.	Foamy	Mono	SMC	Endoth.	P.endoth.	Collag.	Elastin	Fibrinol	Rethr.
1 hour	++	+++	-	-	-	-	-	-	-	-	-	-	-	-
2 hours	++	+++	-	-	-	-	-	-	-	-	-	-	-	++
3 hours	++	+++	+	-	-	-	-	-	-	-	-	-	-	++
6 hours	++	+++	++	-	-	-	-	-	-	-	-	-	-	++
12 hours	++	+++	+++	-	-	-	-	-	-	-	-	-	±	++
24 hours	++	+++	+++	-	-	-	+	-	-	-	-	-	-	++
2 days	++	+++	++	+	+	+	++	+	-	-	-	-	-	++
3 days	+	+	+	+	++	++	+++	++	-	-	-	-	+	++
4 days	+	+	±	±	+++	+++	+++	+++	-	-	-	-	+	++
6 days	+	+	-	-	+++	+++	++++	++++	-	-	-	-	+	++
2 weeks	±	-	-	-	+	++	+++	++++	-	+	-	++	+	++
3 weeks	-	-	-	-	-	-	++	++++	+	+	++	+++	++	++
1 month	-	-	-	-	-	-	+	++++	++	+	++	+++	++	++
2 months	-	-	-	-	-	-	-	++++	++	+	++	+++	++	++
3 months	-	-	-	-	-	-	-	++++	++++	-	++	+++	±	++
10 months	-	-	-	-	-	-	-	++++	++++	-	++	+++	-	++

IV.5

AORTIC ANASTOMOSIS

Macroscopical results

Everting and inverting aortic anastomoses were constructed and the animals sacrificed, after the same time intervals as described in the previous chapter. Table IV.3 shows the number of anastomoses performed at the various time intervals. In this series, more macroscopically visible complications were encountered. Most complications were found in the first group of rats operated upon; the number of complications decreased with increasing surgical skill. These complications were not equally distributed among the two groups (Table IV.4). More postoperative aneurysms, on the suture line, occurred in the inverting anastomosis group. In the group with everting anastomoses, two aneurysmatic aortas were seen, while aneurysms were found in four animals with an inverting anastomosis. In four animals, stenosis of the inverting anastomotic suture line was observed, and one animal (everting anastomosis) showed an organized caval bleeding, at sacrifice. In one animal (everting anastomosis) an infection was present.

One postoperative death was seen in the everting anastomosis group. No other complications, such as paralysis or necrosis of the hind legs, were observed in the two groups.

It was striking to find such a small number of clinically relevant complications after the construction of inverting anastomosis, especially as this anastomosis was deliberately performed in a sloppy and inaccurate way. Most of the animals had no problems at all.

Collateral formation was observed once.

In the anastomosis group the same investigations, except for TEM and SEM studies were performed as in the group with the aortic flap operation. The same staining methods and immunohistochemical procedures were used.

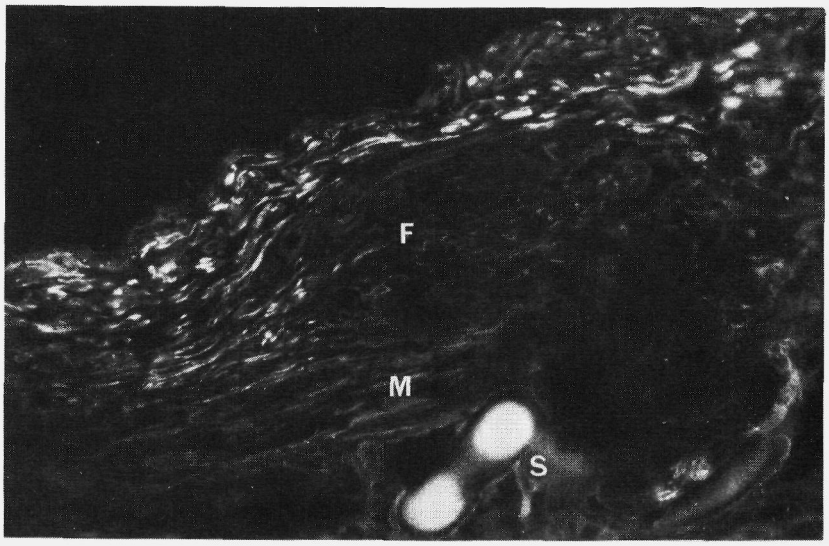
TABLE IV.3

TIME	EVERTING ANASTOMOSIS	INVERTING ANASTOMOSIS
3 months	3	2
2 months	3	2
1 month	3	3
3 weeks	1	1
2 weeks	3	2
6 days	2	2
4 days	3	3
3 days	3	2
2 days	3	2
1 day	3	2
12 hours	3	3
6 hours	3	1
3 hours	3	2
2 hours	3	2
1 hour	3	2
Total number	42	31

TABLE IV.4

MACROSCOPICAL OBSERVATIONS

	EVERTING	INVERTING
postoperative death	1 (2%)	-
aneurysm on the suture line	2 (5%)	4 (13%)
aneurysm	-	-
stenosis	-	3 (10%)
infection	1 (2%)	0
paralysis hind legs	-	-
collateral formation	1 (2%)	-
postoperative bleeding	-	1 (3%)
	5 (12%)	8 (26%)



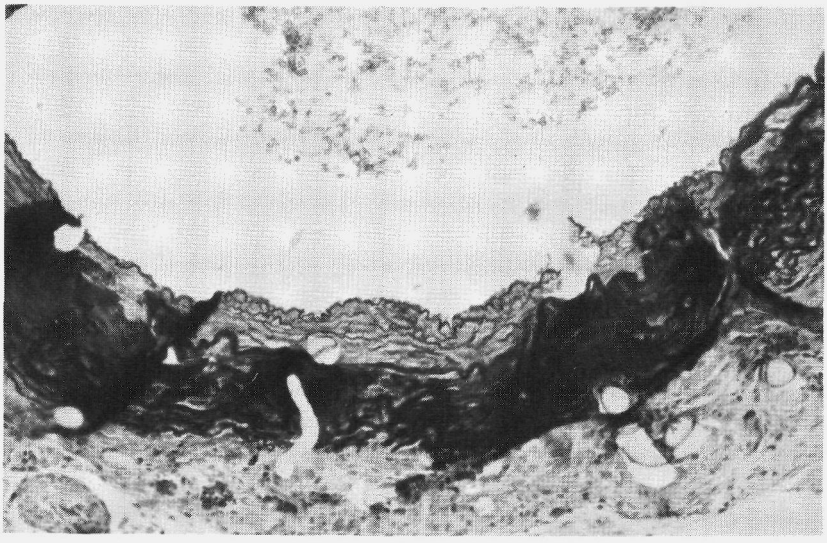


FIG. 37 Demonstration of smooth muscle cells in a completely organized lesion. The pattern of fluorescence closely resembles that seen in a normal vessel wall. Note that the flap (F) and the egdes of the original media (M) are devoid of smooth muscle cells. Brightly fluorescent areas at the bottom are suture threads (S). Frozen section, indirect immunofluorescence. Two months. (magn. 400x)

FIG. 38 Cross sectioned suture line of a two-week old inverting anastomosis. Note multiple flap-like protrusions; in some areas the healing of the wall is in progress as judged by elastin deposition. Lawson MSB. (magn. 200x)

IV.6

Microscopical results

A thrombus was always found, when an aortic anastomosis had been constructed, mostly confined to the spaces between the stitches only. Some specimens still showed perfused channels in the aortic lumen explaining why, in spite of the firm thrombus masses formed on the anastomotic line, the major part of the animals did not show any macroscopical complications after surgery. The more accurately the sutures were placed, the less thrombus formation was observed. The suture material itself did not cause thrombus formation. In everting anastomoses, smaller amounts of thrombus were found than in the series with inverting anastomoses. In these inverting anastomoses, part of the adventitia and media were brought into the lumen, thus forming multiple aortic "flaps" (fig. 38).

In spite of the formation of large thrombi on these inverting anastomoses, no gross microscopical damage was seen; this observation was in accordance with the macroscopical findings.

At the early time intervals, everting aortic anastomosis showed pictures typical for thrombosis, in a short stretch of the aorta; thrombus formation was found just adjacent to the anastomotic line, between the stitches, with spurs in distal direction. Proximal to the anastomotic line no thrombus formation was found.

The thrombi were round and laminated in structure. The composition of the thrombus was similar to that of thrombi formed on aortic flaps, although at three hours more PMNs were seen in these thrombi.

Many places with rethrombosis and fresh apposition of platelets and fibrin on the thrombus mass were observed. The total picture made a more violent impression than the one of the flap thrombosis studies. At these early time intervals, the inverting anastomosis also showed mural thrombosis, but was less organized. The thrombus was more loosely arranged and did not seem to originate from between the sutures. Even after two hours, PMNs were already observed. In the two-hour specimens of the flap procedures, this was not observed.

In the two and three-hour specimens, the aorta proximal to the anastomotic line, was always patent and did not show thrombus formation. The six and

twelve-hour specimens showed thrombi characteristic for that time. Apart from these thrombi, fresh thrombi were found as well. The form of thrombi was different in the everting and inverting groups, the latter protruding further into the lumen.

After one to three days, mononuclear cells invaded the thrombus, while the early stages of investment of the thrombus by these cells was observed. The thrombi in the suture niches became more organized, the lumen in the everting anastomosis group remained mostly open, in spite of the narrowing due to thrombosis on the suture line. The inverted anastomotic lines showed more occluding thrombi. No reaction to suture material was observed. The original vessel wall showed no alterations.

After three days, an increased investment of the thrombi had been achieved. The difference between thrombus formation in an everting and inverting anastomosis became increasingly evident. In the inverting anastomosis group, the protruding thrombi were threatening the patency of the vessel, while in the everting specimens the mural thrombi promoted the healing of the suture line.

In everting anastomoses, a thrombus mass of corresponding age was found after four days. Due to a strong tendency towards rethrombosis, this thrombus was practically everywhere accompanied by younger thrombus masses. Three to four-hour old thrombus masses were generally found on older thrombosis. On the surface of the thrombus masses, a mononuclear investment was seen.

After four days, the rate of rethrombosis was even higher in the inverting series. The vessel itself was always patent, once even in spite of a false aneurysm. In this aorta (inverting anastomosis), the old vessel wall had partially disappeared; a thick thrombus mass had replaced it and bulged outwards, corresponding with the macroscopical observation in this animal.

In the older parts of the thrombus, SMCs were observed.

After six days, thrombus formation, typical for that time interval, was observed. Thrombus formation was again more pronounced in the inverting anastomoses. A high rate of rethrombosis was also seen in these specimens, an increased amount of smooth muscle-like cells and foamy cells was evidence of organization of younger thrombi. The aortas were patent. In all time intervals, thrombus formation, distal to the anastomotic line, was constantly observed.

After two weeks, the thrombi in the everting anastomoses were present in a mural position. The vessel itself was patent in all investigated specimens. Thrombus organization, typical for two weeks, was found, whereas, even in the patent vessels, rethrombosis was a common finding.
In the organized thrombus, different stages of organization could be observed. Moreover, different forms and stages of elastin formation were found; the amount of elastin varying in all parts of the lesion. Small amounts of newly formed collagen were also found. In one specimen, a thrombus was found proximal to the suture line; this was an exceptional observation. A change in form of the smooth muscle-like cells was apparent; near the lumen, the nuclei became more darkly stained and smaller, while in the deeper parts, a more hazy, lightly stained form of nucleus was present.
After two weeks, large thrombus masses were seen in the series with inverting anastomoses. Thrombi of different ages were present.
Newly formed elastin was only present in granular deposits; no formation of elastin strands was seen. One vessel appeared not to be perfused, while proximally the beginning recanalization was suspected.

After three weeks, a still more pronounced difference between the inverting and everting anastomoses was seen. A nearly total healing of the vessel wall had occurred in the everting anastomoses while, in different specimens with inverting anastomoses, thrombus masses with rethrombosis were encountered. No rethrombosis was seen in the neatly healed everting anastomosis. In elastin staining, the new elastin strands and granules were beautifully displayed. Between the newly formed elastin and collagen, apparently normal smooth muscle-like cells were seen in their natural orientation.
In the specimens with inverting anastomoses, the organization was irregular. Younger organization forms could frequently be found, indicating a high rate of rethrombosis; some fresh platelets were seen on non-endothelialized surface areas.

Re-endothelialization:
While in the everting anastomoses a nearly complete endothelialization was found; this re-endothelialization was far from complete in the inverting specimens. In the centre of the thrombus (in some specimens the occluding part)

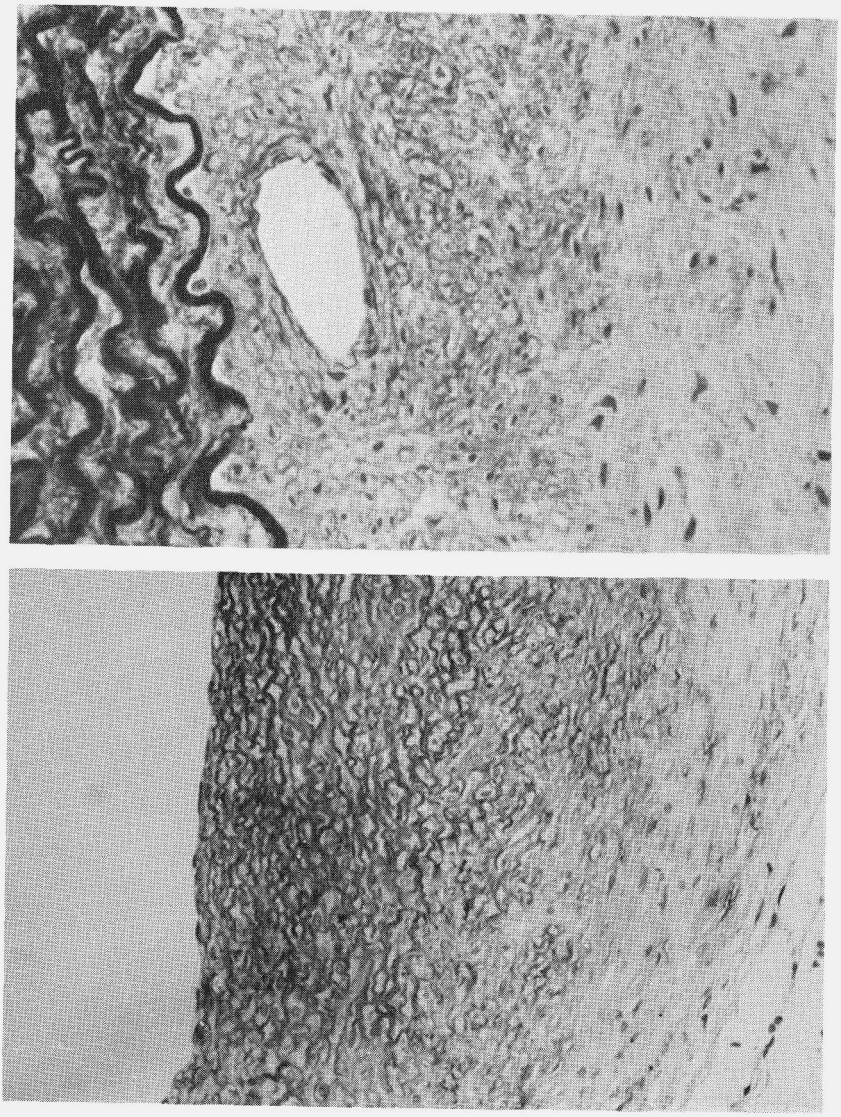


FIG. 39 Recanalization of a one-month old thrombus mass. Note the differences in structure of the elastic components. Strand-like elastin can be seen around the vessel, while more granular deposits are seen away from the lumen. Lawson MSB. (magn. 650x)

FIG. 40 Totally healed part of the suture line from a three-month old everting anastomosis. Note complete absence of original vascular wall. Lawson MSB. (magn. 400x)

attempts at recanalization were seen. These little canals penetrated the occluding thrombus mass from the proximal side (fig. 39). In one specimen, with a large thrombus, the occluding mass existed of thrombus masses of different ages. The oldest thrombi were found in the centre, near the anastomotic line.

After one month, healing had progressed. In most specimens of the everting series, the aorta was patent and the lumen was invested with new endothelial cells. SMCs seemed to have taken their normal position and elastin strands were neatly positioned around them. More collagen was deposited in the deeper layers of the newly formed vessel wall.
On cross-section, every vessel wall showed a clear, ovoid shape. The thrombus mass formed a permanent ridge on sagittal sections of the vessel.
In one specimen of the everting series, the original vessel wall on the suture line had disappeared. In spite of this, the aorta was patent due to a circular organizing lesion, forming an entirely new wall. In this specimen, some areas of rethrombosis were observed, even very young ones, containing many PMNs.

After two and three months, it was occasionally difficult to recognize the lesion (fig. 40). In the everting series, all aortas showed an open lumen, except one which was totally occluded with thrombi of different ages.
In the proximal part of the thrombus, recanalization was found. The elastin stained more intensely, and was laid down in neatly arranged strands around the recanalization channels.
In the deeper parts, a new adventitial layer had been formed. SMCs had a normal shape and position.
The inverting series showed a further organization; in some areas, rethrombosis was seen.

Histochemical investigations with immunofluorescence stainings confirmed these results.
Platelets were present in the time intervals up to four to six days, while fibrin was found up to two weeks. At later time intervals, fibrin was sometimes observed in areas of rethrombosis. Fluorescence studies showed more fibrin than was seen in the immunoperoxidase staining. After two weeks,

fluorescing SMCs were recognized in the lesions. Furthermore, endothelial cells, stained with anti-Factor VIII, were found. The staining intensity of SMCs increased with the progression of time. (In these anastomosis experiments, the cut ends of the media also showed a deprivation of SMCs.)
After three months, no difference between the original wall and the organized lesion could be seen in immunofluorescence.
Anti-platelet and anti-Factor VIII fluorescence often showed an irregular staining pattern, corresponding with the high rate of rethrombosis and the frequency of interruption of the endothelial lining.

Fibrinolytic studies were also performed on the anastomotic specimens. No regular pattern could be determined in this study. No clear fibrinolytic activity of the lesion on the suture line could be found. In the lumen of the vessel, a very weak lysis zone could sometimes be observed. The adventitia showed more fibrinolytic activity than was found intraluminally.
No differences in fibrinolytic activity were seen between the inverting and everting series.

IV.7

RESULTS HUMAN MATERIAL

Macroscopical results

During the collection of human material, it became clear that the age of the arterial thrombotic or atherosclerotic material generally could not be determined. Most human specimens were surgical material, obtained during elective or emergency surgical procedures. If the case history was a short one, or if a sudden occlusion had taken place, a guess could be made about the age of the collected material.

Most of the human material, however, came from reconstructive vascular surgical procedures on patients with long case histories, who had most frequently no specific aggravation of the complaints. The patients belonged to the categories Fontaine III and IV, suffering nightly pains and with threatening or present gangrene. Sometimes, arteries were taken from amputation material. A number of venous bypasses, and one bovine bypass graft were also collected. The majority of specimens consisted of atherosclerotic material from endarterectomies and sections of totally or partially occluded arteries.

During a first macroscopical inspection, a rough division was made in the obtained specimens.

The same microscopical investigations were performed on human material as on the experimental material.

The material was divided into:

1) young thrombi, obtained from embolectomy material
2) totally occluded or partially occluded vessels
3) material from endarterectomy procedures
4) coronary arteries obtained from autopsy material

IV.8

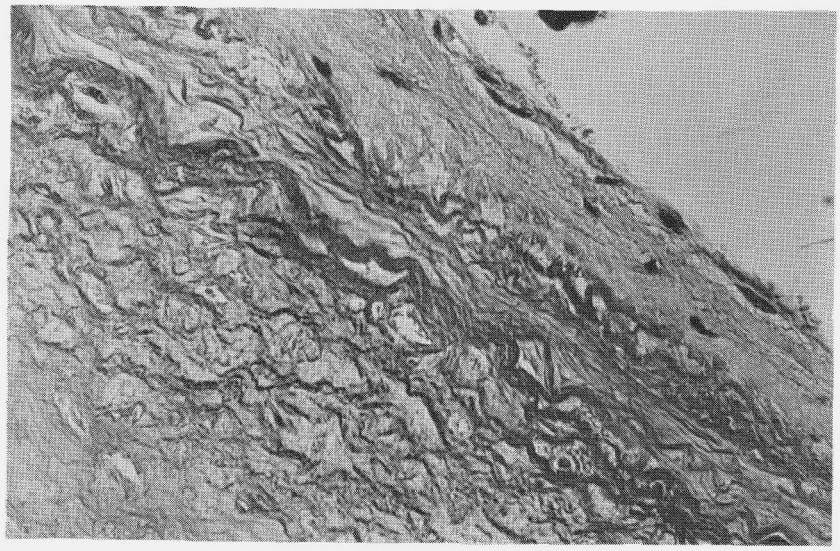
Microscopical results

Group 1: Embolectomy material. Embolectomy material consisted of young organized arterial thrombi with a long tail of stasis thrombus.

Fibrin and platelets were found in LMSB-stained sections. Most of the fresh red thrombi looked like venous thrombi, consisting mainly of erythrocytes, interspersed with fibrin. Platelets could not easily be recognized in this material. In the small, older part of the thrombus, some organization was found. These thombus parts consisted mainly of platelets and fibrin, resembling the experimental thrombi formed after one and two hours.
In small areas of the thrombus, leucocyte infiltration could be found, indicating the age of the thrombus mass. No collagen or elastin was seen. No smooth muscle cells or other mononuclear cells could be found in these thrombi. In studies with the Todd slide method, no fibrinolytic activity was found.
Immunohistochemical studies with anti-fibrin monomer serum showed positive reactions in peroxidase as well as in fluorescence staining. FITC-stained frozen sections generally showed more fibrin than could be found in peroxidase stained sections. Immunofluorescence with anti-platelet serum was positive in these fresh thrombi. No positive reactions were found with anti-Factor VIII:RAG and anti-SMC serum, in immunofluorescence studies.

To summarize: the embolectomy material consisted of a long thrombus mass; the head composed of a young platelet-fibrin thrombus, the tail mainly of a fibrin-erythrocyte mass.

Group 2: Totally occluded and partly occluded arteries.
Most of these arteries were superficial femoral arteries, as this vessel is the most diseased artery in the human leg. The composition of the occluding mass in the vessel was very constant: areas with arteriosclerosis, in combination with atherosclerotic lesions, were often covered by much younger arterial thrombi.
In the deeper layers of the occluded arteries, typical atheromatous lesions were found, while the media showed no or only slight abnormalities. Calcified spots and lipid deposits were observed in fields of connective tissue. Many fibroblasts were seen in this area.
These typical atheromatous plaques were covered with arteriosclerotic lesions and arterial thrombotic material of different ages in most of the specimens. Directly on top of the deep deposits, a thick layer of arteriosclerotic material



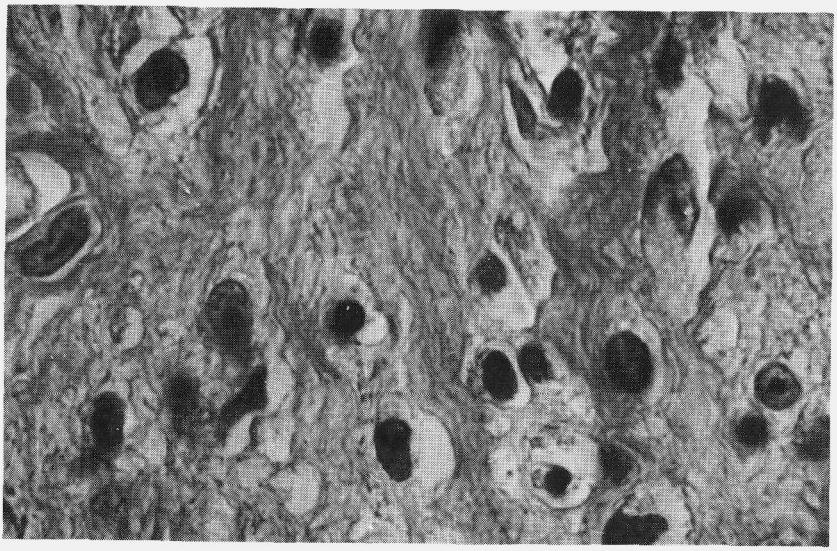


FIG. 41 Smooth muscle cells and elastin in a partly occluded human peripheral artery. Lawson MSB. (magn. 450x)

FIG. 42 Mononuclear cells of various types infiltrated into a platelet-rich area of human thrombus in a peripheral artery. Lawson MSB. (magn. 1650x)

was generally seen. In LMSB staining and in Levanol staining, the matrix consisted of collagen and newly formed elastin. SMCs were found within this material (fig. 41). When not exposed to the pulsatile blood flow, elastin was deposited in a fine granular form. Close to the surface of the arteriosclerotic lesions, elastin strands were observed. Incomplete covering with endothelial cells was generally seen on the surface of the lesion. In human specimens rethrombosis on the surface of the older lesions was common.

Virtually all cell types, such as PMNs, monocytes, smooth muscle-like cells and macrophages, as described in the section on experimental thrombus organization, were also found in the human specimens (fig. 42). The lesions, however, were more complex. Generally, arterial thrombus organization was secondary to lesions already present in the arteries. In some arteries, the lesion was not atheromatous, but arteriosclerotic. The composition of the lesions was practically identical to that found in our experimental study. In most vessels, fibrin was a common finding, generally situated as a rim on the luminal side of the vessel. Fresh thrombus formations stained brown in the immunoperoxidase staining for fibrin. No evidence of fibrin was found in the deeper parts of atheromatous lesions, while, in the more superficial parts of organized arterial thrombi, remnants of fibrin could be found. Immunofluorescence studies with anti-fibrin serum gave the same results as the immunoperoxidase studies; more fibrin, however, seemed to be present in the latter staining. Immunofluorescence studies with anti-platelet serum gave no clear-cut results. Thin rims on the surface of the luminal side of the vessel wall sometimes indicated fresh platelet apposition. In fresh thrombi, diffusely positive reactivity was seen. No platelets were found in organized thrombi.

In the original vessel wall, smooth muscle cells were present in their normal position between the elastin strands, as shown by anti-SMC serum.

A positive reaction to this serum was not regularly observed in the atheromatous parts of the occluded vessels, whereas an abundant number of SMCs was seen in the organized thrombotic masses. With anti-Factor VIII:RAG, endothelial cells were seen often covering the lumen of partly occluded vessels. Wide gaps, corresponding to rethrombotic areas were, however, present. Sometimes, it was necessary to use a high magnification to localize the slight fluorescence of the endothelial cells with anti-Factor VIII:RAG.

The organized thrombi differed in composition from the experimental thrombi by the presence of more collagen. In the atheromatous lesions, much collage-

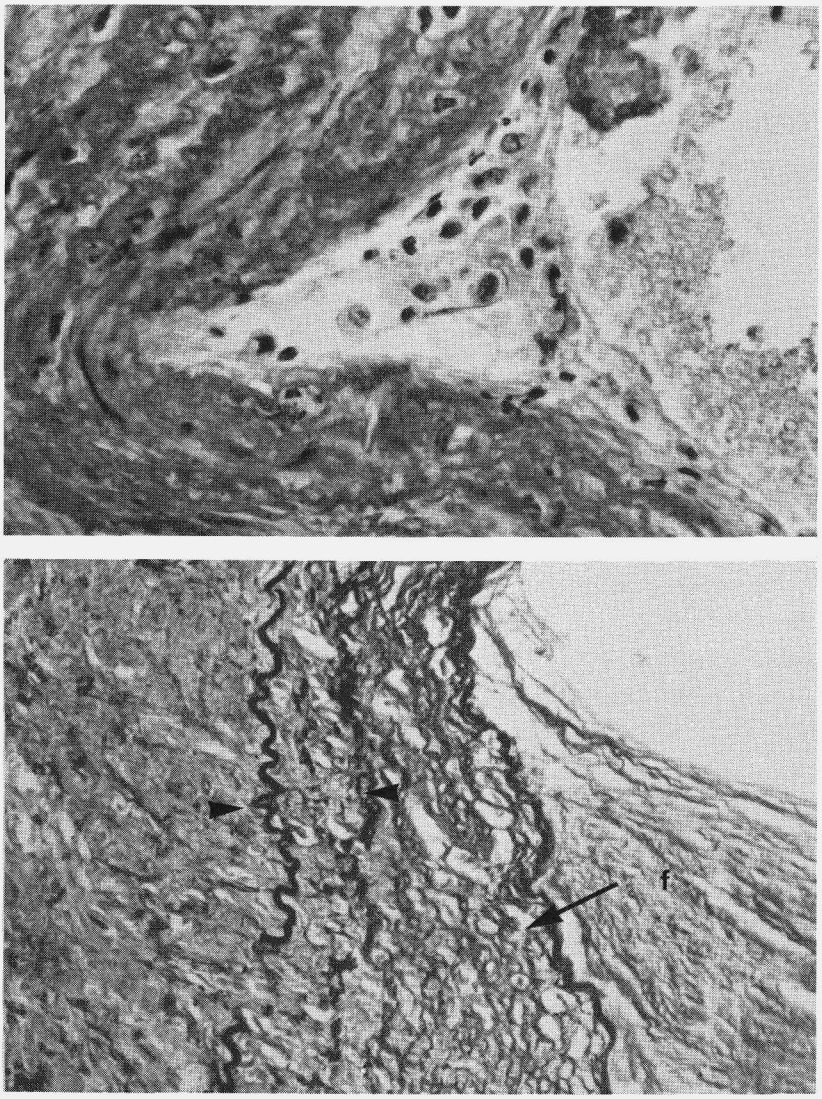


FIG. 43 Organizing mural thrombus in a venous bypass. Estimated age: six days. Lawson MSB. (magn. 750x)

FIG. 44 Thickened intima of a human coronary artery, rich in newly formed elastin strands (arrow), resembling the organized lesions of rat aorta. Original media between arrowheads. On top of the lesion an acellular fibrous plaque (f). (magn. 450x)

nous material was present, while the human arteriosclerotic lesion also showed definitely more collagen than was seen in the rat.
In the collected material, two venous bypasses were included. In one specimen, the vein interposition must have been functioning, because no thrombotic material was found. Extensive fibromuscular intimal hyperplasia was present. Many smooth muscle cells were seen in hyperplastic parts of the vessel wall in LMSB staining, as well as in Levanol staining.
Collagen and elastin were also abundantly present in the hyperplastic parts. In this specimen no fibrin was observed. (No immunofluorescence studies were performed.) Another bypass was clinically occluded by thrombosis; microscopically, an arterialized vein with an arterial thrombus was seen (fig. 43). A laminar composition, represented by different ages of thrombus organization in the vessel, was present. In the hyperplastic intima, SMCs were found. Foamy cells were present in the younger thrombus parts, and only a small lumen was left. With immunoperoxidase staining no fibrin was found in the wall. On the surface, however, fibrin was present. This finding corresponded with the results of the immunofluorescence studies.
Immunofluorescence studies with anti-SMC serum showed many SMCs in the organized thrombus part (age: more than two weeks). A new lamina elastica interna was being formed. In the original vessel wall, no immunofluorescent SMCs were observed. In some parts of the vessel wall, the lumen showed positive fluorescent reactions with anti-Factor VIII:RAG, indicating a more organized and already invested thrombus mass.
To summarize: in an occluded vessel a combination of atherosclerotic, arteriosclerotic and arterial thrombotic material can be found.

Group 3: Material from endarterectomy procedures.
A large part of the collected clinical material consisted of material from endarterectomy specimens. During an endarterectomy, a part of the vessel wall and the occluding mass is bluntly removed, preferably in cleaving planes. The aim is to remove the intima and to leave the media intact.
In the material thus obtained, larger and smaller parts of the vessel wall are seen. For the greater part, the material consists of atheromatous plaques, with or without fresh thrombi. Generally, young arterial thrombi were found on the removed material. All stages of organization could be found in these thrombi; starting with fibrin and platelets, followed by cellular infiltration

and organization, investment of the thrombus and formation of elastin and collagen.

Immunoperoxidase staining for fibrin showed a positive reaction in virtually all specimens, in agreement with the immunohistochemical fluorescence procedures for fibrin. In the arteriosclerotic parts, SMCs were also visible with fluorescence methods. Factor VIII, representing the presence of endothelial cells, could usually not be seen.

Anti-platelet serum showed platelets along the surface of the thrombus and in clusters inside the newly formed intraluminal thrombus masses.

No great differences between group 2 and 3 were found.

The atheromatous lesions proved to be the same, consisting of so-called hyalin material, interspersed with calcified areas and lipid-filled necrotic cell masses. The hyalin material consisted mostly of collagen with a sparse disposition of elastin and a varying amount of cells, depending on its localization. These cells looked like fibroblasts; some smooth muscle-like cells were also seen.

In the atherosclerotic lesions, many haemorrhages were seen, as well as areas with intense iron pigmentation. The haemorrhages were sometimes long-stretched and seemed to be located between two layers of the atherosclerotic lesion. Iron pigment was seen in various isolated spots. Haemorrhages located just under the luminal surface of an atherosclerotic, or arteriosclerotic lesion, were also a common finding in the human material, but not in the experimental material.

Fibrinolytic studies were performed on twelve specimens from each of the groups. In a fresh thrombus, obtained by embolectomy, no fibrinolytic activity could be found. In five of the other eleven specimens from group 2 and 3, fibrinolytic activity at the intimal side of the thrombus or vessel wall was present.

In two cases, a very strong fibrinolytic activity was found inside the thrombus mass itself. Fibrinolytic activity in the adventitia was present in all specimens.

Group 4: Coronary arteries.

Thirty coronary arteries, taken from autopsy material, were also studied. The striking difference with the other peripheral material was the invariable presence of arteriosclerotic lesions without degenerative changes. Three

staining methods were performed on this material (LMSB and Levanol staining and immunoperoxidase staining for fibrin).

In this material, the same types of lesions were found, as well as organized and young arterial thrombosis in different stages of organization.

Many sites with rethrombosis were seen. The thickened intima often resembled the organized lesions seen in the experimental study (fig. 44). Large fields of red-pink background material, interspersed with cell bodies and sparsely laid down elastin, could be seen. In the deeper parts some collagen was seen. In the Levanol staining, the matrix of these fields was stained yellow, and contained cells with a dark blue cytoplasm and red nuclei, indicating the presence of smooth muscle-like cells. In coronary arteries, still perfused at the time of death, newly formed strands of elastin were seen near the surface of the organized thrombus masses.

Fibrin staining indicated the presence of fibrin in the diseased intimas in 45% of 38 unselected cases.

To summarize the results from the human material, it can be stated that in the human peripheral vessels atherosclerotic lesions, as well as arteriosclerotic lesions, were generally found; much fresh arterial thrombosis was seen on these lesions; places with rethrombosis were common; the coronary arteriosclerotic lesions were not entirely comparable with peripheral lesions.

IV.9

Transmission and scanning electronmicroscopical results

In electronmicroscopical studies at various representative time intervals, the results were obtained confirming the lightmicroscopical data. In the early time intervals (one and two hours), the greyish thrombus mass was composed of platelets and fibrin. More fibrin was situated near the surface of the mass, whereas diffuse fibrin strands were seen between the platelets (fig. 45).
Platelets did not have a stable form. After two to three hours, groups of degranulated platelets and platelet ghosts were seen, forming an even more diffuse mass (fig. 46). Erythrocyte ghosts were also present.
More clearly than in lightmicroscopical studies, an early appearance of PMNs, mostly attached to the surface, was seen (fig. 47). Electronmicroscopically, they could easily be identified as normal PMNs, mostly with a typical form of the nucleus. In later time intervals, PMNs infiltrated the thrombus and mixed with the platelets, erythrocytes and fibrin. Observations in LM and EM-studies showed no discrepancies up to six days.
After these six days to two weeks, a starting investment was seen by endothelial-like cells. Some parts were really covered by these latter cells (fig. 48), while other parts were only partly covered (fig. 49). The nature of these cells could not positively be identified, not even in EM studies.
Smooth muscle-like cells, inside the thrombus, showed a large amount of endoplasmatic reticulum, indicating synthesis. After two weeks, granular deposits of elastin were observed around these cells (fig. 48 and 49).
At later time intervals, this elastin became more laminar, while the smooth muscle-like cells showed less endoplasmatic reticulum and more fibrillar structures, similar to normal SMCs (fig. 50).
After three months, a more regular composition of the wall was found, with normal appearing endothelial cells (fig. 51)
Collagen was not found in perceptible amounts in these time intervals. Recurrent thrombosis was seen after six days on uncovered areas. Loose platelet-fibrin formations were observed on the denuded wall (fig. 52).
In conclusion, it can be stated that no differences could be observed in LM and EM-studies. It was not possible, however, to confirm every microscopical observation by ultrastructural study.

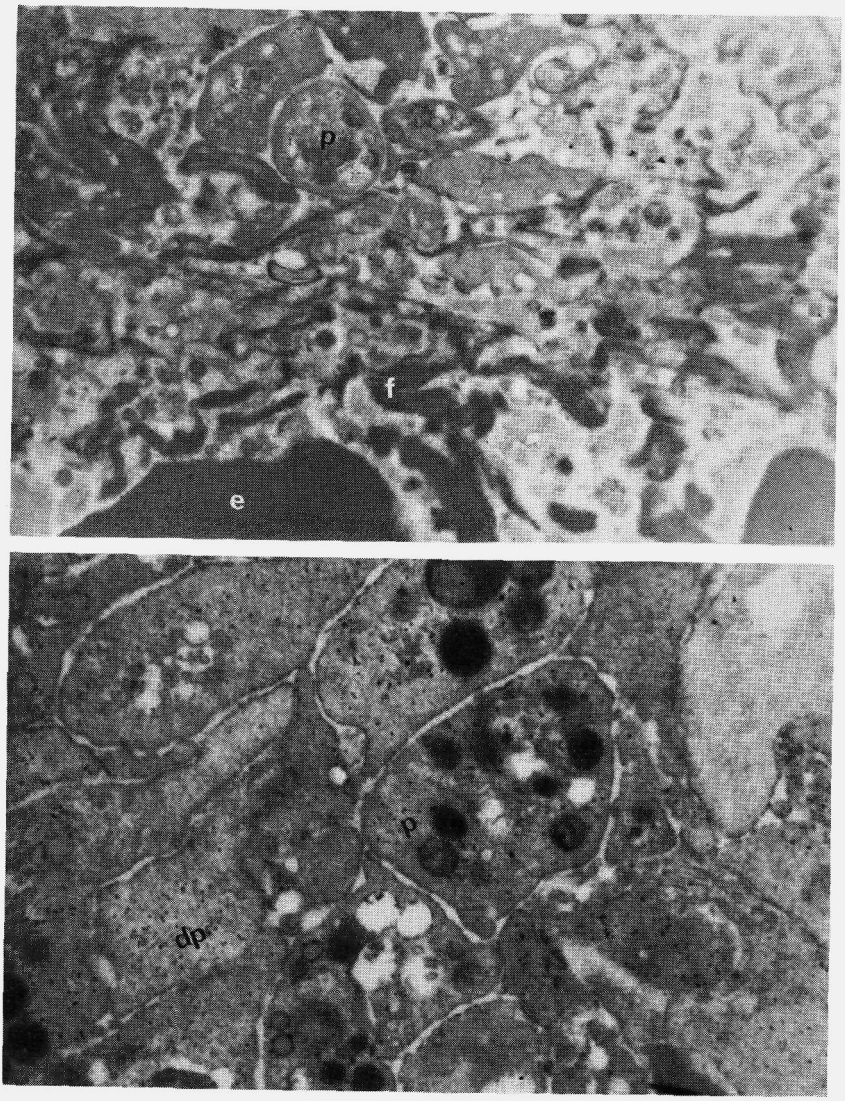


FIG. 45 Electron micrograph near the surface of a fresh arterial thrombus of two hours. Fibrin (f) and platelets (p) are closely opposed. An erythrocyte (e) is also seen. (magn. 14.800x)

FIG. 46 Demonstration of a mixture of platelets (p) and degranulated platelets (dp) after three hours, forming a diffuse mass. (magn. 9600x)

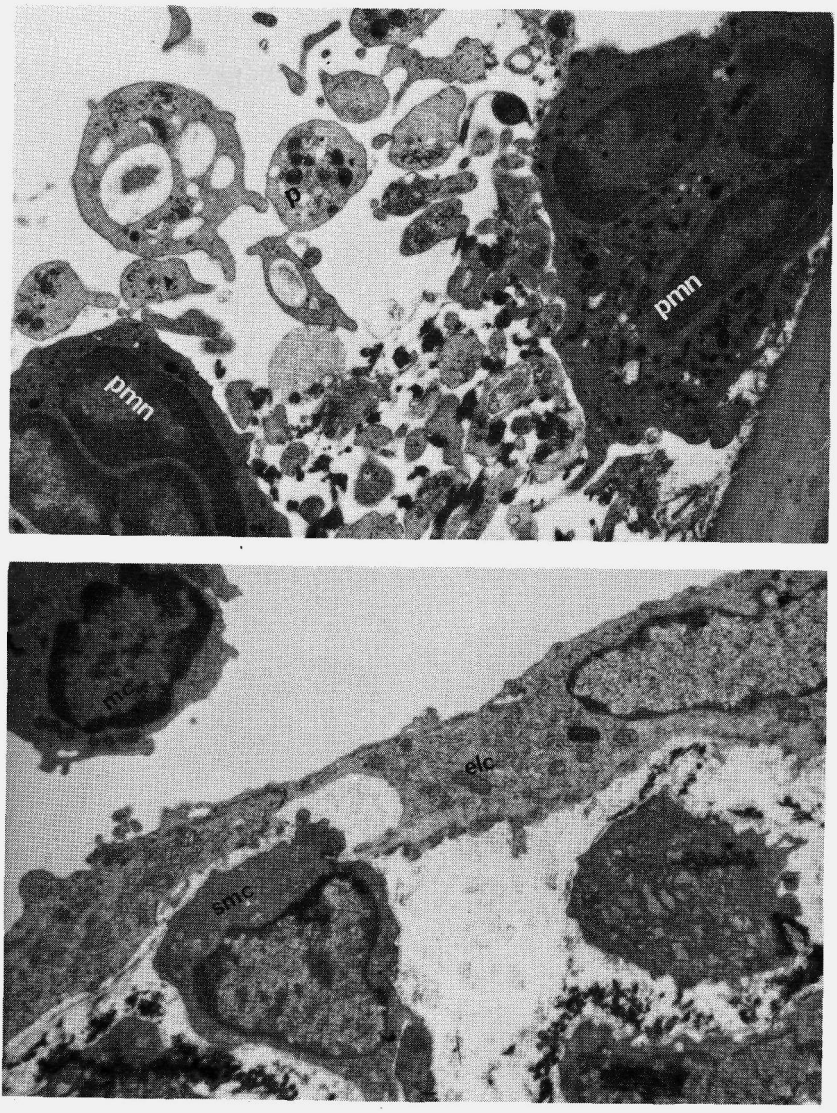


FIG. 47 Neutrophilic polymorphonuclear leucocytes (PMN) between loosely aggregated platelets and fibrin along the elastica interna of a deendothelialized vessel wall. Three-hour old thrombus. (magn. 9600x)

FIG. 48 Investment of an organized thrombus mass by endothelial-like cells (elc). Under the lining smooth muscle cells (SMC) and elastin are present. In the lumen a monocyte (mc). Six days. (magn. 6800x)

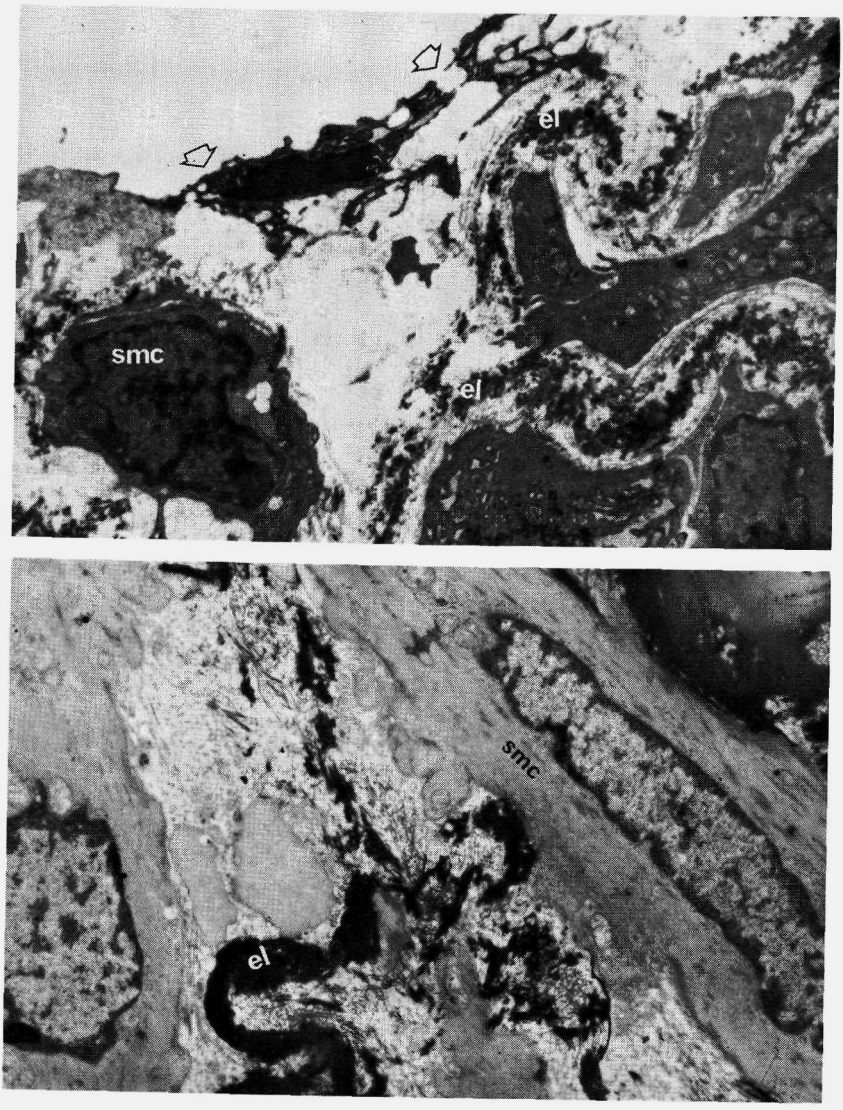
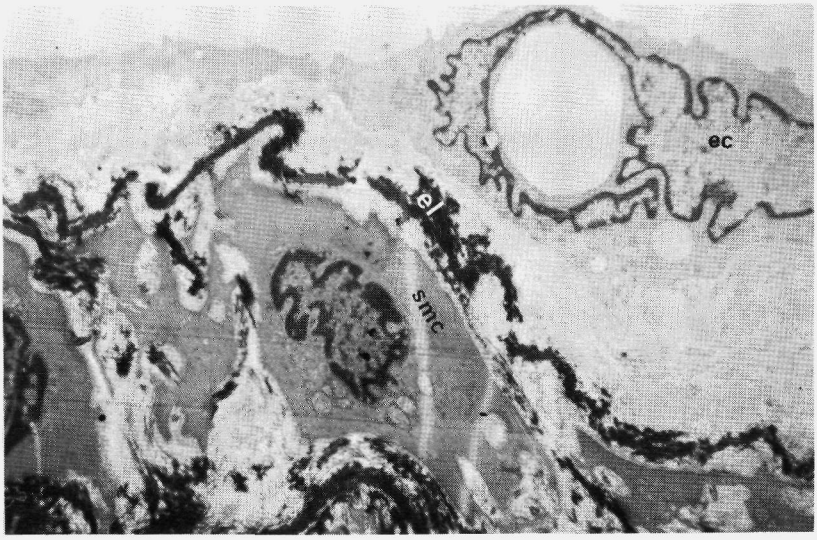


FIG. 49 Dead or damaged cells (open arrows) in the lining of an organized thrombus. Smooth muscle cells (SMC) are surrounded by granular elastin deposits (el). Six days. (magn. 6800x)

FIG. 50 Smooth muscle cells (SMC) surrounded by laminar elastic tissue (el) in a normal vessel wall. Note the paucity of endoplasmatic reticulum, and the large amounts of fibrillar structures in these cells. (magn. 9600x)



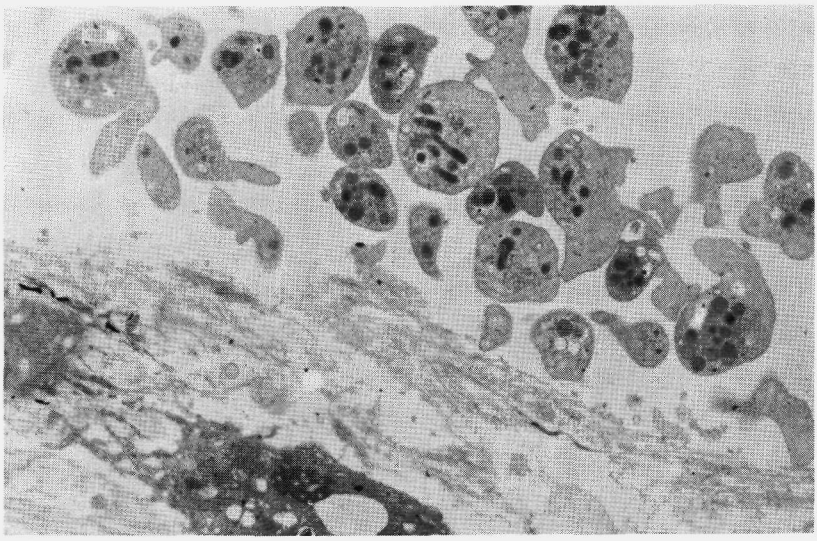


FIG. 51 Organized thrombus after three months. The smooth muscle cells have a fibrillar cytoplasm, and are surrounded by laminar-elastin. Large amounts of basement membrane are present below the endothelium (EC). (magn. 6800x)

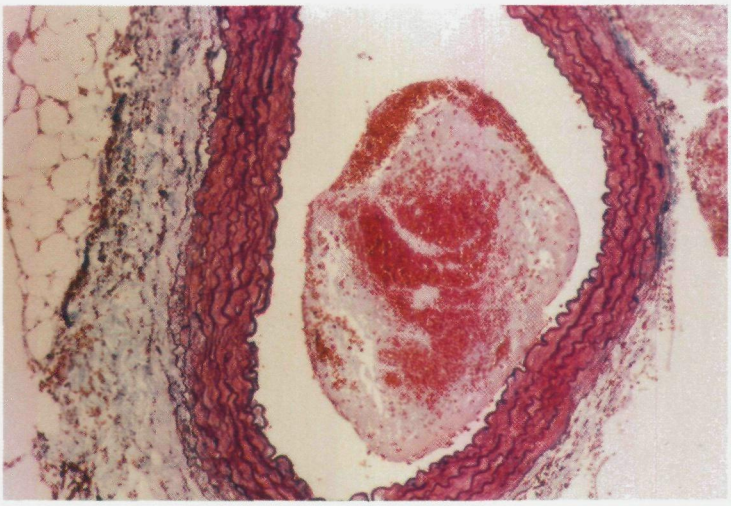
FIG. 52 Loose platelet aggregate on the (denuded?) surface of a six-day old thrombus. Rethrombosis area. (magn. 9600x)

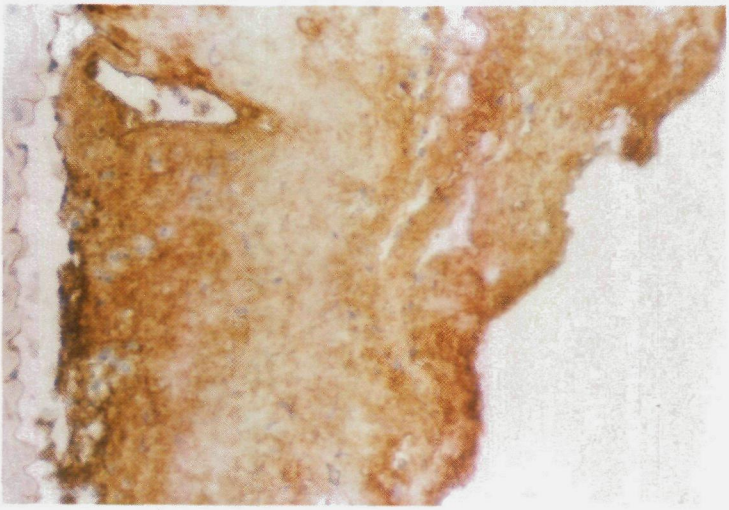
A good stereoscopical idea of the position of the flap in relation to the vessel wall could be obtained by scanning electronmicroscopical studies. Evenso, at later time intervals, increasing thrombus growth was seen, which obscured the flap almost immediately.

No additional results, concerning the composition and organization, were obtained with this method of investigation. Shortly after induction, distinct platelets, fibrin and erythrocytes were seen, quickly disappearing in the large amorphe mass, growing on the flap.

No serial scanning electronmicroscopical investigation was further performed.

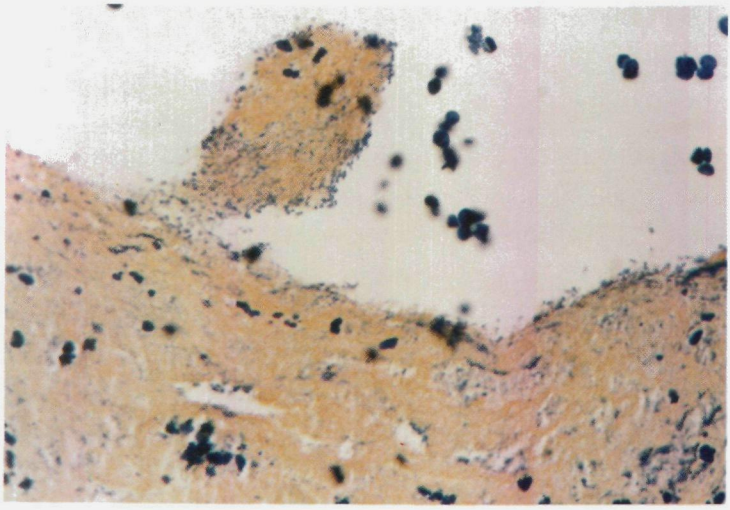
FIG. 53 Low power scanning electron micrograph of an aortic flap immediately after construction (zero hours), showing the suture line (sl) and the position of the aortic flap (F). Micrograph courtesy Dr. W.C. de Bruyn.

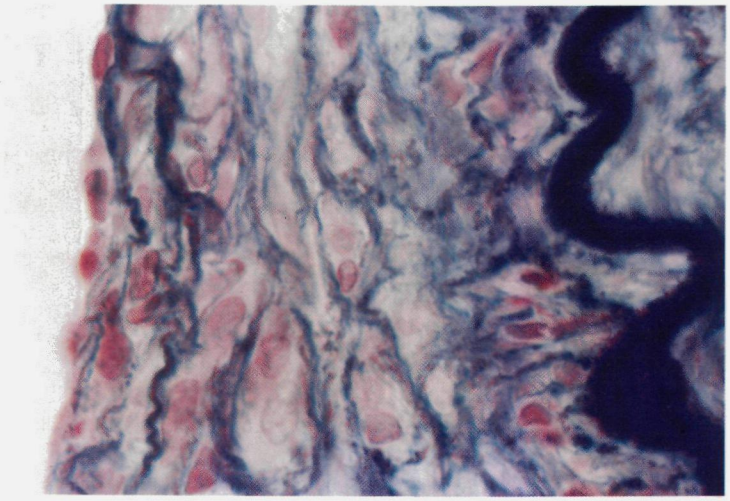




Color plate 1 Survey of a three-day old thrombus. Lawson MSB. (magn. 125x)

Color plate 2 One-day old thrombus stained for fibrin by immunoperoxidase histochemistry. Note the blue granulocytes inside the thrombus. (magn. 350x)





Color plate 3 Fresh platelet apposition on a twelve-hour old thrombus. Levanol staining. (magn. 350x)

Color plate 4 Newly formed lamellar and granular elastin in a two-week old lesion. Lawson MSB. (magn. 1250x)

CHAPTER V

DISCUSSION

Haemodynamical aspects. Based on the results of investigations on this newly developed model, an excellent, reproducable formation of an arterial thrombus can be claimed. The thrombus can always be found on the intraluminally inverted flap. The size of the thrombus can vary, but clear arterial thrombosis is always present. A specific pattern in thrombus formation is also observed. The largest thrombus volume is found just on the inverted flap and in distal direction. The aortic flap is frequently embedded in thrombotic material. Proximal to the flap less thrombosis is found. In the arteriotomy area, small amounts of thrombosis are encountered. The arteriotomy is a longitudinal incision, closed by interrupted sutures. Engler (1961) found a distinct difference in thrombus formation on transverse and longitudinal arteriotomies, dependent on the size of the artery. The small thrombus formation, in the course of the longitudinal arteriotomy, gave the impression of a normal healing process after a vascular incision. Thrombus formation was never found in places where microvascular atraumatic clamps were placed during surgery. This is confirmed by the results of the sham operated animals which showed no thrombus formation at all. Thrombogenic action of the used suture material was never observed.

The localization of thrombus formations on the flap and distally of the flap was confirmed by quantitative investigations. The amount of thrombus material was similar in time intervals of four and twenty-four hours. Because of the standard surgical conditions (qualitative as well as quantitative), the causes of thrombosis were evident. Haemodynamical causes do play an important part in causing thrombosis. Subendothelial tissue thromboplastins are important too (Astrup and Coccheri - 1962, Astrup and Claassen - 1967, Astrup - 1967, Huggins - 1969). A change in composition of clotting factors can be the origin of imbalance in the natural situation. Except for the change caused by tissue thromboplastins and fibrinolytic activity by tissue activators, prostacyclin and thromboxane production have a great influence on the local situation. The function of blood platelets must be stressed in the sequence of events (Chung-Hsin et al. - 1981).

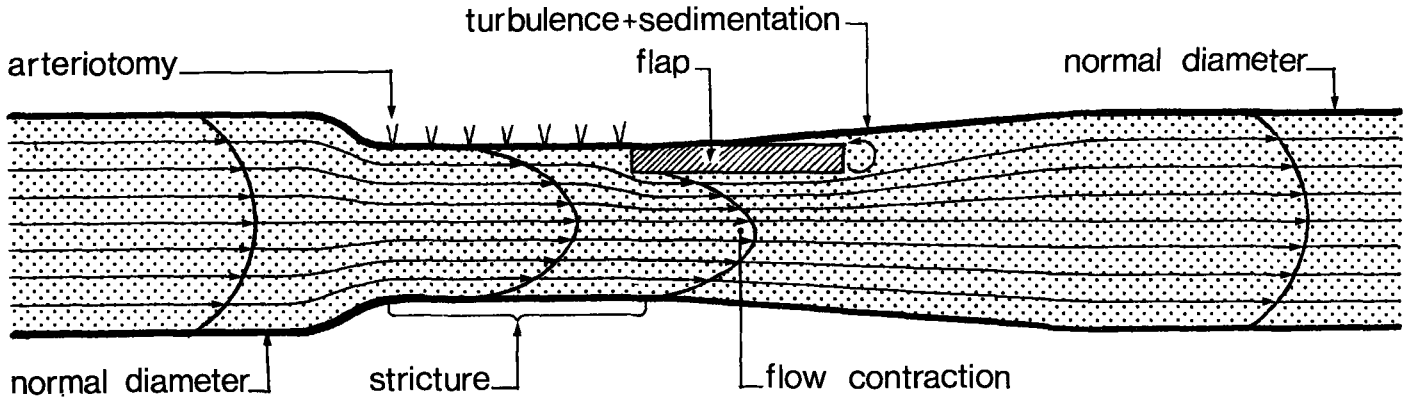


plate 11

dv/dr shear stress ↑
reynolds nr. ↑

intimal damage
turbulence ??

In considering the purpose of this investigation, a crucial question has to be answered: is this model for production of an arterial thrombosis comparable with the human situation and can the causes of thrombosis be extrapolated to man?

Bearing the haemodynamical considerations (chapter I) in mind, the following conclusions can be drawn from the experimental situation.

The flow alterations, caused by this flap procedure are threefold: firstly, the laminar flow in the aorta will encounter a gradual slight stenosis, caused by the closing of the arteriotomy; secondly, behind this stenosis, an abrupt narrowing will take place; thirdly, because of the intraluminal presence of the flap, the distal end of the flap is the start of the normal diameter of the aorta again. To summarize, the following haemodynamical changes will take place. (plate 11).

When a regular laminar flow enters a gradual stenosis, this will not normally cause great change. However, an increased velocity and an increase in pressure gradient will occur; a change in shear stress will, therefore, be present at this part of the lesion in the aorta.

The shear stress will become higher because of the increased velocity and the decrease in diameter. Hence, the Reynolds number will also increase.

$$T = \eta \frac{dv}{dr}$$

$$Rey : \frac{Vav.d}{v}$$

In the next part of the lesion, the flap will cause an abrupt narrowing of the vessel, which may cause a slight contraction of the flow, producing a more pronounced increase in velocity and pressure gradient. Consequently, the shear stress and the Reynolds number will show an increase.

It is not certain that this increase in Reynolds number will produce a turbulent fase, but the possibility exists. In this part of the lesion, the shear stress varies, and the blood is exposed to the subendothelial tissue of the adventitia.

At the end of the flap, a sudden divergence is present, causing a number of haemodynamical changes. The contracted flow will show a divergence, causing a dead corner, just at the end of the flap. Here, sedimentation and stasis are

likely to occur. Due to the change in diameter, the Reynolds number and the shear stress will alter.
Turbulence could occur as the result of the increasing diameter. Decrease in velocity, however, counteracts this.
To summarize, stasis and sedimentation can occur at the end of the flap; even turbulence can occur by a change in Reynolds number. A varying shear stress will also occur here. So, in the course of the lesion, the shear stress varies constantly, and the velocity and the pressure gradient follow the law of Bernoulli.
In the normal aorta, the fluid front has a parabolic shape. According to the law of Poiseüille, the shape of the flow front will become more convex in the course of the lesion.
It is not sure, what will occur in the course of the lesion. Theoretically, however, the varying shear stresses and changes in Reynolds number are established facts. Consequently, it is assumed that haemodynamical changes will occur in this situation. Low shear stresses (Matsuda - 1978), high shear stresses (Fry -1968) and varying shear stresses (Goldsmith - 1974) have been described as having a damaging effect on the endothelium.
Boundary layer separation, caused by varying shear stresses, produces a zone of sedimentation and stasis. These zones correspond with the predilection sites for atheromatous plaques (Fox and Hughes - 1966, Caro - 1972, Padmanabhan - 1980).
Gerrity and Naito (1980) found altered endothelial cell morphology after experimental coarctation. Bomberger et al. (1981) confirmed these observations by scanning and transmission EM, and described an enhancement of atherosclerosis distally to a subcritical arterial stenosis in monkeys fed an atherogenic diet for six months. Severe stenosis inhibited the distal development of atheromatous lesions. Gertz et al. (1981) performed experiments on dog coronary arteries and rabbit carotid arteries. They saw endothelial cell damage and thrombus formation distal to a partial ligation of the vessel, without altering the flowrate. A second ligature, aimed at reducing the blood flow, placed proximally to the first one, diminished the changes of the vessel wall distally to the first ligature.
In conclusion, a subcritical stenosis can be of high haemodynamical importance and can be the cause of endothelial cell damage and arterial thrombosis.

In our model this subcritical stenosis is also present.
Turbulence is known to be harmful to the intima. Many authors have reviewed this subject (Weslowski - 1965, De Boer - 1978, Nerem and Cornhill - 1980, Stein and Sabbah - 1974, 1980).

Tissue thromboplastic aspects. Apart from these haemodynamical causes, thromboplastins are also supposed to play an important part in the formation of arterial thrombosis. Huggins (1969) mentioned the importance of thromboplastins in the development of early thrombosis after vascular surgery. In dogs, he experimentally demonstrated a difference in the amount of thromboplastin in medium-sized and large arteries. Medium-sized muscular arteries have more thromboplastins than large arteries. Moreover, the solubility of thromboplastic substances from intact muscular vessels, was twenty to forty times greater than from elastic vessels. By cutting through all layers of the arterial wall, in our model, a lot of thromboplastic material is released into the bloodstream. At the same time, a relatively large surface of adventitia is brought into the lumen. The position of the flap in the aortic lumen will provide a long-lasting imbalance in the local composition of clotting and fibrinolytic factors, causing a genuine arterial thrombosis and not a haemostatic model, because there is no open continuity between the vessel wall and the surrounding tissue. Platelets are supposed to be influenced by thromplastic and thrombogenic activity of these inverted parts of the arterial wall, perhaps also effected by the presence of prostacyclin and thromboxane.
These factors fit the triad of Virchow, causing an arterial thrombosis. At the site of the arteriotomy, a number of changes in clotting factor composition, and a change in haemodynamics (as mentioned above) can be observed.
Many models of arterial thrombosis are solely based on endothelial damage. Is endothelial damage the only cause of arterial thrombosis? If, by whatever reason, the endothelium is damaged, a slight haemodynamical widening in the vessel is evoked. At the same time, subendothelial exposure to the bloodflow is accomplished. The extent and depth of such a lesion to the endothelium, as a result of repeated damage, determines the development of platelet adhesion or the formation of a platelet-aggregate (Davies - 1968, Stemerman - 1972). The introduction of foreign material only causes more confusion as to the real cause of thrombosis.

In conclusion, an arterial thrombosis in this model was formed by endothelial damage, local haemodynamical changes, and a change in local composition of the blood, without the use of exogenous material.

Surgical importance. From a surgical point of view, this model is important too. It stresses the necessity of avoiding the inverted aortic anastomosis. By exposing the various components of the arterial wall to the flowing blood, a subsequent thrombosis is likely to occur, due to a change in haemodynamical factors and/or a change in composition of clotting factors.
Basically, the model for arterial thrombosis causes the same alterations in the lumen of the vessel, although in lesser degree, as will similarly a badly performed aortic anastomosis. An aortic anastomosis is considered inaccurate, whenever a part of the vessel wall is inverted by a suture.
From the results obtained from histological, histochemical and immunohistochemical studies, it must be concluded that in an accurately performed (everting) anastomosis, the formed thrombus masses promote the healing process of the arterial wall. Whenever an aortic arterial anastomosis is performed inaccurately, the formed thrombus masses can be the cause of more thrombosis.
Huggins (1969) proposed that local thromboplastic concentration is an important factor, determining the immediate success of vascular anastomosis, graft or endarterectomy, rather than mechanical considerations based on the size of the vessel.
Inokuchi et al. (1961) developed a mechanical suture apparatus and noted that, in a perfectly performed everting anastomosis, about 1/3 of the regular amount of thrombus mass was found on the suture line.
There are more and more microvascular investigations to be found in current neurosurgical literature. Neurosurgeons tend to be more aggressive in curing and preventing strokes by intracranial, vascular microanastomosis.
The early causes of thrombosis, after anastomosing two arteries, are of interest. Piepgras (1976) also goes back to the triad of Virchow as the main cause of arterial thrombosis:

- change in coagulability of the blood (change in composition of clotting factors);
- disturbances of the bloodflow (haemodynamics), and
- abnormalities of the vessel wall (thromboplastins, subendothelial tissue).

He, too, concludes, as Van Gelder did in his thesis (1980), that a perfectly performed anastomosis excludes or diminishes the occurrence of Virchows' triad.

Rosenbaum (1977) suggests that the beginning stages of thrombosis, occurring during the first thirty minutes after revascularization, are due to technical errors.

Kletter (1979) wrote an interesting paper about the histology of microvascular anastomosis. In this study, he found thrombus masses due to: excessive use of suture material, extensive narrow end-to-end anastomosis and inaccurate suturing (Terpstra - 1966).

From the literature and from the results of our experimental study it can be concluded that:

1. The main causes of early thrombosis are:
   a. abundant presence of thromboplastins
   b. exposure of subendothelial tissue to the blood
   c. inaccurate surgical technique
   d. use of thrombogenic suture material
2. The model can serve as an example of a badly performed anastomosis in arterial vessels.

Much attention was paid to the thrombogenic properties of the suture material used (Dermalon -Davis and Geck- with a C4 needle). In the anastomosis, as well as in the flap series, the material proved to be athrombogenic. Sometimes little chronic reaction was found. No giant cells, attracted by foreign body reaction, were seen in the surrounding of the suture material. These observations were in accordance with the results of Van Gelder (1980) and Kletter (1979).

Klaritzky (1954) and Bertin (1977) published papers on tissue reaction to nylon suture material. They concluded that the material was not totally inert, but merely caused little reaction, which is confirmed by this study. The suture material did not influence the patency of the vessel.

The microsurgical method is not developed for microsurgically skilled personnel only. After training and adequate instructions, anyone with surgical experience can master this method.

The surgery, performed with a tenfold magnification, must be atraumatic. By frequently grasping the vessel wall with a forceps, one could easily produce

intimal trauma, and thus initiate thrombosis. Blunt dissection of the retroperitoneal area is a necessary procedure.

It is not known how the exact amount of tissue thromboplastins can be measured in the circulating blood of rats after this manoeuvre. It is not known in what proportion the surgical procedure causes disregulation of the rat thrombogenic and/or thrombolytic system.

The size of the rat aorta may be another disadvantage in processing procedures. In this study, however, this point was not at stake. There were sometimes problems with the sectioning on the levels with sutures.

The use of microsurgical atraumatic vascular clamps (Aesculap microclips - 20-30 grams/cm^3) did not cause visible damage to the vascular wall. Anyway, no thrombosis was seen in the control sham series, nor were intimal lesions found in the vessel wall, directly proximal or distal to the aortic anastomosis.

Van Gelder used Schwartz clamps, with a pressure of more than 30 grams/cm^3. He observed clear intimal damage without any evidence of occlusion of the vessel due to these lesions. Van Gelder and Kletter both observed a constant necrosis of the vessel wall on the suture line.

The same observation was made in our study. After five to six days, the original vessel wall sometimes seems to disappear, although the vessel is patent. Proximally and distally, a normal perfused vessel can be found. Instead of the normal vessel wall, masses of organizing thrombus are found, evidently strong enough to withstand the aortic bloodpressure. The presence of the blood pressure must be a stimulus in further organizing the arterial thrombus mass. The necrosis of the vessel wall, in the suture line, must be caused by the knots of the suture material. This necrosis was not important concerning the patency of the vessel (Kletter - 1979, Van Gelder - 1980).

No further comment has to be given on the results of the sham operations. Placing the vascular clamps or manipulating the vessel wall never caused arterial thrombosis.

Although no experiments were performed solely with an arteriotomy, the results obtained in flap operations show a purely haemostatic reaction on the arteriotomy proximal to the flap. It is, therefore, likely that a properly closed arteriotomy alone will not give rise to thrombus formation.

With regard to the methods used for tissue processing, the following remarks can be made.

Before harvesting the aortas from the experimental animals, perfusion with saline at a pressure of 130 cm H_2O was used. With perfusion through the left ventricle of the heart, the vessel was rinsed in order to remove all blood or bloodclots. This method proved satisfactory, because the formed thrombus was uninfluenced by this manoeuvre. Few postmortal clot formations were encountered. The specimens were primarily fixed with perfusion in situ with polymerized formaldehyde, followed by postfixation with the same fixative for 24 hours.

Specimens for scanning EM and transmission EM were submitted to the same treatment with glutardialdehyde (1,5%) in 0,067 M cacodylate buffer. The results of these fixative procedures were satisfactory.

Rinsing the specimens in order to remove remaining bloodclots with a syringe under manual pressure is not to be recommended. Insufficient pressure control is obtained by using a syringe and needle; the danger of blowing the formed thrombus away, especially the young ones, is present. The natural position of the specimen is another advantage of total body perfusion. The formed thrombus mass cannot be damaged by manipulating the entire specimen in order to rinse it clean.

Staining methods. Standard staining was performed with the LMSB staining; first described and used by Lindeman (1976). With this staining method, fibrin stained red and platelets greyish-blue; an advantage of this method is the colour contrast, so elastin and collagen are easily recognizable and beautiful staining of the nuclei is present.

All specimens were, therefore, screened with the LMSB staining.

Staining for lipid and iron were done in the first investigations; as no lipid or iron was present in the thrombi, these stainings were not used in the results.

The second general staining was the Levanol method (Van Pelt-Verkuil - submitted- 1982), staining the muscle protein.

A correlation could be made between the suspected smooth muscle cells in the LMSB and in the Levanol staining. Smooth muscle cell bodies stain blue, while the nuclei take a reddish colour. In this staining, platelets take a blue colour and are easily recognizable, especially in fresh appositions.

Because of the new formation of collagen and elastin, special staining for these substances was used. The development of elastin was observed speci-

fically, while the formation of collagen in the experimental series was not very spectacular. In human material, more collagen was formed in the lesions.
Immunoperoxidase staining for fibrin was extremely helpful in determining the nature of some invading cells in the thrombus. The nuclei of various cell types were beautifully displayed with counterstaining by haematoxylin. The presence of fibrin in the peroxidase staining was easily recognized and confirmed by immunohistochemical fluorescence techniques, as well as in LMSB and Levanol staining.
Craane et al. (1978) described this method for detecting fibrin during diffuse intravascular coagulation in rats, using antisera against rat fibrinogen and fibrin (as used in this study). Specifically fibrin is stained by this method, as described by Emeis et al. (1981).
Immunohistochemical fluorescence studies were performed, using FITC-conjugated antisera against fibrin, SMCs, platelets of the rat, and Factor VIII. By using these methods, the results obtained from the histological methods and the results of the immunohistochemical methods, could be compared.
Antiserum against SMCs was obtained from patients with an autoimmune-disease. When tested on rat stomach, liver and aorta, by indirect immunofluorescence, the antiserum specifically demonstrated the SMCs of the rat. The SMCs are beautifully displayed by this method; the results from this study were comparable with the observations in the Levanol and LMSB staining. Anti-platelet serum did not give the same specific results, as the ones obtained with, for instance, anti-SMC serum, but when a difference occurred, these immunohistochemical investigations proved to be important. Studies with anti-platelet serum were used especially for rethrombosis. After absorbing the antibodies against rat fibrinogen and some other serum proteins, good specifity and valuable results could be obtained with this method.
Due to the sensitivity of the platelets to fixation procedures, this method was only used on frozen sections and indirect immunofluorescence.

Rabbit anti-Factor VIII:RAG was obtained from the Haematology Department of The Netherlands Red Cross (Amsterdam). Factor VIII is only present in the vessel wall in the endothelial cells (Hoyer et al. - 1973, Jaffe - 1973 and Gruson - 1974). Because of the specific localization of Factor VIII, this anti-Factor VIII:RAG is important in distinguishing endothelial cells from SMCs. The investment of the lesion with new or other endothelial cells, and the time

that is necessary for the total investment can easily be studied with this method. The high rate of rethrombosis raised a new perspective. It was interesting to see whether rethrombosis occurred on normal surfaces, covered by endothelial cells, or only on surfaces invested with other types of cells, or totally without cells.

Van Pelt-Verkuil et al. (1981) tested the specificity of this antiserum for Factor VIII; no antibodies against fibronectin, fibrinogen or other serum proteins were detected.

Rabbit anti-human lysozyme was used to study the behaviour of leucocytes in the thrombus. No reliable results were obtained from these investigations, presumably due to the lack of cross-reactivity.

No further anti-lysozyme fluorescence was performed because it was clear, from regular histological studies, that many kinds of leucocytes were present and easily recognizable.

Pre-immune rabbit serum was used for control studies; only a small amount of background fluorescence was found on these slides.

In order to get an idea of the real situation in the vessel, scanning EM was performed. The investigation into the development of an arterial thrombosis on this level, was not a serial one; in order to follow the development and growth of the thrombus during the first three days, three aortas were examined.

A good view of the intra luminal situation is given by scanning EM, making it possible to see the flap lying in the lumen, and partially obstructing it. After three hours, a thrombus mass is present; at a higher magnification the mass existed of platelets and fibrin strands.

This observation was additional to the results already obtained. The only aim was, however, to get a proper stereoscopical view of the intraluminal situation.

Transmission EM was used to get a better determination of some cells: were these cells really those referred to in this thesis as SMCs? Using a very high magnification, one can be fairly sure of the nature of some cell types which are difficult to determine by other methods of investigation.

One of the reasons this study was performed, was to establish whether arterial thrombosis is the start of more vascular pathology (specifically arteriosclerosis and atherosclerosis).

It is important to investigate the thrombogenicity of the lesion and the formed thrombus masses. Many papers dealing with the organization and investment of arterial thrombosis are available. A detailed description of arterial thrombosis can be found in every handbook of pathology. However, it has not been definitely established, whether there are fibrin and platelets present at the inception of the thrombotic mass. No further attention will be paid to the lipid deposits, only arteriosclerotic lesions will be considered important, whereas atheromatous lesions will be mentioned without further explanation.

Thrombus morphology.

Platelets/fibrin. The results of the experimental study showed arterial thrombosis, during the first one to three hours to be composed of platelets and fibrin. In reviews, Wharton-Jones (1851) and Zahn (1875) mentioned that blood cells were necessary in the thrombus mass of traumatized arteries and veins. Bizzozero (1882) could prove that the blood elements in these thrombus masses were platelets. The important role of platelets in the early stages of experimental arterial thrombus formation was again confirmed by subsequent observations, including those made by Fulton et al. (1953) on vessels in the hamster cheek pouch and by Honour and Russel (1962) on vessels in the cerebral cortex and mesentery of rabbits. In his published theories, Duguid mentioned the important role of platelets in the formation of arterial thrombosis.

Macroscopically, venous thrombi are easy to distinguish from arterial thrombi. When formed during life, venous thrombi have a rather dry granular appearance, whereas arterial thrombi have a more compact structure. Venous thrombi have a predominantly red colour and arterial thrombi are usually pale, sometimes interspersed with dark red areas. The microscopical image of arterial and venous thrombi is also described by French (1964). Chandler supported the opinion of French by stating that thrombi are formed by a mixture of blood elements. Platelets, fibrin and leucocytes are important at this point. No clear distinction is made between arterial and venous thrombi. The proportion of the constituents may vary with the origin of the clots. Thus, the structure of thrombi and clots is related to the mechanism of their formation. In 1970 and 1971, French wrote reviews on the subject of arterial thrombosis. He tried to relate arterial thrombosis to atherogenesis. The role of platelets and fibrin became more and more important. The stimulus for

platelet aggregation is being discussed. Collagen, not elastin, is a stimulus for platelet aggregation, as well as for ADP-release. In his description of the pure thrombosis, he mentioned that he could not demonstrate the presence of fibrin between the platelet masses by normal histological techniques. Fibrin was only detected on the surface of a platelet mass and did not seem to play an important role in the start of an arterial thrombus. The origin of an arterial thrombosis is extremely important because the real origin of atherosclerosis is unknown.

Many authors tried to relate arterial thrombosis to atherosclerosis, for instance Spaet - 1964, 1970, 1977, French - 1971, Haust - 1971, Woolf - 1978 and Moore - 1979.

In 1971 Haust stated that three basic lesions, i.e. fatty dots, grey-gelatinous elevations and microthrombi, can be regarded as potential early atherosclerotic lesions. In the results of our experimental study, arterial thrombosis was found in the flap series, as well as in the anastomosis series. Platelet masses were seen with cellular elements in between the strands of fibrin, while the surface of these platelet-fibrin thrombi were often covered with a fibrin rim. With normal histological techniques, the fibrin in the thrombus could not be seen, but only suspected. With immunohistochemical methods, however, fibrin could easily be shown inside the platelet mass. The ratio platelets-fibrin was in favour of the platelets. The reason why the thrombus mass was not swept away as an embolus, is speculative. Probably, in the first moment of thrombus formation, the thrombogenic stimulus is extremely high, so a thrombus is rapidly formed. Parts of this yet not organized thrombus must embolize. The observed thrombus is likely to be a remnant of the original thrombus. French (1971) refers to the observation of Mustart and co-workers, that a platelet-fibrin thrombus is unstable. After thrombus infusion in swine, they observed a decreased number of platelets and formation of platelet-fibrin thrombi in small vessels. These thrombi tend to break up over a period of two hours, and the platelets return to the circulation, as is shown by platelet counts. The quantitative measurements in our study, showed practically no increase in thrombus volume in the first four to twenty-four hours. Due to the high thrombogenicity of the thrombus this is a peculiar observation. Thus, the probability of embolization is very high, but is not likely to be discovered. The emboli must be small and are probably of no clinical importance to the experimental animal. If there had been no embolization, the thrombus would

have grown faster. No clear evidence of embolization is present; in order to establish this, the hind legs of the rat would have had to be dissected in minute sections.
A difference in thrombosis induction exists; perhaps the thrombi found on the damaged parts of the vascular wall are more stable than thrombus-induced thrombi. It is not certain what caused the arterial thrombi to break-up. Speculations on high fibrinolytic activity are made. In our experimental study, no resolutions of platelet-fibrin thrombi were ever observed, but it is likely that in a number of cases only remnants of thrombi were found and investigated.
Zahn (1875) made the laminated structure of the platelet-fibrin thrombus generally accepted. Laminated structures remain a common finding in thrombus formation, although they are more clearly visible in a venous thrombus when more fibrin is incorporated. The formation of an arterial thrombus is probably due to the mechanism of rethrombosis, which is quickly repeated in the early stages. In arterial thrombosis, platelets predominate over fibrin. The presence of platelets and fibrin in early time intervals was confirmed through EM studies. Large masses of composed material, gradually infiltrated by PMNs, were found. In the early time intervals, the presence of fibrin between platelets was clearly demonstrated (fig. 45). Fresh arterial thrombosis, therefore, does not consists exclusively of platelets, but is a combination of two components. In EM pictures, the laminated structure was also demonstrated. The crucial question is, whether the arterial thrombus mass will stay attached to the wall, or will it embolize? By staying attached to the wall, and then becoming a firm and stable arterial thrombus, organization will follow.

PMNs. The migration of various cell types could be followed throughout the different time intervals. After three hours, migrating and superficial PMNs could generally be seen in flap thrombosis, confirmed in EM observations. In some specimens, even in one and two-hour time intervals, PMNs could be sporadically observed, which were mostly neutrophilic granulocytes. In the series with the aortic anastomosis, however, leucocytes were more frequently seen, beginning after two hours; the amount of fibrin and platelets did not show any change. The reason of leucocytes infiltrating the thrombus mass is not exactly known. Hofmann et al. (1980) published an ultrastructural study on arterial thrombosis in the rat carotid artery. Thrombus formation was

evoked by chilling (described by Meng and Seuter - 1977). Platelet agglutination occurred in the centre of the platelet aggregate within five minutes and led to thrombocytorrhexis after thirty minutes. He saw an increasing fagocytosis of monocytes and leucocytes, stimulated by the resulting cellular "waste". A massive leucocytosis was found after four hours, as a result of early thrombocytorrhexis. A viscous metamorphosis occurred after twenty-four hours, both in fibrin-poor and fibrin-rich platelet aggregates. In this study, objections could be made to the method of thrombus induction. According to the author, the triad of Virchow is also achieved, but the exact origin of the thrombus is unknown. The most probable cause is the cold stimulus.

In 1965 Henry devoted a paper to the role of leucocytes in thrombosis. He showed that leucocytes can no longer be considered as trapped elements, but as active elements in thrombus organization. According to the author, they appeared to derive from the passing blood, and not from the arterial wall. Leucocytes have been shown to be lytic to fibrin (Riddle - 1964) and other proteins, thus contributing to the thrombus dissolution. Rebuck and Crowly (1955) presented clear evidence that neutrophilic-released fragments were ingested by mononuclear leucocytes, while Richardson et al. (1976) showed chemotaxis for human monocytes by fibrinogen-derived peptides. This transportation of aminoacid residues, lipids and carbohydrates was considered to be a cytotrophical stimulus for proliferating cells during thrombus organization. Henry also mentioned the fibrinolytic activity of eosinophilic cells, carrying the precursor profibrolysin. Kwaan and Hatem (1978) published a short paper about the early release of fibrin by eosinophilic cells. They described an accumulation of these cells six to twelve hours after thrombus formation, followed by a quick disappearance of these cells when fibrin was transformed into long, striated, dense bundles and the platelets had disappeared. In their opinion, eosinophilic cells are attracted by the early formation of fibrin, associated with platelet aggregates. Neutrophilic and eosinophilic cells have no lytic function in the already formed thrombus. Gottlob et al. (1978) stressed the property of leucocytes to digest fibrin; they contain elastolytic and collagenolytic enzymes.

In his paper, Gottlob demonstrated the possible thrombolytic promoting activity of desintegrated leucocytes. Comparative studies showed an increased thrombolysis after incubation of the clot with leucocytes and streptokinase. The mechanism whereby leucocytes potentiate the action of streptokinase, is

not yet understood. Some authors believe that eosinophilic leucocytes contain plasminogen (Riddle and Barnhart - 1964). Anyway, leucocytes were able to penetrate and dissolve the formed thrombus mass.
In our study, we observed an increasing amount of leucocytes after three hours of thrombus formation; this observation corresponds with the literature. No fibrinolytic activity was ever observed in these neutrophilic or eosinophilic leucocytes. After two days, a few, quickly disappearing eosinophilic leucocytes were found. More eosinophils were seen in human material.
The results of our study tend to support the view that these leucocytes have a strong infiltrating impulse, come from the passing blood, and adhere to the rough surface of the thrombus. After twenty-four hours, their number stopped increasing. By that time, gaps in the platelet-fibrin mass are seen. When the amount of polymorphe leucocytes diminishes, mononuclear cells start to appear in the thrombus mass. This may very well correspond with the cytotrophical function, found by Rebuck and Crowley. It is possible, even probable, that during thrombus formation some kind of stimulus must exist, giving a signal to other cell types to infiltrate. Polymorphe leucocytes herald the organizing mass of cells.

Monocytes. The stimulus for other types of cells to migrate into the thrombus mass, may very well be the leucocyte-disintegration. When the number of polymorphe leucocytes decreases, the number of monocytes, smooth muscle-like cells, macrophages and foamy cells increase. These little monocytic cells, with a large darkly stained nucleus, stay mostly on the surface of the thrombus, and seem to initiate the investment of the mass, which takes place around the third or fourth day of thrombus organization. This may explain the source of the new endothelial cells. Various mononuclear cell types were present after four to six days. The small mononuclear cells, mentioned above, did not have the characteristics of lymphocytes; they looked more like monocytes with a large, dark, round nucleus and primarily a round-shaped cell body. Sometimes, a few intracellular granules were visible. Repeated observations were made to ascertain the ability of these cells to arrange themselves in an endothelial-like way on the surface of the thrombus. The cell body flattened out and partly covered the thrombus mass. In EM studies, at six days and two weeks, endothelial-like cells covered the thrombus mass. The cells seemed to be smaller than normal endothelial cells, but appeared to be built

up the same way. The way these cells covered the thrombus mass, gave the impression of normal endothelial cells; they were, however, not reactive to anti-Factor VIII:RAG immunofluorescent studies. A reason to believe these mononuclear cells are monocytes, is the increasing presence of macrophages inside the thrombus mass. It is assumed, that monocytes are young blood cells, later on becoming macrophages with a large fagocytotic capacity (Ham - 1965). The monocyte grows and its nucleus assumes a less regular shape. In EM observations, a large Golgi apparatus develops in the cytoplasm. Whenever the macrophages are visible inside the thrombus mass, foamy cells are also seen. Many speculations have been made concerning the origin of the foamy cells in the arteriosclerotic and atherosclerotic lesions. With the increasing presence of foamy cells and macrophages, smooth muscle-like cells also seem to move at high speed. Is there any relation between foamy cells and macrophages or smooth muscle-like cells?

Smooth muscle cells (SMCs). Many hypotheses and studies were carried out on the function and origin of smooth muscle-like cells. Many studies were performed on the myointimal thickening in atherosclerotic lesions. These studies were performed on human and experimental material. Haust (1969-1971) stressed the importance of SMCs in the development of atheromatous lesions; she is still working on the specific properties of these cells. Ross and Glomset (1976) came to the same conclusion, when they produced their response to injury theory. Jurukova (1977) stated that thrombosis is essentially a healing process, since thrombi cover endothelial defects following vascular injury. She found, in an EM study on experimental thrombi in the rat carotid artery, that arterial SMCs are entirely responsible for the organization of thrombi. Maybe this is too strong an assumption, but SMCs do seem to play a very important role.

Fishman (1975) produced an endothelial denudation by passing a stream of air through the common carotid artery of the rat; SMC-proliferation was seen in all cases. Spaet and Stemerman (1973), Hasler (1976), Jørgensen (1978) and Woolf (1979) confirmed the role of SMCs in the formation of arteriosclerotic and atherosclerotic lesions.

In our study, the presence of smooth muscle-like cells is first seen after two days. These cells are not found in the superficial layers, but always in the deeper parts of the thrombus. From histological studies it is apparent that

these cells do not come from the blood, but from the vessel wall or the flap. When these SMCs migrate, they probably behave differently. The nucleus takes a lighter colour and the inside of the nucleus is slightly vacuolated. In LMSB, the nucleus stained light red to pink, while in the Levanol staining, the nucleus took a reddish colour. The cytoplasm stained light blue in Levanol and light red in LMSB staining. The presence of the SMCs was confirmed by immunohistochemical techniques in the flap and the original vessel wall. At this stage, the SMCs in the thrombus could not be seen with fluorescence techniques. The presence of SMCs was confirmed by EM studies, even in early time intervals. After six days, these cells were, however, also detectable in the thrombus mass with immunohistochemical fluorescence staining.
This phenomenon is probably caused by the pluripotence of this cell.
After six days, the cells are incorporated in the thrombus mass and feel "at home", starting to produce muscle protein again, acting as a reactive antigen to anti-SMC serum. These cells are already present in the time interval from two to six days, but are not detectable with anti-smooth muscle cell serum.
The pluripotent character of the smooth muscle cells from the medial part of the arterial wall was investigated by Ross and Glomset (1973), in vivo as well as in vitro. They concluded that arterial SMCs play a fundamental role in atherosclerosis. Their function is to react whenever there is a damaging endothelial injury. The migrational tendency of SMCs is evoked by the presence of plasma protein and other substances. They migrate into the intima, produce collagen-elastin, and play an important role in lipid deposition. In 1976 Ross and Glomset emphasized the importance of the SMC. They concluded, at that time, that SMC proliferation is a conditione sine qua non in atherogenesis.
These cells can accumulate intracellular lipids in the presence of increased concentrations of extracellular lipoprotein, and they may also promote the deposition of lipid in the extracellular matrix. Proteoglycans seem to play a very important role.
More et al. (1977) described the property of intracellular lipid accumulation of these arterial SMCs. SMCs have been shown to accumulate lipids in the uterine wall in human toxemia in pregnancy (Haust - 1977). More showed intracellular lipid accumulations of these cells in catheter-induced arterial thrombi in aortas of rabbits with a normal blood cholesterol.

The idea thus formed about a structural change of SMCs in the healing process of injured arteries was confirmed by Murray (1966). These cells become the foam cells of the plaques (Sumiyoshi et al. - 1973).
Bhawan (1977) permanently occluded the carotid artery of the rat. Smooth muscle cells of this part of the isolated vessel degenerated and died. New undifferentiated cells, however, entered the thrombus mass, and matured to new SMCs, producing elastin and collagen around them. After one month, they accumulated fat droplets inside, which, thereafter, multiplied. All these events occurred in the absence of bloodflow.
Smith et al. (1979) stated that the amount of lipoprotein in endothelialized lesions is larger than in uncovered mural thrombi. There is a strong possibility that SMCs have influenced this.

Foam cells. To return to the point at which we left the discussion on the organization of thrombi: are foam cells SMCs with fat droplets, or are these cells macrophages filled with fagocytized platelets?
In this lesion, they all appear at the same time interval, so some relation between these cells has to exist. With the aid of LM and EM observations, the indications are strong that the first foamy cells are macrophages filled and still filling with degranulated platelets. It is a remarkable fact, that these foamy cells are often seen in large platelet-fibrin masses.
In a monograph, French (1971) mentioned the possibility of macrophages to digest red bloodcells and platelets (Chandler and Haust - 1971 and Shirasawa and Chandler - 1971).
This process may transform them into highly vacuolated foam cells. In our present study, however, no regular pattern of empty zones around these cells was seen, so a definite conclusion can not be made. It is unlikely that these cells are lipid droplets producing ischaemic SMCs, because a description of these cells was made after a longer time interval in arterial thrombosis (Bhawan - 1977)). Due to their earlier appearance, macrophages are more likely to be the first foamy cells.
The disappearance of the foamy cells was observed in our histological study, while normal SMCs and monocytes were present for a prolonged period of time. It is possible, that two types of foamy cells are present: perhaps at the beginning of the organization, the foamy cells represent the platelet and erythrocyte-fagocytizing macrophages, while the SMCs produce little droplets,

giving it a foamy appearance. The predisposing factor causing them to produce fat, may be ischaemia, although no fat droplets were found with fat staining methods.
A reproducable observation from about four to six days to two weeks, was the disappearance of medial SMCs from the cut ends of the original arterial wall and the flap segment. Thus, conforming the above-mentioned theories about a cytothrophical stimulus, strands of, possibly, dedifferentiated SMCs were seen moving towards the thrombus area. The reason for this cell movement has to be some stimulus emanating from the thrombus mass. The stimulus has to be related to the breakdown of some type of cell, or it must come from a substance produced by a cell, trapped in the thrombus mass. The component of the arterial thrombosis to cause this most likely is the platelet (Ross and Glomset - 1974, Moore - 1979, Woolf - 1979).
Foamy cells appear and SMCs are seen for the first time at the same stage. Although they cannot be detected with immunofluorescence studies, as mentioned above, these observations confirm the results of others, emphasizing the important role of SMCs in the organization of arterial thrombosis.
Murray (1966) gives an explanation concerning the structural change of the migrating SMCs. According to his observations, SMCs assume some of the features of fibroblasts and appear to migrate into the injured zone. This explains why these cells are not detected by fluorescence studies and do not stain with the Levanol method; there is not enough muscle protein present to give this cell its normal identifying features. In Murray's results, the cell became metabolically active and started to produce protein. Electromicroscopically, a large Golgi apparatus and an increasing, rough endoplasmatic reticulum were seen. He found collagen fibrils around these cells, while no elastin was seen.

Collagen/elastin. In our LM studies, no collagen is found around the migrating SMCs after six days. Within two weeks, the first signs of active production were observed. Around the cells in the deeper layers, fine granular elastin production is found around the cells, which had lost their length-wise orientation. In collagen staining, some collagen can also be found, although in small amounts. The cells produce more elastin in the areas close to the surface of the lesion, probably due to the pulsatile blood pressure, exerted on the arterial wall. In the superficial areas of the lesions, elastin is formed in a

more strand-like pattern, giving elastic strength to the new arterial wall. At the same time, the cells arrive at the superficial parts and the cell structure changes. The nucleus stains more darkly and the cell body becomes smaller. Orientation along the streamline of the flowing blood is observed.
In three months, a virtually normal vessel wall is formed with elastin strands, joining to form elastin lamellae. Between these lamellae SMCs are found in the same pattern as in the normal vessel wall. Structural different SMCs can still be found in the deeper parts of the lesion, surrounded by granular elastin and collagen. No foamy cells were seen in this time interval. After ten months, no foamy cells or macrophages were seen. These LM observations were confirmed by EM studies.

Thrombus investment/endothelial-pseudoendothelial cells. Whether the SMCs play a role in the covering of the lesion is not certain. No immunofluorescence with anti-SMC serum could be observed on the surface of these lesions. It is possible, however, that the endothelial-like cells are dedifferentiated SMCs, not containing muscle protein, and, for the lack of it, do not react in immunohistochemical studies. Endothelial and pseudo-endothelial cells are found in specimens after the time interval of two weeks. No real endothelium was found, but after three weeks endothelial cells could be confirmed by immunohistochemical studies with anti-Factor VIII:RAG.
What is the role of the endothelium in thrombus formation and in thrombus breakdown? What is the origin of these new endothelial cells?
The uncovered areas of the lesions in our specimens appear to be highly thrombogenic, judged by the rate of rethrombosis. Areas, covered with endothelial cells, showed less rethrombosis. A protective and barrier function must therefore be attributed to these cells.
Fibrinolytic activity can be found in the endothelial cell. This will be discussed later.
The origin of the endothelial cells is a problem of many years' standing. A lot of experimental work has been done on this subject.
A number of speculations have been made on the origin of this cell. In studies with experimentally induced lesions, Florey et al. (1961) found indications that replacement of the endothelium proceeded by slow ingrowth from the peripheral parts. Later work suggested that leucocytes and monocytes were the source of these new cells (Ghani and Tibbs - 1962, Baumgartner -

1970). More recent studies of regeneration, following injury with a balloon-tipped catheter, have led to the proposal that dedifferentiation of underlying SMCs results in the formation of new endothelial cells (Ross and Glomset - 1973, Spaet and Stemerman - 1973).

Davies et al. (1968) noted endothelialization from intact sheets of endothelial and, possibly, from mononuclear cells.

Fishman (1975), however, developed a new endothelial denudation model in large arteries, and concluded that endothelialization can be attributed to rapid endothelial ingrowth from the ends of the injured segment of the vessel. During the time, necessary for ingrowth of these endothelial cells, the area of the lesion is covered by pseudoendothelial cells, most likely deriving from SMCs.

The explanation must be somewhere in between. In the present study, it was observed that the covering endothelial cells first react to anti-Factor VIII: RAG after three weeks. After two to three days, other covering cells are seen, forming a nice mononuclear lining on the thrombus mass. Perhaps this last investment is only temporary and is superceded by slowly ingrowing, real endothelial cells, arriving after three weeks. As already described above, endothelial-like cells are seen in EM studies, while no immunofluorescence to anti-Factor VIII:RAG could be noticed.

From these data, it can be concluded that some cells, probably monocytes, can temporarily take over the function of endothelial cells. It is, however, theoretically possible too, supported by EM observations, that these covering cells are young endothelial cells, without the normal Factor VIII contents of the mature cell.

The pseudoendothelium could just as well be a temporary covering by SMCs, lying on the surface. This first covering of the thrombus seems to be done by small mononuclear cells from the blood, confirmed in EM-studies. When the organization proceeds, a myo-intimal thickening occurs, showing the ingrowing SMCs, also lying on the surface and not covered by any other layer of cells. Platelets and a small amounts of fibrin seem to mainly adhere on these places. In both LMSB and Levanol staining, some large cells, containing darkly stained nuclei, seem to cover the lesion. These cells were called pseudo-endothelial because they did not have the specific properties of a normal endothelial cell, and did not take a flattened position, but usually kept a

round to polygonal form, while the nucleus also showed some normal endothelial-like features.
In the areas, where fluorescence was observed with anti-Factor VIII:RAG, a normal endothelial covering could be found in histological slides.
No rethrombosis was found on locations where these cells were present in their normal morphology.
Ashford (1959) ascribed two properties to the endothelium, namely the ability to stimulate fibrinolysis, and the ability to form a barrier, separating the procoagulants and platelets of the blood from the underlying tissue. These two properties are present anyhow, but the influence of these properties on the thrombus still remains unclear.
In conclusion can be noted, that the origin of the endothelial cells in expermental work probably depends on the kind of lesion used for producing thrombosis. Endothelial cells are, however, very important in the entire organization of the thrombus; thus, their function is worth further study.

Fibrinolytic aspects. The fibrinolytic activity of the vessel wall has been the subject of many studies during the past few years. Fibrinolytic activity of the wall, as well as the blood, may play an important role in the appearance of arteriosclerosis and thrombosis (Kluft - 1978).
Human arteries and veins have a varying fibrinolytic activity. Generally, arteries show little or no fibrinolytic activity in the intima and media, but strong activity in the adventitia. Veins showed the same strong activity in the outermost layers of their adventitia. The intima and the media of the veins showed little activity, depending on the localization of the veins. Above the diaphragm, intimal fibrinolytic activity was higher than it was below.
Increased fibrinolytic activity was seen after sudden death, liver cirrhosis and intracerebral haemorrhages. Decreased fibrinolytic activity was found in cases with endotoxin shock and hyalin membrane disease (Noordhoek Hegt - thesis 1977).
Noordhoek Hegt and Brakman (1974) found a definite relationship between the inhibition of fibrinolysis in the human vascular wall and the presence of SMCs in this wall. This action of the SMCs implicates the inhibition of plasmin. The local balance between activation and inhibition of fibrinolysis will eventually be useful in determining the fate of the fibrin deposits on the vascular wall.
Meade (1979) showed a relationship between fibrinolytic activity of the blood

and atherosclerosis, especially in ischaemic heart disease. He noted a lot of features effecting fibrinolytic activity and plasma fibrinogen concentrations. Experimental fibrinolytic studies on bloodclots in vitro and on thrombosis in veins and arteries in vivo (Sandritter and Gottlob - 1954) have shown the importance of lytic activity in contemporary disease.

Many experiments and studies on the fibrinolytic field were performed by Astrup (1967). He stated that arteriosclerosis can be formed by connective tissue, forming a scar in the vessel wall lesion. This connective tissue-forming is a healing process, regulated by thromboplastic and fibrinolytic agents. The regulation is activated by the local release of cellular components (tissue thromboplastin and tissue plasminogen activators), which act on enzyme precursor (prothrombin, plasminogen) of humoral origin and convert them into fibrinoplastic (thrombin) and fibrinolytic (plasmin) enzymes.

In veins, fibrinolytic activity in the endothelial cells prevents an accumulation of fibrin, whereas the lack of fibrinolytic activity of endothelium in arteries can be responsible for the development of a pathological reaction of the vessel wall.

Astrup believes fibrinolytic activity in the blood to be a major factor determining the amount of fibrin deposited on the intimal surface, as a process of normal tissue repair. He thus stated his awareness of the multifactorial origin of vessel wall disease, of which fibrinolysis is one component. So, the prevention of arterial vessel wall disease must be sought in diminishing or regulating the healing connective tissue formation on the endothelial surface as a reaction on the damaged vessel wall. Scar formation must be kept to the minimum.

Fibrinolytic activity of the rat arteries differs from activity in human arteries. Guinan and Astrup (1980) published a paper on this subject. In rats, arteries show a higher fibrinolytic activity in the endothelium than in the corresponding veins, which were less active or not active at all with the Todd slide method. Their results showed the presence of plasminogen activator in the endothelium of some large arteries and veins of the rat. In twenty samples of rat aorta, they found twenty fibrinolytically active endothelial surfaces, of which fourteen were strongly active.

The results of our study appear to contradict these findings. In time intervals from one hour to two days practically no fibrinolytic activity was found. The weak fibrinolytic activity, measured in and around the lesion, could be

observed on the intima, while adventitial fibrinolytic activity was present in almost every animal. The highest fibrinolytic activity was seen after the three-week and one and two-month time intervals, diminishing after three and ten months. After the ten-month time interval, no fibrinolytic activity could be seen at all. The absence of normal high fibrinolytic activity in rat aortas must have an explanation. It is possible that this activity is disturbed by the surgical procedure. By inverting the aorta flap, many thromboplastins are brought in contact with the blood and the activated, normal concentrations of tissue plasminogen activator. The amount of thrombus, found in the study, also shows little effect of plasmin activity.

Another explanation may be the total consumption of tissue plasminogen activator by the large thrombus formation. These observations may well be the result of fibrinolytic and thromboplastic activity.

Noordhoek Hegt described the fibrinolytic inhibitory effect of SMCs in the walls of human arteries. In rats, however, the increased activity and ingrowth of the SMCs in the thrombus, coincide with the presence of fibrinolytic activity. In the rat, Smokovitis and Astrup (1978) also found that SMCs had an inhibiting effect on fibrinolysis.

Therefore, the increased fibrinolytic activity of the lesion after three days, must have another explanation. The first ingrowing SMCs probably do not have the same properties as matured medial SMCs and they are accompanied by many other white blood cells. Possibly, fibrinolytically active substances are produced by neutrophils and eosinophils (Kwaan - 1978, Gottlob - 1978).

With the disappearance of the pseudo-endothelial cells, the fibrinolysis on the intimal surface disappeared too. In areas with these latter cells, no specific fibrinolytic activity was ever observed. A definite pattern could not be found. Guinan and Astrup (1980), however, did not find a definite pattern either.

The conclusion of our study is that the fibrinolytical activity is reactive to the thrombus formation, and merely is an attempt to dissolve the formed mass.

The normal balance in the arterial vessel wall is probably disturbed by the formation of the flap in the surgical procedure.

Despite these results, human fibrinolytic activity must be important in the development of arteriosclerosis and, probably, atherosclerosis.

The normal low fibrinolytic activity in human arteries is essential for the healing process hypothesis. If the activity of the arterial vessel wall was high, there could be no thrombus formation on damaged parts of the luminal side of the vessel. Repeated thrombosis on damaged parts, could have taken place before organization had time to begin. The healing action of the vessel wall would be inhibited by high fibrinolytic activity. With low arterial activity, a swift and smooth healing of the vessel can take place by organization of the small thrombus. Fibrinolytic activity in large thrombus formation, can be caused by ingrowing capillaries. In the human specimens, more fibrinolytically active foci were encountered. Apart from the nearly always positive reaction of the adventitial layer, more intimal and intrathrombotical fibrinolytic zones were seen. Again, in our series, no permanent observation was made. Although in a smaller amount than in the experimental work, the vessels showed little or no fibrinolytic activity. In the older, organized, occluded vessels, recanalization areas sometimes posessed strong fibrinolytic activity. In the environmental large field of SMCs, no strong fibrinolytic activity was seen. The impression exists that the normal distribution of thrombolytic and thromboplastic agents is disturbed by thrombosis, so it is impossible to make an exact comparison with a normal vessel.

Two venous bypasses were also submitted to fibrinolytic studies: little or no fibrinolytic activity was found on the intimal side of the occluded venous transplants, while the adventitia showed strong fibrinolytic activity. This observation can be regarded as normal, although no literature exists on the normal distribution of thrombolytic and thromboplastic factors in venous bypass surgery.

Even in very young thrombi, intralesional foci of thrombolysis were found; probably due to the active surroundings of the already occluded vessel.

Fibrinolysis and arterial wall repair. The importance of fibrinolysis concerning the repair of intraluminal vascular damage is clear, and the importance of thrombus formation is evident. When no thrombus formation is present, no repair can take place. When no fibrinolytic activity is present, the natural balance between thrombosis and fibrinolysis is locally disturbed, resulting in an uncontrolled thrombosis and agglutination. It, therefore, seems contradictory that thrombosis should be beneficial to the vessel wall, but only as the primary step in the repair of the vessel wall. Thrombosis and fibrinolysis

are of great importance in the development of atherosclerosis, as has already been stressed by Duguid in his early publications.
The ultimate stadia are formed by rearrangement of the vascular wall. This new composition consists of new elastin and some collagen formation in a specific pattern. In the deepest layers of the vessel wall in old thrombus areas, some collagen can be seen, whereas elastin is formed throughout the entire vascular wall. Elastin is produced by active SMCs, seen in LM studies and confirmed in EM investigations. The new elastin is laid down in a granular way. In the superficial layers, the elastin is similarly formed, but laid down in a strand-like position. This must be due to the influence of the pulsating blood flow, passing this part of the wall. The elastin formation and positioning is thus dependent on the bloodflow. In some specimens, the difference in formation at the same time interval can clearly be seen (fig. 27).

Rethrombosis. It must be kept in mind that the organized lesion still forms a haemodynamically important obstruction in the lumen of the vessel. In microscopical observations, denuded areas of the inner vessel wall could repeatedly be observed. Signs of rethrombosis on these parts were practically always present. Rethrombosis was suspected when fresh platelet-thrombi, fibrin and PMNs were present. Sometimes, an organizing thrombus of an advanced time interval was recognized. Nonetheless, free thrombi of speculative origin were frequently encountered in the vessel lumen.
Considering the theoretical aspects in the introduction, this observation did not come as a surprise. The thrombogenicity of an organized lesion is present for a long time, because an elevation of the surface will always be there.
Many of the normal histological and immunohistochemical observations show a regular pattern of rethrombosis. In the first time intervals of the flap procedures, a typical laminar pattern of fibrin and platelets is found. In later time intervals, clear-cut cases of rethrombosis are observed; sometimes on many places on the surface of the organizing thrombus.
Loose floating, free thrombus masses are no exception in the various time intervals either; even at very late time intervals, signs of rethrombosis were seen. No specific literature was found on this subject.
Some ideas and considerations are developed while studying the results. In the first time intervals, no investment of the thrombus has taken place. The surface of the thrombus is rough and thrombogenic. This is probably the

reason for platelets and fibrin to adhere on the surface, thus adding another layer to the growing thrombusmass, which is probably the normal way for an arterial thrombus to develop. When investment of the thrombus has started after one to two days, the rate of rethrombosis, however, diminishes, but does not disappear. From this time on, haemodynamics are believed to play an important role. The organizing thrombus forms a permanent irregularity in the vessel wall. The laminar flow will collide with an abrupt stenosis and will loose its stability. A change in shear stress and a change in Reynolds' number will occur, damaging the surface of the thrombus again, and causing adherence of platelets. This situation will not disappear, but will only persist in the course of time. This way it is possible that, even after three and ten months, signs of rethrombosis are found on the surface of the fully organized lesions and in the lumen of the vessel. Whenever the endothelial lining is not intact, rethrombosis is likely to occur.

With the new Levanol staining (Van Pelt-Verkuil - 1982 - submitted), single or clusters of platelets can easily be detected; it is possible to find small indications of rethrombosis.

By this constant observation of rethrombosis, a strong argument in favour of the "thrombogenic theory" is presented. This phenomenon brings forward a restless situation in the lesion. It could be possible that in time the lesion would become even larger, and by this growing (because of rethrombosis) the ischaemia in the centre of the lesion would cause ischaemic changes in the SMCs (Bhawan - 1977).

On theoretical grounds, it must be kept in mind, however, that the greater the lesions are, the more rethrombosis should occur; no evidence, is found on this point.

Organization of the thrombus rules out the presence of thromboplastins as a cause of rethrombosis. When no investment by endothelial cells is present, the subendothelial tissue can act as a thrombogenic centre.

In recently occluded human vessels, a striking resemblance to the experimental lesions could be found in thrombus composition. Large fields of platelets and fibrin were interspersed by many leucocytes and, later on, with an increasing number of monocytic cells. The laminated structure of these thrombi was encountered in practically every thrombus.

Thus, platelets played an important role in the formation and organization of these thrombotic processes. So, the present problem is, to what extent pla-

telets are responsible for arteriosclerotic and atherosclerotic pathology.
As already mentioned in the introduction, the last decade showed a revival of interest in the platelet. The renewed interest in the physiology of the platelet was also stimulated by the detection of the effects of prostacyclin on platelets (Moncada - 1976, 1980). The role of both thromboxane and prostacyclin in arterial thrombus formation has been, and still is, the subject to many clinical and experimental investigations.
Honour and Mitchell (1963) concluded from their studies, that a presence of ADP and ATP in high concentrations at the injured side of the vessel wall, caused platelets to clump together and adhere to the vessel wall at the site of the injury. The fundamental role of the platelet in thrombus formation in the flowing blood was described by Mustard et al. (1966). He found the initial adherence of platelets to the vessel wall and to each other, independent of blood coagulation. Subsequent growth and stabilization, however, is largely dependent on blood coagulation.
Mason et al. (1976) and Friedman et al. (1978) confirmed the role of platelets in the early formation of arterial thrombosis. They also stressed the necessity of platelets in the proliferative response of the injured artery and, possibly, (Friedman) in the development of arteriosclerosis and atherosclerosis. Its adherance to the subendothelium and artificial surfaces is an important property of the platelet.
White and Heptinstall (1978) discussed the chemical behaviour of platelets, because they, too, believed in the fundamental role of platelets in arterial thrombus formation.
Meuleman et al. (1980) measured the reduced survival time and the number of labelled platelets in the circulating blood, after having caused arterial thrombosis by an indwelling canula. After removal of the canula, the platelet survival time normalized within a few hours.
Besides the importance of platelets in the initial arterial thrombus formation, the importance of fibrin is also stressed. Olson (1972) described reduced development of arterial and venous thrombi after defibrination by Arvin(R).
Sanchez (1976) and Brown et al. (1977) performed radio-isotopical studies on fibrin incorporation. Although a portion of the labelled fibrinogen is deposited in the vasa vasorum, it appears from these studies that the major part is deposited on the luminal surface; at the same time suggesting that interaction of platelets and red cells with polymerizing fibrin in vivo, is an important

factor, contributing to the development of thrombi (Smith - 1981).
Tomikawa et al. (1980) investigated the effect of fibrinogen on ADP-induced platelet aggregation. Fibrinogen is known to enhance platelet aggregation, induced by agents such as ADP, collagen, thrombin and arachidonic acid.
Varying shear stresses cause platelet aggregation, as was recently published by Rieger (1980), Stein (1980) and Stevens (1980). In his studies, Stevens investigated the role of platelet prostaglandin synthesis in shear induced platelet alterations in vitro. He concluded that in platelet-rich plasma, shear induced release from platelets of dense and α-granule contents is associated with, and potentiated by stimulation of thromboxane A_2-synthesis. At the same time, in shear-induced platelet aggregation, thromboxane A_2-synthesis plays no appreciable role when platelets were damaged in these investigations. This may be an indication that the varying shear stresses may be solely responsible for platelet aggregation.
Another aspect of platelet function, is the role platelets are assumed to play in promoting SMC-proliferation. From his studies in thrombocytopenic animals, Friedman et al. (1977) concluded that the SMC-proliferation in arteriosclerotic lesions was inhibited. Clowes (1977) confirmed this observation with studies performed in rats with an inherited platelet defect. In 1978, he investigated the effect of heparin on SMC-proliferation. Compared with controles, he observed a suppression of the proliferation in heparinized rats. This effect may be due to the enhancement of the inhibition of anti-thrombin III by heparin, and suggests an unsuspected role in injury-induced SMC-proliferation of these substances (Lancet - Jan. 1981). Castellot (1980) stated that endothelial cells produce a heparin-like inhibitor for SMC growth, confirming the observation of Clowes, although more recent results suggest that this effect of heparin is not due to its anticoagulant properties (Guyton - 1980). Considering all available data, fibrin may play an important role, but platelet aggregation may also be suppressed by heparin.
The influence of the pituitary gland in arterial intimal proliferation in the rat, is extensively investigated by Tiell et al. (1978) and by Drouet et al. (1977). In hypophysectomized rats, the proliferative response of SMCs is temporarily inhibited. The suggestion is that this proliferative response may be induced by a platelet-connected mitogen, possibly derived from the pituitary. Besides the pituitary, there have to be other sites where the platelet-bound mitogen can be composed; this accounts for a transient inhibition of the proliferative

response. Drouet et al. (1977) performed studies on fawn hooded rats (animals with a platelet-storage defect), showing a non-platelet bound mitogen as the origin of proliferating SMCs. The function of these platelets can probably be taken over by other elements. The inhibitory effect on proliferative action of SMCs in hypophysectomized rats is counteracted by a non-specific, induced inflammation (Tiell et al. - 1978). The platelet-derived growth factor may in vivo increase prostacyclin synthesis of the endothelial cell, as a part of a feedback mechanism controlling platelet aggregation (Coughlin et al. - 1980), and thus also controlling SMC proliferation.

To summarize, platelet functions from all available data, are:

- haemostatic plug function
- function as an important element in arterial thrombosis
- mitogenic release or carrier function
- thromboxane A_2 production

Considering all results from recent studies on platelet function and the basic elements of the thrombogenic theory, one must conclude that the development of arteriosclerosis is dependent on the repair function of the platelets. Data from the present study support these observations. The exact function of the platelets in the organization of the thrombus is not proven in this study.

Correlation between the results of the experimental study and human arteriosclerotic and atherosclerotic lesions.

History

In the course of the seventeenth century, Bellini and Thebesius, anatomists from Italy and Germany, working independently, found calcifications on the bifurcations of large coronary vessels. A clear connection with angina pectoris, a term introduced by Heberden in 1768, was not yet made. In 1761, Morgagni was the first to connect the symptomatology of angina pectoris with coronary sclerosis, thus giving a further stimulus to investigate vessels and vessel walls.

In 1773 Jenner suggested that angina pectoris was caused by calcifications in the coronary arteries. His colleagues Parry and Heberden, rejected this interpretation, also because of piety for their tutor Hunter, who himself suffered from anginous pain. Jenner based his ideas on data of two autopsies of patients with angina pectoris.

Early in the nineteenth century, Hodgson described a pathogenic connection between coronary sclerosis and and the occurrence of angina pectoris, with or without cardiac decompensation.
In the second half of the nineteenth century, the first theories about the pathogenesis of atherosclerosis were published, independent of earlier publications about coronary sclerosis: the interest in arteriosclerotic aberrations extended over all arteries of the human body.

The nomenclature of the pathology of vessels is rather confusing.

Arteriosclerosis (Lobstein - 1833), when it was used for the first time, meant a thickening of the arterial vessel wall with pultaceous softenings and calcification.

The term atheroma was used to describe a local fatty degeneration of a vessel wall.

Atherosclerosis was first used by Marchand (1904) to emphasize that proliferation of connective tissue very often coincides with degenerative fatty changes.

There is still much confusion about the use of these terms. The following definitions will be used in this thesis:

Arteriosclerosis - Focal, fibrous changes in the intima of the vessel wall in large to medium sized arteries, without degenerative changes.

Atherosclerosis - A focal change of the vessel wall of arteries, irrespective of their diameter, with fatty degeneration and hardening.

Atheroma - A focal and/or calcified degenerative change in the vessel wall.

Opinions as to the origin of atherosclerosis were often of a contradictory kind. With the first publications by the great pioneers of this pathology (Rokitansky, Virchow, Zahn), a controversy immediately developed.
Rokitansky claimed, in his "Handbuch der pathologischen Anatomie" (1842), that atherosclerosis (vascular stricture or obstruction) developed because material from the blood stream settled on the endothelium, macroscopically giving the intima a hypertrophic aspect; the causes of this deposition on the vessel wall were left unexplained.

Rokitansky emphatically stated that apposition of material on the vessel wall was the point at stake. According to his findings, there was no material under the endothelium. Rokitansky made his observations mainly in the large vessels, notably on bifurcations and inlets. The changes, he saw, had a laminar structure, varying in size, and had a mural and mainly fibrinous character.
These lesions lead to a narrowing in the blood vessel. Though the occurrence of predilection sites for these appositions is mentioned, Rokitansky leaves its cause undecided. He clearly notes the importance of age, whereas he rejects inflammation as a possible cause. The degree of vascularization of the vessel wall did not escape his attention. The increased degree of vascularization of the adventitia is described as being of doubtful importance (many of the aortas involved in his research had been affected by syphilis).
He also describes a system of small canals through the plaque, stating that through these canals, nutrition can take place from the lumen to the plaque. He did not observe any continuity between the above-mentioned canals and the blood vessels of the adventitia.
After the initial formation of the aberration (plaque), a change takes place, on account of an atheromatous process and ossification. By this atheromatous process, Rokitansky means a softening and forming of fatty globules in the plaque area, and also the deposition of cholesterol in crystals and Ca-salts. He denoted the ossification as calcification which, whether or not, was attended with the atheromatous process.

In 1856 Virchow delivered his opinion on the development of atherosclerosis. He strongly emphasized that the changes he saw were situated under the endothelium, notably near the border between intima and media.
Virchow carefully distinguished between fatty infiltration, which he saw as a result of degeneration of connective tissue cells under the endothelium, and the atheroma, which he regarded as a fatty change, taking place in the deepest layer of the intima, separated from the lumen of the vessel by a layer of connective tissue.
He rejected Rokitansky's theory about a superficial deposit, because this layer of connective tissue was continuous with the adjoining intima (he clearly was unaware of the regenerative capacity of endothelial cells and other cells of the vessel wall).

The cellular or infiltration theory, as Virchow's theory is called, is based on a passage of blood elements through the vessel wall. This might cause a chronic inflammatory reaction in the vessel wall, with a proliferation of cells and hypertrophy of the vessel wall. The latter might lead to fatty infiltration and degeneration. He called this pathological picture: "endarteritis chronica deformans".
Virchow criticized Rokitansky's hypothesis, the latter a modest pathologist, in a way that was not so gentle. At the time, Virchow's authority had been generally accepted to such an extend, that Rokitansky sidetracked his own theory.
A pupil and admirer of Virchow, Zahn, described in 1857 the fact that Virchow's findings were final in the following words: "Der nächsten Folge hiervon war, das von der früheren Beobachtungen, welche er (Virchow) zugleich einer strengen Kritik unterwarf, der gröszte Teil überflüssig wurde, und neue Untersuchungen hierüber nicht geboten erschienen". These words illustrate the way of thinking at that time.
Rindfleisch (1867) finally completed Virchow's theory. He thought that mechanical factors possibly played a part in defining the predilection sites for atherosclerosis. Most aberrations were localized, where the blood stream exercises the greatest force on the vessel wall. This mechanical trauma first causes a fibrous growth in the intima, possibly of an inflammatory origin. As the intima is avascular, this layer becomes ischaemic because of the pressure from the vessel wall, resulting in a fatty degeneration, atheromatous liquefaction, formation of abcesses and finally ulceration.

In 1883, Thoma published quite another view on the development of atherosclerosis. He thought that changes in the intima were caused by a pathological process in the media. Because of the pulsatile force of the blood, the media bulges out at certain places, thus forming a kind of niches in the vessel wall. In the course of a few months, these niches are filled up by a thickening of connective tissue from the intima, in an attempt to recreate the vessel's orginal shape; this pathological aberration is progressive and, on account of further stretching, necrosis develops in the plaque thus formed. The plaque also swells because of the absorption of liquid and then bulges out in the lumen of the vessel concerned. Consequently, the inevitable atheromatous softening and ulceration follows. Bork (1926), Crawford and Levene

(1953) were the only ones who paid attention to this illogical and unappreciated theory.

The difference in opinion between Rokitansky and Virchow concerns the way in which the atherosclerotic lesion of the arterial vessel wall develops. Rokitansky's hypothesis is called "the thrombogenic or apposition hypothesis", whereas Virchow's hypothesis is called "the infiltration hypothesis". In 1862 Virchow admitted that an apposition of material from the blood on to the vessel wall could take place; he, however, stuck to his original theory. This infiltration theory dominated atherosclerosis research well into the twentieth century, especially influencing the construction of experiments with animals.

One of the main causes of this prolonged influence was that Ignatowski (1909) was able to induce atherosclerosis in the aorta of rabbits by feeding them a diet of milk, meat and eggs. The yolk seemed to be the most important ingredient of this diet.

Anitschkow (1913) identified cholesterol as the atherogenic factor. The atheromatous lesion thus induced, was described as a generalized or local accumulation of lipid in the arterial wall; the accumulated lipids subsequently being increasingly organized by fibrous tissue, giving rise to the typical atheromatous plaque.

Virchow's theory and Anitschkow's experiments caused an avalanche of experimental and epidemiological investigations into the role of lipids in atherogenesis. From the epidemiological investigations (reviewed by Gotto - 1976), it appeared that there was a positive correlation between cholesterol concentrations in the blood and the incidence of coronary disease. Moreover, the larger part of cholesterolesters, present in the developed lesions, appeared to be derived directly from plasma lipoproteins (Smith - 1974). This is in contrast with the cholesterolesters in the so-called "fatty streaks", which contain locally synthesized oleic acid as the principal fatty acid. In arteriosclerotic lesions, the arterial vessel wall would be more permeable and would offer a free passage to intact lipoproteins.

Endothelial cells as a natural barrier against a cholesterol influx in the arterial wall was postulated by Smith (1974). The action of this barrier would gradually decrease in relation with age. If an endothelial lesion is effected, an accumulation of lipids in the intima can be evoked, even with a very low concentration of lipids in the blood. Moore (1973) and Bhawan (1977) even described a fatty degeneration in the evoked endothelial lesion in studies

performed in normolipaemic animals with low blood cholesterol levels (rabbits and rats). The accumulated lipids would originate from cells present in the lesion. This would imply that, in a ischaemic situation, the smooth muscular cells organizing the lesion would produce the lipids themselves.
Such a fatty degeneration of SMCs under ischaemic circumstances is also known from other studies (e.g. Haust - 1977).
According to Chandler and Hand (1961), these "foamy cells" might also partly be macrophages which have phagocyted much lipid-rich material.
An intermediate form of hypothesis, which could be applied to both the infiltration theory and the thrombogenic theory, was postulated by Rössle in 1944. This so-called "insudation-theory" was further elaborated by Haust (1970). In this theory, the vessel wall is subject to intimal and subintimal lesions because of noxious agentia, followed by a local serous inflammation in the intima. At this place, an insudation would develop from the blood. When the lesion is small, the insudate will be serous and is then easily absorbed. When, however, a bigger lesion has developed, it is organized by avascular connective tissue.
This latter theory can be seen as a compromise between the thrombogenic and infiltration theories, and has evolved because of lack of conclusive evidence in support of both theories. Anitschkow's findings with rabbit aortas were generally seen as being a confirmation of Virchow's infiltration theory. Anitschkow himself saw his findings as a result of both general and local changes, and not as the final proof of the infiltration theory. Owing to the focal nature of the changes and the striking irregularities of the arterial walls and because of dissatisfaction with arbitrary variation in permeability of the vessel wall for lipoproteins, the apposition theory has again aroused interest.
As early as 1904, Marchand mentioned the existence of smaller and thicker appositions, which, because of organization, formed new plaques on the lesion. In 1912 Mallory wrote that fibrinous plaques can be formed in the aorta by the organization of fibrin. In 1936, Clark et al. studied plaques in the aortas and coronary arteries in detail. They noticed that a pattern of repetition could be found with regard to the presence of fibrin in the plaques. From this, they concluded that a plaque could be formed by frequent apposition of blood elements, which are organized and caused the plaque to grow. These findings had little impact on the opinion of leading pathologists.

After the second world war, Duguid attacked the infiltration theory; in a number of publications, he tried to explain the development of the subintimal accumulations of lipids and of atherosclerosis, in connection with the thrombogenic hypothesis (Duguid - 1946, 1948, 1949, 1954, 1955 and 1976).

Relation between arterial thrombosis and athero/arteriosclerosis in view of the thrombogenic theory.

In 1976, Duguid published a monograph based on his former papers (1946, 1948, 1949, 1954, 1955) called "The dynamics of atherosclerosis" in which he fights for acknowledgment of the thrombogenic theory. He starts with a mural thrombus which is formed on the vessel wall. This mural thombus will organize, and will thus become a part of the vessel wall. During organization, it becomes covered with endothelium, resulting in a fibrous thickening of the intima. The mural thrombosis is formed on endothelial lesions, caused by mechanical trauma. Haemodynamical changes are, in this case, among the noxious agents. With increasing age, the compliance of the vessel wall decreases; if the pulse maintains the same pressure, a slackened extension of the vessel wall may occur. As the layers of the vessel wall no longer form a mutual unit, they may shift in respect of each other, due to pulsatile forces. This may cause rupturing of the endothelium, but also, for instance, ruptures between intima and media. With this theory in mind, Duguid clearly adopted the haemodynamical basis of atherosclerosis. Especially the vessels (aorta and aa. iliacae), that were most subject to this, showed many atherosclerotic changes in his investigations.
In 1948, Harrison supported Duguid by injecting rabbits with fibrin clots. Fibrin emboli, overgrown with endothelium, developed in the pulmonary arteries, were incorporated in the vessel wall and, there, formed fibrous plaques. In 1952, these experiments were succesfully repeated by Heard.
Crawford and Woolf (1968) effected mechanical lesions to vessel walls in the aortas of pigs. The mural thrombi, formed on this lesions, were organized into aberrations that very strongly resembled atheromatous plaques.
Geiringer (1951), McLetchie (1952) and Crawford and Levene (1952) studied the human aorta, and in 1949 Heard described the changes in the arteries of the kidney, in this context. On account of the descriptions by these authors, the hypothesis that atherosclerosis could be the development and organization

of mural arterial thrombi, was more generally assessed.
Duguid described, in "The dynamics of atherosclerosis" (1976), how thrombi in various sizes and stages of organization can be seen in coronary arteries. The thrombi vary from miniscule thrombi to occlusive ones, mainly in the extramyocardiac arteries; these thrombi especially occur on bifurcations and sinuous parts of the artery. Recently formed thrombi are generally found on places were fibrous thickenings are present (possibly formed from older mural thrombi). In this way, many thrombi also develop in the aorta, which are not macroscopically visible; even with a microscope it is often difficult to detect them. A reason for this is, that even larger thrombi are subject to very fast changes, or disappear and thus escape detection. In older persons, whose aortas are considerably affected by the atherosclerotic process, several transitional forms can be better recognized. Owing to the repeating character of the process, several layers are found. Crawford and Levene (1952) indicate that, in about 42% of the lesions they studied, old fibrin thrombi can be seen.
Many observations have shown fibrin-like deposits in lipid-rich lesions. It was determined that fibrin exists in a very early stage of lipid deposition. This suggests a small mural thrombus undergoing further organization and transformation; subendothelial deposits of fibrin have been unanimously demonstrated as well. (Crawford and Levene - 1952 and Bleyl - 1969).
Many authors found fibrin with the aid of the fluoresence antibody techniques in early, superficially located "fatty streaks" and in early, fibrous plaques (Woolf, Crawford - 1960, Wyllie, More and Haust - 1964).
This is affirmed in the series of autopsies done by Haust (1956) and Woolf (1969). Chandler and Pope (1975) affirmed these findings and alleged them to be in favour of the thrombogenic theory. Most fibrin material is located in the thickened intima and, ocassionally, in the media (Bleyl - 1969). Bleyl even indicates that fibrin can be found in the adventitia. The occurrence of fibrin in atheromatous lesions pleads in favour of this theory, because these atheromatous lesions are essentially the result of repeated appositions of mural arterial thrombi. The known laminar construction fits into this picture.
The view that fibrin deposits in raised lesions, due to thrombus incorporation, is strongly supported by the observation that platelet antigens can be found at the same site as fibrin (Carstairs - 1965, Woolf and Carstairs - 1967, Hudson and McCaughey - 1974, Woolf - 1978).

The frequent occurrence of small thrombi, even in the blood vessels of young people, now seems to be accepted (Movat - 1959, McMillan - 1965, Chandler - 1972 and Haust - 1971, Jørgensen - 1972). Acknowledging this took a very long time, because it was difficult to recognize these microchanges in the vessel wall; the more so, when one is unaware of their existance. Small fibrin appositions are found in autopsy material of individuals of all ages, notably in the aorta, the coronary arteries, and the medium-sized arteries; this is rather the rule than the exception. Most fibrin deposits were seen on organized thrombi, but small depositions could also be seen on the apparently normal vessel walls of young children (Haust - 1971). In 1969, Likar reported that it was also an ordinary phenomenon in the coronary arteries of cows. To find a plausible explanation for the fact that development of thrmobi appears to take place without immediate fatal results, a detailed description of fibrinolysis is introduced (Astrup - 1967).

Duguid's theory is based on the constant balance between the formation and breakdowm of microthrombi. In young people, a balance between thrombosis and fibrinolysis would be normally present. In later (middle-aged) life, these microthrombi are incorporated in the vessel wall because of a shift of this balance, resulting in a delayed breakdown.

Mead et al. (1979) found in an epidemiological research that the fibrinolytic activity decreased as one aged. In males, a decrease was seen up to the age of 58, after which a slight rise could be observed.

Astrup and Claassen (1957) found low fibrinolytic activity in the intima and media of the human aorta; a high activity was observed in the adventitia. The distribution of the thromboplastic activity showed a reverse order.

Human veins do have a higher level of fibrinolytic activity in the intima (Astrup and Coccheri - 1961), as is already mentioned.

With his histochemical technique (the Todd slide method), Todd (1960) found fibrinolytically active endothelial cells in the arteries of an ischaemic leg. In veins containing thrombi, from whatever cause, the fibrinolytic activity was low. At places of recanalization in these veins, he ascertained an increase in fibrinolytic activity. The differences between arteries and veins, regarding their ability of fibrinolytic activity, could be part of an explanation of the differences in organization and incorporation of fibrin depositions. The incorporation into the vessel wall brings us back to Rokitansky's and Duguid's thrombogenic theory.

Astrup and Coccheri (1962) gave another explanation of the cause of tissue recuperation, as they found an increased fibrinolytic activity behind an atheromatous lesion; the degree of vascularization of the vessel wall appeared to be strongly increased.

In the thrombogenic theory, too, the deposition of lipids in the lesion remained unexplained. Duguid (1976) gave the following explanation.

On account of the thickenings in the wall, the vessels have become less elastic and can no longer expand normally. This causes lacerations between the layers and haemorrhages into the vessel wall, which disintegrate and leave fatty deposits, accumulating in the course of years. If the haemorrhages increase, the fibrous cover will become thinner and softer; the superficial layers can then be torn away and cause ulceration of the lesion. These ulcerations cause thrombosis which again will be organized, gradually narrowing the vessel and depriving it of its elasticity. Consequently, the lipids do not cause atherosclerosis, according to this theory, but play a secondary role; the pulsatile motions of the arterial wall are considered to be of prime importance.

Geiringer (1951), Paterson, Mills and Movat (1957) and Winternitz (1938) described multiple micro vessels penetrating the intima. There is, however, still a controversy about the origin of these micro vessels growing into the lesion. The micro vessels most probably grow from the media into the lesion (Geiringer). A haemorrhage from these vessels can possibly be explained as follows. If apposition of platelets and fibrin takes place, the elasticity of the wall diminishes. A period can be perceived, in the pulse, during which the pressure on the wall falls off. At that moment, due to a lack of elasticity, a laceration could develop in the thickened intima, followed by a haemorrhage. In his histological research, Duguid describes, at this stage, haemosiderous accumulations, pointing to a resorbed haemorrhage.

The controversy between infiltration and thrombogenic theory is notably important from a clinical point of view. If the thrombogenic theory is followed, a drug against early thrombosis might have a great impact. Moreover, the natural balance between thrombosis and fibrinolysis will be of great importance.

Lipids can be incorporated into the lesion as part of the healing of tissue, or as a result of a degenerative process.

The degenerative changes can be arranged in:

- shrivelling and maceration
- hyaline changes, and
- fatty changes.

According to Duguid, the lesions in coronary vessels seem to have sometimes played an obstructive role. Because of the shrivelling of the thrombus mass, some parts leave the wall and retract to create space for the passage of blood. These new canals, developed by shrivelling, are covered with endothelium and, due to the blood pressure of the passing blood stream, the thrombus mass will be further compressed. In this way, recanalization of a vessel can develop; consequently, a coronary obstruction is not necessarily irreversible.

Maceration of a thrombus can most easily be seen in red thrombi. If erythrocytes are packed together in a thrombus and no longer participate in the circulation, they will disintegrate and leave a debris that is half fluid, half lipid, which, in respect of the local character, looks like an atheromatous aberration.

The change of fibrin into hyaline (the connective tissue change that develops) is controversial. It is not quite certain whether all the fibrin material changes into structures of a connective tissue nature; fibrin material may also possibly be at the root of it.

The compressed fibrin is also called hyaline; a typical pathological term.

With the aid of an electron microscope, Levene (1955) affirmed the fact that at least a part of the hyalinous material in an atheroma was largely composed of fibrin. It cannot be stated conclusively that all hyaline material really is fibrin; this cannot be ascertained with the well-known immunofluorescence methods either. It is not certain that the fluorescing part only consists of fibrin; there are, however, strong indications that it does.

Most mural thrombi develop fatty changes. In fibrin-rich thrombi, these changes are often sparsely present. In red thrombi, the fatty changes are amply present where the masses of erythrocytes and other cells disintigrate, thus forming macerated sites, consisting of neutral lipids, fatty acids and cholesterol.

This decay alone, however, cannot be the sole reason for the development of an atheroma, because a thrombus does not contain as much lipid material as

an average atherosclerotic plaque. The origin of the fatty streaks is presumably totally different from that of the atherosclerotic plaques.
They can already be observed in young people and are composed of (micro) accumulations of foam cells, situated directly under the endothelium in the intima, causing small, yellow elevations in the vessel. The fatty streaks run parallel to the blood stream. In view of the localization and the occurrence of these fatty streaks, there is no direct relationship between these micro accumulations and the development of atherosclerosis. It is more likely, that a temporary change in a metabolic sense is the point at stake; macrophages from the vessel wall, or deriving from the passing blood, gather lipids that cannot be metabolized.
Also, because of the fact that these fatty streaks occur at a very early age, a direct link with the development of atherosclerosis cannot be made. In 1979 Tracy published an elaborate investigation about the correlation between fatty streaks and atherosclerosis. By virtue of data from autopsies done on 18.000 deceased of various races, he arrives at remarkable conclusions. Fatty streaks are correlated with certain types of atherogenous noxe, for instance hypertension (type A), or smoking (type B). This does not necessarily imply that this fatty streak develops into a flourishing atheromatous lesion. Together with type B, type A can cause progression to atherosclerosis. In principle, a "fatty streak" has no direct correlation with generalized atherosclerosis without atherogenous noxes. The main causes of atherosclerosis, however, did not start to play a role until after the formation of the fatty streaks. Most data in favour of the thrombogenic theory are available from experimental studies. In the last decade, however, many retrospective and prospective studies were performed to determine the role of platelets in coronary disease (for instance the Anturane Reinfarction Trial).
In the early sixties, appreciation of haemodynamical events in the vascular system was the first sign of a renewed interest in the basic cause of atherosclerosis.
In 1957 Texon published a study about haemodynamical causes of atherosclerosis. In one hundred autopsies, he found atherosclerotic changes in all of them, possibly due to haemodynamical causes in persons over forty years old. The distribution of atherosclerotic lesions strongly indicated that there are certain predilection sites, determined by the motion of the blood. The incidence of haemodynamically induced atherosclerotic lesions, as well as the

location and degree, appeared to be mainly age-related. Contributing atherogenic effects were considered, race, sex, habitus, diet, nutritional status, lipid metabolism, drugs, hormones, associated illness and stress.
Further studies on this subject confirmed these first data as mentioned above (Texon - 1957, 1967), not considering changing haemodynamics as a possible cause of arterial thrombosis and subsequent atherosclerosis. Lipids may play a secondary role in the development of an atherosclerotic plaque.
In 1976 Crawford at al. performed a study on the influence of flow upon the intimal surface morphology, in normal and atherosclerotic human arteries.
With increasing degree of intimal thickening (induced by intimal damage) and atherosclerosis, longitudinal ridges in the luminal surface were found.
In complete involvement of the intima, the ridge pattern was replaced by a smooth, grossly irregular surface. The irregular changes of the surface could exist by the presence of local turbulent flow. They conclude that formative interactions may exist between blood flow and wall structure on a local base, thus indicating the importance of haemodynamical changes.
Apart from haemodynamical changes, a local change in composition of clotting factors can be established by ulcerating atheromatous plaques. With the varying shear stress and exposure to subendothelial tissue, essential factors in the formation of arterial thrombosis are present (Baumgartner, Turrito - 1979).
In man, there are strong indications of intimal damage due to haemodynamical changes at predilection sites (Wesolowski - 1965, Fox and Hugh - 1966, Fry - 1972, Caro - 1971, Goldsmith - 1974, Texon - 1976 and Nerem and Cornhill - 1980). This intimal damage causes arterial thrombosis, with or without organization. These lesions can form a permanent disturbance in the architecture of the luminal surface of the vessel wall, producing local haemodynamical changes.
Goedhard (1978) published a study on the elasticity of human aortas. With age, elastic properties decreased and so did the total arterial capicitance, while pulse-wave velocity increased. A change in elastic properties of the aorta will result in haemodynamical changes.
Except for the changes of the wall by aging, early atherosclerotic lesions can be found in young persons. Kjaernes et al. (1981) devoted a study to the results of autopsies on 33 young adults. Many atherosclerotic lesions were already detected in a specific pattern. They saw the early lesion starting at

the outer walls of the aortic bifurcation. They believe their results to be in support of Caro's hypothesis; the latter claims atherosclerotic lesions to be present in areas with a low shear stress (i.e. low velocity gradient), whereas Fry favours the places with high velocity gradients in developing atherosclerotic lesions.
From a haemodynamical, as well as from a thromboplastic point of view, strong indications are present that the thrombogenic theory is, essentially, correct.

With our experimental study, another indication and piece of evidence is presented. The strong resemblance of human arteriosclerotic lesions, laid down upon the old atherosclerotic plaques or on recent intimal damage, with the experimental lesions must be considered important. Prentice et al. (1966) showed an increased thrombogenic activity of atheromatous aortic tissue, the latter enhancing intrinsic prothrombin activator formation and platelet aggregation.
The importance of our experimental model is that the experimental lesions are practically identical to human arteriosclerotic lesions; in this model anti-arteriosclerotic drugs can be tested.
Baba et al. (1980) confirmed the experimental results in coronary arteries, making a difference between uncomplicated plaques showing mature, intimal SMCs and complicated plaques with extensive degeneration of SMCs. In our study, the same data were observed in the coronary arteries.
Smith and Staples (1981) published a paper on the haemostatic factors in the human aortic intima. They stated that accumulation of lipid, in developing fibrinous atherosclerotic plaques, is associated with high concentrations of fibrin; this observation suggests that fibrin may play a key role in the initial lesion development. Coming from this lipid-minded author, this can be considered as a strong support for the thrombogenic theory.
The generally accepted pathogenesis of a myocardial infarction, is the quick development of an occluding arterial thrombosis or a vasospasm.
In a double blind study, The Sixty-Plus Reinfarction Study Research Group (1980) showed the importance of oral anticoagulant therapy in reducing the risk of recurrent myocardial infarction.
In Cardiff, an initial low dose regimen of Aspirin (300 mg daily) led to benefit, but became less effective because the apparent benefit disappeared after prolonged use of the drug.

In America, The Aspirin Myocardial Infarction Study did not prove reduced mortality after myocardial infarction either.
The Anturane Reinfarction Trial, however, showed some promising results, like for instance a decrease in sudden death after myocardial infarction. The recent favourable results of prostacyclin studies (Moncada, Eastcott, Sczceklik - 1976-1980) really do support the thrombogenic theory.
Clinical importance is also present, shown by the many publications on the use of platelet-aggregation inhibition. Many clinical studies have already been devoted to this subject.
Platelet aggregation is the therapeutic goal. With prostacyclin and/or thromboxane inhibitors, as well as with Aspirin-like substances, substantial results are obtained (Lancet - March 1980).
Huygens et al. (1981) published a clear survey on the effect of acetylsalicylic acid in the vessel wall. He concluded that giving 100 mg of Aspirin in twelve hours led to a strong depression of platelet thromboxane synthesis. Hoogendijk (1980) found a cummulative effect of daily low doses of Aspirin on thromboxane synthesis of platelets, while Fields (1977) found no significant improvement in prophylacting cerebral perfusion disorders with normal dosages of Aspirin. Therefore, the hypothesis of Jaffe and Weksler in 1979 was correct. The effect of inhibition of prostaglandin synthesis in platelets is dose-dependent.
Tyson (1981) administered a selective inhibitor of thromboxane synthetase to healthy volunteers. Laboratory tests did not reveal any side-effects or significant abnormalities. Transient increases in bleeding time, corresponding with low levels of TXB_2, a metabolite of thromboxane, were recorded.
Mannucci (1981) has organized the Rokitansky-Duguid project. He wants to collect patients with Von Willebrandts disease (among other features), characterized by decreased platelet adhesion to the subendothelium, in order to study the vessel walls of these patients; less atherosclerotic lesions are expected to be found.

As far as vascular surgery is concerned, the meaning of the thrombogenic theory is very important. With the experimental anastomosis series, it was clearly revealed that identical thrombosis and organization can be expected in a badly performed anastomosis as well as in a flap procedure.

In other words, the flap procedure represents arterial thrombosis in an aortic anastomosis. In arterial vascular surgery, it is known that a badly performed anastomosis will eventually lead to occlusion. When a bypass is not placed in the right angle, this will lead to occlusion. A badly performed endarteriectomy will lead to high thrombogenicity of the vascular wall.

The next step in this investigation will, therefore, be the testing of various platelet-aggregation inhibiting drugs. Bay-g-6575 (Seuter - 1979) has already been tested in this model, and did not show a significant effect on the thrombus growth.

With the new anti-platelet aggregation drug Ticlopidine, a significant decrease in thrombus amount was experimentally perceived.

This is a very promising result, but must be more thoroughly elaborated;
it most probably means that this model really does contribute to the search for new drugs in the struggle against cardiovascular disease.

CONCLUSIONS

- It is possible to produce an arterial thrombosis in rat aorta by changing the arterial wall, the local blood flow and the local composition of the blood.

- A detailed description of an organizing arterial thrombosis is given.

- The resulting vessel wall lesion is comparable to arteriosclerotic lesions in man.

- The newly developed model can be used in testing antithrombotic drugs in general.

- The results of this study give support to the apposition hypothesis of Rokitansky, as well as to the thrombogenic theory of Duguid.

- Smooth muscle cells play a crucial role in the organization of this arterial thrombosis.

Based on the results of this study, one can reasonably expect that platelet-aggregation inhibiting drugs should have a beneficial effect in the prevention of arteriosclerosis.

SUMMARY

Peripheral vascular and cardiac disorders frequently occur in our society. Medical treatment is confined to symptomatic therapy c.q. drug therapy, vascular surgery and convalescence after cardiac infarction and stroke; so, the search for a causal therapy still continues.
Because of the impossibility to perform primary investigations in patients, many experimental models have been developed in order to study the origin and, consequently, a possible therapy for vascular pathology.
It is known that arterial thrombosis plays a fundamental role in the development of cardiac and peripheral vascular disease. Therefore, a number of factors, important in causing thrombosis, will be described and discussed in this thesis.

Chapter I starts with a short, general introduction in chapter I.1.

In chapter I.2, the role of platelets in the thrombogenesis is discussed. The formation of a carpet-like platelet covering (adhesion), and the formation of a platelet-aggregate on a vessel wall lesion, is dependent on the kind of vascular damage.
These platelet reactions are important in the subsequent changes of the vascular wall. In order to prevent vessel wall changes and degeneration, many investigations into adhesion and aggregation of the platelets were performed. Because smooth muscle cell (SMC) proliferation appears to play an important role in the organization of an arterial thrombus, a number of experimental results is discussed. The production of prostacyclin by the endothelial cell, as well as the production of thromboxane by the platelets, is also an important factor in the development of arterial thrombosis. These two substances, formed out of arachidonic acid, have opposite effects: thromboxane is a vasoconstrictor and promotes platelet-aggregation, whereas prostacyclin is a vasodilatator, and inhibits platelet-aggregation. By disturbing the local balance between these two agents, the formation of a platelet-fibrin thrombus can be influenced. The formation of fibrin is always involved in the

development of platelet-aggregates. This thesis will deal with the formation of platelet-aggregates.
Except for changes in the vessel wall (vessel wall lesions) and local changes in the composition of the blood (prostacyclin - thromboxane - tissue thromboplastins), local changes in the blood flow appear to play an important role in the thrombogenesis (based on the triad of Virchow). Haemodynamical changes are important in causing vessel wall lesions in the human, as well as in the experimental situation.

After discussion of the physical principles of the haemodynamics, chapter I.3 deals with the pathophysiological consequences in the human situation; in this form, these physical data can not be applied to the physiological circulation. Because of their frequent ocurrence in human pathology, much attention is paid to vascular stenosis and dilatation.

In chapter I.4, the aims of this investigation are set out:
1. Is it possible to produce arterial thrombosis in rat aorta by changing the arterial wall, the blood flow and the composition of the blood?
2. Is it possible to give a summarized, detailed description of the development and organization of this arterial thrombosis?
3. Is such an organizing arterial thrombosis comparable to arteriosclerotic lesions found in man?

In chapter II, a survey of the different models for arterial thrombosis is given. Arguments for the necessity of developing a new experimental model for arterial thrombosis are discussed. Pilot-study and, eventually, development of the new -aortic flap- method are mentioned.

In chapter III, the microsurgical techniques for the aortic flap procedure, the inverting and everting anastomoses, and the technique for the sham procedure are described, followed by a description of the immunohistochemical and histological techniques, used in the processing of the experimental and human specimens. In short, transmission- and scanning electronmicroscopical processing techniques, are described. The used antisera are set out in a table.

Chapter IV deals with the results, starting with a discussion of the macroscopical results in chapter IV.1.

The normal anatomy of rat aorta, with a number of histological stainings and immunohistochemical techniques, are described in chapter IV.2.

In chronological order, the microscopical results of the flapprocedure are set out in chapter IV.3, with a description of the characteristics of every time interval.
After formation of a platelet-fibrin thrombus, an invasion of polymorphonulear cells (PMNs) can be observed, their number increasing during the first twenty-four hours. After this time interval, some eosinophilic granulocytes, macrophages and monocytes can be observed inside and on the thrombus. Little monocytic cells are seen, covering the thrombus mass in an endothelial-like way. The number of SMCs increases, while the number of monocytic cells decreases. These SMCs play a crucial role in the organization of the thrombus. After two weeks, these cells start to produce elastin; first in a granular form, and in later time intervals in strand-like formations, depending on their position in the vessel wall, and influenced by the pulsatile blood flow. After three weeks, investment with normal endothelial cells was confirmed in immunohistochemical studies.
Rethrombosis can be observed in all time intervals, even after ten months. In the two-week old lesions, fibrin could still be demonstrated in the deeper parts of the lesion, and, evidently, in all rethrombotic areas. After three and ten months, the organized vessel wall lesions closely resembled the human arteriosclerotic lesions. Fatty degeneration was never observed in this study.

The relation of thrombus volume and position is described in chapter IV.4, while thrombus volume in two different, early time intervals is calculated.

The macroscopical and microscopical results of the anastomoses series are described in chapter IV.5 and IV.6, and are compared to the results of the flap procedure. A striking resemblance between the results of the inverting anastomoses series and the flap series is confirmed.

The results of the human material, divided into four groups, are described in chapter IV.7 and IV.8. A close resemblance between the fresh thrombi, organizing into subsequent arteriosclerotic lesions in peripheral and coronary arteries, with the experimental flap results is demonstrated.

In the last chapter of the results, chapter IV.9, the transmission and scanning electronmicroscopical results are described, confirming the histological and immunohistochemical results.

In chapter V, the newly developed model is discussed in relation to the literature, as well as to vascular surgery. The importance of haemodynamical changes and thromboplastic activity in the formation of an arterial thrombus is stressed, while the various stages in thrombus organization are seperately discussed.
A number of speculations is made concerning the role of fibrinolysis in the formation and degradation of the thrombus. With the aid of historical data, a correlation is made between experimental and human vascular pathology. The theory and results of Duguid are extensively discussed, and compared with the experimental results of this thesis. The causes and manifestation of an arterial thrombosis in man strongly resemble those in the experimental animal model.

Based on this thrombogenic theory, the possible importance of platelet-aggregation inhibiting drugs is mentioned, together with a number of clinical and experimental investigations.

In conclusion, it can be stated that this model represents vessel wall changes in man, following organization of arterial thrombosis. Subsequently, this model can contribute to the investigations into the prevention of cardiovascular disease.

SAMENVATTING

Cardiale en perifere vaatziekten komen veelvuldig voor in onze samenleving. De medische behandeling van deze ziekten is beperkt tot symptomatische therapie c.q. medicamenteuze therapie, vaatchirurgie en revaliderende therapie na een hartinfarct of cerebrovasculair accident. Het onderzoek naar een causale therapie vindt derhalve voortgang.
Aangezien het niet mogelijk is experimenteel onderzoek te verrichten bij patienten, zijn veel experimentele diermodellen ontworpen om het ontstaan en een mogelijke bestrijding van vaatpathologie te bestuderen.
Arteriële thrombose speelt een fundamentele rol bij in ontwikkeling van cardiale en perifere vaatziekten.

Hoofdstuk I begint met een korte, algemene inleiding in hoofdstuk I.1.

In hoofdstuk I.2 wordt de rol van de bloedplaatjes in de thrombogenese besproken. De vorming van een tapijtachtige plaatjesbedekking (plaatjesadhaesie) en de vorming van een plaatjesaggregaat ter plaatse van een vaatwandlaesie is afhankelijk van de soort vaatwandbeschadiging. De plaatjesreacties zijn van belang in de daaropvolgende vaatwandveranderingen.
Veel onderzoek naar plaatjesadhaesie en aggregatie werd reeds verricht teneinde vaatwandveranderingen en vaatwanddegeneraties te voorkomen. Een aantal resultaten van experimenteel werk aangaande de gladde spiercelproliferatie worden besproken, aangezien deze cel een belangrijke rol speelt in de organisatie van een arteriële thrombose. In de ontwikkeling van arteriële thrombose spelen prostacycline, door de endotheelcel gevormd, en het door de bloedplaatjes gevormde thromboxane een belangrijke rol. Deze, uit arachidonzuur gevormde, actieve stoffen hebben een tegengesteld effect; thromboxane is een vasoconstrictor en bevordert plaatjesaggregatie, terwijl prostacycline een vasodilatator is en plaatjesaggregatie tegengaat.
Door het verstoren van het locale, natuurlijke evenwicht tussen deze twee stoffen kan de plaatjes-fibrine thrombusvorming worden beinvloed. Fibrine is altijd betrokken bij de vorming van plaatjesaggregaten. Deze dissertatie zal handelen over de ontwikkeling van plaatjesaggregaten.
Gebaseerd op de Triade van Virchow spelen, naast vaatwandveranderingen

(vaatwandlaesies) en locale veranderingen in de bloedsamenstelling (prostacycline - thromboxane - weefselthromboplastines), veranderingen in de bloedstroom een belangrijke rol. In de mens en in de experimentele situatie zijn haemodynamische veranderingen in de bloedstroom van belang bij het ontstaan van endotheelbeschadigingen.

Na een bespreking van de haemodynamische physica, worden in hoofdstuk I.3 de pathophysiologische gevolgen hiervan voor de mens beschreven.
De physica kan in deze vorm niet op de normale physiologische circulatie worden toegepast. Veel aandacht wordt besteed aan de vaatwandstenose en vaatwanddilatatie, twee frequent voorkomende beelden in de menselijke vaatpathologie.

In hoofdstuk I.4 wordt de vraagstelling uiteengezet.

1. Is het mogelijk om een arteriële thrombose op te wekken in een ratteaorta door de vaatwand, de bloedstroom en de samenstelling van het bloed te veranderen?
2. Is het mogelijk een samenvattende, gedetailleerde beschrijving te geven van de ontwikkeling en organisatie van deze arteriële thrombose?
3. Is deze organiserende arteriële thrombose vergelijkbaar met arteriosclerotische laesies bij de mens?

In hoofdstuk II wordt een overzicht gegeven van de verschillende arteriële thrombose-modellen. Argumenten worden aangevoerd, teneinde de noodzaak voor de ontwikkeling van een nieuw experimenteel model voor arteriële thrombose aan te tonen. De ontwikkeling van het nieuwe model wordt besproken vanaf de Pilot-study tot de "aorta flap".

In hoofdstuk III worden de microchirurgische technieken voor het aanleggen van een aortaflap, van de inverterende en everterende aorta-anastomose, en van de sham-operatieprocedure besproken. Een opsomming van histologische en immunohistochemische technieken, welke zijn gebruikt in het verwerken en beoordelen van de experimentele en menselijke preparaten, wordt gegeven. De bewerkingen van de preparaten voor beoordeling in transmissie- en scanning electronenmicroscopie worden in het kort beschreven. De gebruikte antisera worden in een aparte tabel vermeld.

Hoofdstuk IV behandelt de resultaten. In hoofdstuk IV.1 worden de macroscopische resultaten vermeld. In chronologische volgorde worden de microscopische resultaten van de flapprocedure uiteengezet, waarbij de karakteristieke veranderingen per tijdseenheid worden beschreven.
Na de vorming van een plaatjes-fibrine thrombus, kan ingroei van polymorphe mononucleaire cellen (PMNs) worden waargenomen. Het aantal PMNs neemt toe gedurende de eerste 24 uur. Na dit tijdsinterval kunnen enkele eosinophiele granulocyten, macrophagen en monocyten in en op de thrombus worden aangetroffen. De thrombusmassa wordt bedekt door kleine monocytaire cellen. Het aantal gladde spiercellen neemt toe, terwijl het aantal monocytaire cellen afneemt. Na twee weken beginnen de gladde spiercellen elastine te produceren; de elastine wordt afgezet in granulaire vorm. Naarmate de ligging van deze elastine-producerende cellen dichter bij het doorstroomde lumen is gesitueerd, wordt de elastine onder invloed van de pulsatiele bloedstroom in lamellaire vorm afgezet.
Ware endotheelcellen kunnen met behulp van anti-Factor VIII:RAG na drie weken op het oppervlak van de thrombus worden gezien.
Rethrombose op de gevormde arteriële thrombose is een verschijnsel wat in alle tijdsintervallen terug is te vinden, zelfs nog na tien maanden. Fibrine kan, tot twee weken na het ontstaan van de thrombus, worden aangetoond in de diepe lagen van de laesie; uiteraard is fibrine altijd aanwezig op de rethromboseplaatsen.
Na drie en tien maanden gelijken de georganiseerde vaatwandlaesies op de menselijke arteriosclerotische afwijkingen. Vettige degeneratie werd in dit onderzoek nooit waargenomen.

In hoofdstuk IV.4 wordt de verhouding tussen thrombusvolume en positie vermeld, terwijl tevens het thrombusvolume op twee vroege tijdstippen wordt berekend.

De macroscopische en microscopische resultaten van de anastomose-series worden besproken in hoofdstuk IV.5 en IV.6. Deze resultaten worden vergeleken met die van de flapserie, waarbij een opvallende gelijkenis wordt gevonden tussen thrombusvorming bij de flapseries en de inverterende anastomose-series.

In hoofdstuk IV.7 en IV.8 zijn de resultaten van het menselijk materiaal, verdeeld in 4 groepen, vermeld. Er bestaat een sterke gelijkenis tussen de verse thrombi en de daaropvolgende organisatie tot arteriosclerotische afwijkingen met de resultaten van de experimentele flapseries.

Het laatste hoofdstuk van de resultaten, hoofdstuk IV.9, vermeldt de transmissie-en scanning electronenmicroscopische resultaten, die de histologische en immunohistochemische resultaten bevestigen.

In hoofdstuk V wordt het nieuw ontwikkelde model besproken tegen de achtergrond van literatuurgegevens, alsmede in het licht van de vaatchirurgie. Het belang van haemodynamische veranderingen en van thromboplastische activiteit wordt benadrukt. De verschillende stadia van thrombose-organisatie worden apart besproken.
Speculatieve beschouwingen over het belang van fibrinolyse in de ontwikkeling en afbraak van een thrombus worden gegeven.
Aan de hand van histologische gegevens betreffende het thrombose-onderzoek wordt een verbinding gelegd tussen de experimentele en humane pathologie. De theorie en de resultaten van Duguid worden uitgebreid beschouwd en vergeleken met de resultaten van de experimentele flapseries. Uit deze beschouwing komt naar voren, dat de oorzaken en manifestatie van arteriële thrombose bij de mens sterke gelijkenis vertonen met deze dierexperimentele situatie.

Het mogelijke belang van plaatjesaggregatie-remmende medicamenten wordt vermeld, ondersteund door deze thrombogene theorie. Een aantal klinische en experimentele studies worden dienaangaande genoemd.

Concluderend kan worden gesteld, dat het model representatief is voor vaatwandveranderingen bij de mens, volgend op arteriële thrombose.
Daarom kan dit model bijdragen in het onderzoek naar de voorkoming van cardiovasculaire ziekten.

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