HISTOCHEMICAL STUDIES ON VASCULAR FIBRINOLYSIS

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HISTOCHEMICAL STUDIES ON VASCULAR FIBRINOLYSIS

by

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to my parents

to Annet

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INTRODUCTION

The deposition of fibrin on a mass of platelets adhering to an injured vascular wall plays an important role in physiological haemostasis following rupture of a blood vessel (MacFarlane 1976).

However, when injury involves only loss of endothelial cells, the 'haemostatic plug' may merely consist of platelets (MacFarlane 1976). Without the added support of fibrin, the platelet plug is inherently unstable and is easily swept away by the blood stream. Only a basal layer of platelets then remains adherent to the exposed subendothelial tissue, providing a surrogate vascular lining while restitution of a true cellular covering takes place (MacFarlane 1976). A new cellular lining may be produced by smooth muscle cells migrating from the vascular tunica media (Spaet et al. 1975) or by endothelial cells arriving from the periphery of the injured area (Poole et al. 1958).

In case of abnormalities of the vessel wall the blood or the flow, the haemostatic mechanisms may induce an excessive increase in the size of the haemostatic plug which then becomes a pathologic lesion, ultimately developing into the 'thrombus' of Virchow (1856). Thrombi generally consist of platelets and fibrin enmeshed with leucocytes and red cells in various proportions (Chandler 1969). The fibrin provides tensile strength and structural integrity to the thrombus (Rodman 1969).

Thrombi may persist on the vascular wall until ultimate disposal is accomplished by endothelial overgrowth and organization by smooth muscle cells (Chandler 1970). In this way, organized mural thrombi may produce focal intimal thickenings indistinguishable from arteriosclerotic lesions (Haust 1975). Such observations have led to the supposition that persistent mural thrombi are of great importance in the genesis or progression of arteriosclerotic lesions (Duguid 1946).

Alternatively, thrombi may grow further and finally occlude the vascular lumen. This occurs frequently in cardiac and cerebral infarctions, of which the mortality is very high, even exceeding that of cancer (Hampton and Mitchell 1976). Moverover, thrombosis frequently results in peripheral venous or arterial occlusion crippling and disabling many of the patients. Fortunately, however, most thrombi do not lead to such dramatic manifestations, since they are usually cleared from the circulation by fibrinolysis (Sherry 1969).

Fibrinolysis is generally believed to play an important role in haemostasis and thrombosis, determining whether, in a particular instance, a surface

deposit held together by fibrin will break up or persist to undergo further growth or organization (Astrup 1969). Too extensive fibrinolysis, however, may result in a premature dissolution of a haemostatic plug and haemorrhagic diathesis (Hirsch et al. 1968; Chesterman 1975). Reduced fibrinolysis, on the other hand, may lead to extensive fibrin deposition (Ashford et al. 1968; Diffang and Saldeen 1974) and to an increased tendendy for thrombosis (Brakman et al. 1966; Nilsson and Isacson 1973).

To anticipate the fate of fibrin deposited on a blood vessel, it is important not only to know the systemic fibrinolytic activity but also the local fibrinolytic activity of the vascular tissue itself. Many investigators have, therefore, studied the fibrinolytic properties of the human vascular wall. Using Todd's (1959) fibrin slide technique, most workers found fibrinolysis promoting activity in relation to vascular endothelial cells (Astrup 1966; Pandolfi 1972). This activity is due to a fibrinolytic activator which is able to convert an inactive precursor, plasminogen, to plasmin, a proteolytic enzyme which is capable of digesting fibrin into several soluble fragments.

Studies of the distribution of the plasminogen activator in the human vascular wall have shown that great differences exist in endothelial activator activity when different human blood vessels are examined on fibrin slides.

Therefore, it was decided to undertake a systematic study of the endothelial activator activity in human arteries and veins employing a standardized fibrin slide technique with a standardized substrate.

During this investigation, however, it was discovered that the observed activity was influenced by fibrinolysis inhibiting material present in some human tissues. To determine the localization and extent of this inhibition of fibrinolysis in tissue sections, a 'fibrin slide sandwich technique', as described in detail in the Appendix, was devised.

The present histochemical studies are concerned with the activation and inhibition of fibrinolysis in the human vascular wall. They attempt to answer the following questions:

1. Does the human vascular wall contain material which inhibits fibrinolysis?
2. What is the source of the inhibiting material within the human vascular wall?
3. Does the inhibitory material influence the degree of the fibrinolytic activity of a vascular tissue section on fibrin slides?
4. What is the distribution and variation in fibrinolytic activity in the walls of human arteries and veins when examined by the 'fibrin slide technique'?

5. What is the distribution and variation in fibrinolysis inhibition in the walls of human arteries and veins when examined by the 'fibrin slide sandwich technique'?
6. What are the relationships between activation and inhibition of fibrinolysis in the walls of human arteries and veins?

These questions are dealt with in the following Chapters in the same sequence.

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Chapter I

HISTOCHEMICAL STUDY OF AN INHIBITOR OF FIBRINOLYSIS IN THE HUMAN ARTERIAL WALL

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The formation of fibrin is a fundamental biological repair mechanism, but the fibrin has ultimately to be removed by fibrinolysis. This is accomplished by the proteolytic enzyme plasmin, which is converted from plasminogen, an inactive precursor in blood, by an activator. Astrup and Permin (1947) have demonstrated that the fibrinolytic activity of tissues is due to such an activator of plasminogen.

To localize fibrinolytic activity in different tissues, Todd (1958) developed a histochemical method in which frozen tissue sections are covered by a thin layer of fibrin rich in plasminogen. During incubation at 37°C, structures containing the plasminogen activator caused lysis which appeared as clear zones in the subsequently stained fibrin. By means of this fibrin slide technique, several investigators (Todd 1959; Kwaan 1966) found the plasminogen activator in human tissues to be concentrated predominantly in endothelial cells of capillaries and veins.

The endothelial cells of the lumen of human arteries usually showed no activity, however, in contrast to the vasa vasorum of their adventitia. This remarkable lack of activity in endothelial cells of human arterial intima prompted us to carry out a renewed investigation of human muscular and elastic arteries, as well as veins, by means of the fibrin slide technique. For discrimination between plasminogen activator activity and non-specific protease activity, we prepared slides with bovine fibrinogen with and without plasminogen (Brakman 1967).

We confirmed the original finding that fibrinolytic activity due to an activator of plasminogen is related to endothelial cells of most veins and capillaries, but not to those of arterial intima. During this investigation, however, our attention was drawn to an outstanding recurrent phenomenon: the

initially small round areas of lysis due to plasminogen activator in the vasa vasorum of the arterial adventitia flatten towards the site of the media, while they extend during prolonged incubation. Consequently, their centres shift to lateral (Fig. 1).

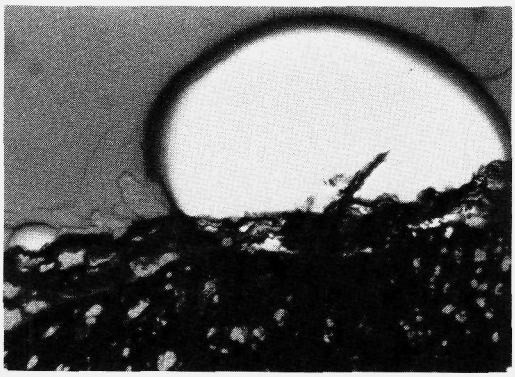
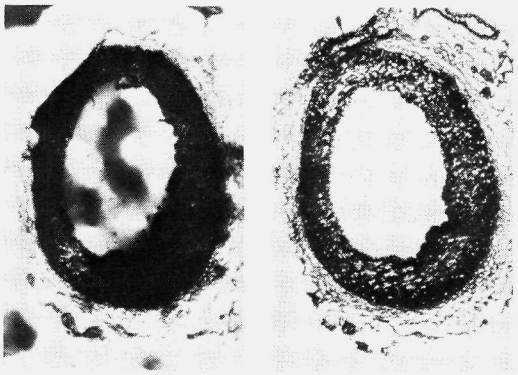


FIG. 1 Lysis produced by the vasa vasorum in the adventitia of the hepatic artery. Left, an initially small zone of lysis; right, an extended zone with flattening towards the media. Harris' alum haematoxylin (x 75).

This feature was more pronounced in human muscular than in elastic arteries, while the distortion of lytic zones was hardly noticeable around large human veins. A possible explanation for this could be that medial tissue contains an inhibitor of fibrinolysis which may diffuse into the fibrin layer above it, thus preventing lysis at those sites.

Evidence for this hypothesis was given by demonstrating the inhibition of fibrinolytically active compounds placed on top of the medial site of human arteries. This was achieved by modifying the fibrin slide technique as follows: frozen sections of the tissue to be examined were placed on a microscope slide, covered by fibrin, and left for at least 2 h in a moist refrigerator, to allow diffusion of inhibiting components from the sections into the fibrin layer. Fibrinolytically active frozen sections were then placed on top of the fibrin film after which the preparations were incubated for various lengths of time at 37°C, fixed, and stained. We called this technique the 'fibrin slide sandwich technique' (Noordhoek Hegt and Brakman, 1973). When used on fibrin films rich in plasminogen, the fibrinolytically active layer of the 'sandwich' consisted of a frozen section of human lung known to be rich in plasminogen activator, or a section of a frozen urokinase solution (human urokinase, Leo Pharmaceuticals, Copenhagen, dissolved to 7 Ploug units ml^{-1} in saline barbital buffer with 15% gelatin). Human plasmin (Sgouris, Inman, McCall, Hyndman and Anderson, 1960) (Michigan Department of Public Health, Michigan, diluted to 1.5 U ml^{-1} in saline with 15% gelatin) was used on plasminogen-free fibrin.

By means of the sandwich technique using both activator and plasmin as the active layer, a clear inhibition of fibrinolysis was demonstrated in the medial area of fresh human arteries but not in the adventitial region. This inhibitory effect was indicated by a dark-stained fibrin strand remaining on top of the media while the active top layer had lysed most of the fibrin which it contacted. Usually some fibrin is observed remaining in the lumen at the moment when the fibrin adjacent to the adventitia is already lysed (Fig. 2a). Inhibition of both activator and plasmin by human elastic arteries was not as effective as by muscular ones; the walls of large veins, for example the superior vena cava, showed very little inhibition.



a b

FIG 2 Cross sections of the splenic artery unheated (a) and heated (b) assayed on plasminogen-free fibrin with the fibrin slide sandwich technique using plasmin as active compound. After incubation for 140 min, a dark-stained fibrin strand remains on top of the media of the unheated section, while on the heated section all fibrin is lysed. Harris' alum haematoxylin (x 25).

During further attempts to confirm our evidence, it was discovered that heating tissue sections in a dry oven at 100°C for 12 h before covering with fibrin abolished the inhibition effect. The absence of a dark-stained fibrin strand on top of the media of a heated section (Fig. 2b) shows the loss of inhibition in contrast to its presence in fresh sections (Fig. 2a).

Our results thus indicate the presence of an inhibitor of fibrinolysis in the human arterial wall which is able to diffuse into fibrin. This provides a natural explanation for the distortion of lytic zones to the medial site, and possibly for the absence of fibrinolytic activity in the arterial intima as seen with the fibrin slide technique. The physiological significance of this inhibitor in human arteries remains to be discovered, but its presence should be kept in mind when considering the role of fibrinolysis in vascular disease.

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Chapter II

INHIBITION OF FIBRINOLYSIS BY THE HUMAN VASCULAR WALL RELATED TO THE PRESENCE OF SMOOTH MUSCLE CELLS

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Key Words. Inhibition of fibrinolysis - Human vascular wall - Smooth muscle cells - Fibrin slide sandwich technique

Abstract. The fibrin slide sandwich technique is a histochemical method for the detection and localization of inhibition of fibrinolysis in tissues. By this technique it was demonstrated that plasmin inhibition is present in the human vascular wall. The great diversity in the pattern and the capacity for inhibition among different parts of the vascular system appeared to be in close association with the localization and number of smooth muscle cells present in this system. The postulated relationship between fibrinolytic inhibition and smooth muscle cells was strongly supported by the demonstration of a similar inhibition of fibrinolysis in other human tissues containing a high number of smooth muscle cells.

INTRODUCTION

Although not commonly known, the presence of an inhibitor of plasmin in the human arterial wall seems to be rather well established (Benzer et al. 1966; Isidori et al. 1963; Neri Serneri et al. 1965; Noordhoek Hegt and Brakman 1974). Inhibition of plasmin has also been demonstrated in connection with large human veins (Neri Serneri et al. 1965) and, moreover, it occurs in the supernatant of cultures of human peripheral veins (Bernik and Kwaan 1971). The latter investigation made it likely that a certain cell type produces such an inhibitor.

The localization of the inhibition of fibrinolysis was made recently possible by means of a modified fibrin slide technique (Noordhoek Hegt and Brak-

man 1973). In a preliminary report (Noordhoek Hegt and Brakman 1974), it was demonstrated that inhibition of fibrinolysis could be detected in the medial area of human arteries, while no inhibition was present in the adventitial region with this so-called fibrin slide sandwich technique. It was observed that this inhibitory effect was more pronounced in the media of arteries of medium caliber than in the media of large arteries, while large veins such as the superior vena cava showed the least inhibition. The aim of the experiments reported in this article was to examine different types of human blood vessels more systematically by use of the fibrin slide sandwich technique in order to classify these vessels as to their inhibitory capacity towards plasmin. Parallel frozen tissue sections were stained histologically in order to relate the inhibitory effect to tissue structures present in the vascular wall, e.g., collagen, elastic tissue, and smooth muscle cells.

MATERIALS AND METHODS

Human blood vessels to be examined for inhibition of fibrinolysis were taken from autopsy and biopsy material and immediately placed in isopentane cooled with liquid nitrogen. The hepatic artery (4), the splenic artery (7), and the right coronary artery (6), were taken as examples of arteries of medium caliber; the aorta (10) and the common carotid artery (4) as large arteries; the great saphenous vein (10) and the umbilical vein (4) as veins of medium caliber; and the vena cava (6) and internal jugular vein (5) as representatives of large veins. After freezing, the specimens were stored in aluminium air-tight bags at a temperature below -20°C until use. Eight frozen sections of 16 μm thickness were cut from each blood vessel with a cryostat microtome, individually placed on eight precleaned microscope slides, and covered by a plasminogen-free fibrin film. The fibrin was spread over the tissue in the same manner and over the same area as described for the fibrin slide technique devised by Todd (1959) 15 years ago. To obtain a clear-cut inhibition pattern, the fibrin film must be 120 μm thick. This is accomplished by spreading a mixture of 100 μl of a solution of 0.7% bovine plasminogen-free fibrinogen (Poviet, Amsterdam) in a phosphate buffer (pH 7.75; ionic strength 0.15) and 20 μl of a solution of bovine thrombin (Leo Pharmaceuticals, Copenhagen) in saline (20 NIH U/ml) over an area of 2.5 x 4 cm. After clotting, the preparations were left for 1 h in a moist refrigerator to allow diffusion of inhibiting compounds from the tissue section into the overlying fibrin. An 8 μm thick section of a previously frozen plasmin solution was

then placed on top of each fibrin film. Human plasmin (Sgouris et al. 1960) from the State of Michigan Department of Public Health (USA), diluted to 1.5 CU/ml in saline with 15% gelatin, was used. The general construction of the sandwich so formed is illustrated in Fig. 1a.

Finally, the preparations were incubated in a moist environment at 37°C for varying periods of time ranging from 40 to 180 min with intervals of 20 min, fixed in a 4-percent formaldehyde solution for 15 min, rinsed in running tap water for another 15 min, and stained in Harris' alum haematoxylin stain for 12 h. During incubation, the top layer thaws and, subsequently, the liberated plasmin will attempt to digest the underlying fibrin. This digestion will be prevented, however, at the sites where a plasmin inhibitor has previously diffused from the tissue section into the fibrin. As a result of this inhibition, a fibrin strand will remain at those sites. A general schematic illustration of such inhibition is shown in Fig. 1b.

Parallel tissue sections which were heated at 100°C in a dry oven for 12 h prior to the coverage with fibrin were used as a control.

FIG 1 The fibrin slide sandwich technique on cross-section before (a) and after (b) incubation. A = fibrinolytically active top layer; B = fibrin film; C = microscope slide; D = tissue to be examined for inhibition of fibrinolysis; E = lysed fibrin.

In order to correlate the plasmin inhibition patterns with a certain tissue structure present in the vascular wall, histological staining was done on 16 µm thick parallel frozen tissue sections with the elastic tissue stain of Lawson (1936) and the picro-Mallory staining method of Lendrum et al. (1962) especially adapted for muscle determination. This adaption consisted of staining with fuchsin for 10 min, with 2% phosphotungstic acid also for 10 min, and with 1% light green for 30 sec.

In addition, three different trichrome-type staining techniques were used to exclude fibrin-like material in the lumen or wall of the blood vessels onto which any inhibitor of the fibrinolytic system from the blood might have absorbed: the MSB method of Lendrum et al. (1962), the picro-Mallory stain of Lendrum et al. (1962) and Ladewig's (1938) modification of the Mallory--Heidenhain method. On the basis of these staining experiments, the presence

of fibrin-like material in the lumen or wall of the investigated vessels was excluded.

RESULTS

When using plasmin as the active top layer on slides without tissue sections, massive lysis of the underlying fibrin was observed after some 80 min of incubation. Slides with tissue sections, but without active top layer, never showed any activity on plasminogen-free fibrin. When examining cross--sections of arteries of medium caliber with the sandwich technique, complete lysis was observed on top of the adventitia of the vessel after 80 min of incubation, while the fibrin on top of the medial/intimal and in the luminal area remained unaffected. This inhibition pattern is schematically illustrated in Fig. 2.

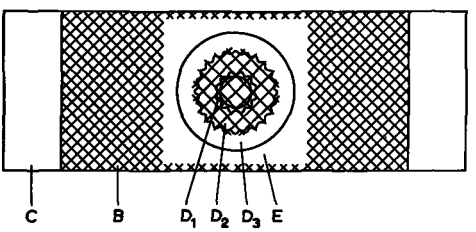

FIG. 2 Schematic top view of a cross-sectioned human artery of medium caliber examined for plasmin inhibition by means of the fibrin slide sandwich technique. After 100 min of incubation, a fibrin layer remained on top of the medial/intimal area and in the lumen of the vessel, while the fibrin on the adventitia was already completely lysed. B = fibrin film; C = microscope slide; D_1 = tunica intima; D_2 = tunica media; D_3 = tunica adventitia; E = lysed fibrin.

Between 100 and 140 min of incubation, the fibrin in the luminal region was also lysed, while the fibrin above the medial/intimal area was not lysed until after about 160 min of incubation in the majority of cases. Fig. 3 shows an actual slide of an artery of medium caliber after 100 min of incubation with a fibrin layer remaining on top of the media and intima and in the lumen.

Longitudinal sections of these blood vessels, when examined with the fibrin slide sandwich technique, showed no difference in lysis time between the fibrin on the adventitial area and that in the lumen.

Hardly any inhibition of the plasmin-induced fibrinolysis was observed when the preparations were not left in a moist chamber at 4°C before incubation, allowing no time for diffusion; on the other hand, the inhibitory

effect was strongly reduced when the slides were stored at 4°C for 12 h or more.

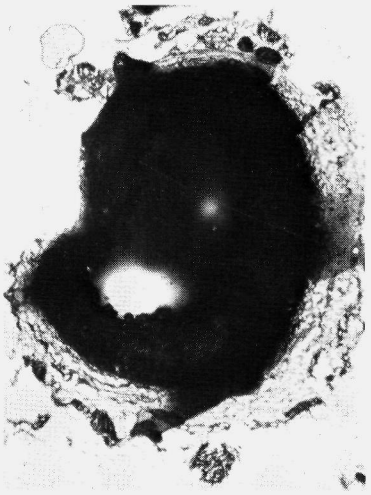

FIG. 3 Cross-section of the human splenic artery assayed on plasminogen--free fibrin with the fibrin slide sandwich technique using plasmin as active compound. After incubation for 100 min, a dark-stained fibrin layer remains on top of the medial/intimal area and in the lumen of the vessel, while on the adventitia nearly all fibrin is lysed. Harris' alum haematoxylin. x 20.

Dry heating of the tissue sections at 100°C for 12 h prior to the coverage with fibrin abolished the inhibition in all instances, as evidenced by the fact that no remaining fibrin was ever seen on top of a heated vascular tissue section after 80 min of incubation.

From these data, it can be concluded that a plasmin inhibitor, which is able to diffuse into fibrin, is present in the media of human arteries of medium caliber. Since the intima and media of these blood vessels are closely associated, it was not possible at that stage of the study to distinguish whether the inhibitor was also present in the intima itself. The inhibitory effect was, however, definitely not located in the adventitia.

Frozen sections of human veins of medium caliber, when examined with the fibrin slide sandwich technique, also showed a fibrin strand remaining on top of the medial/intimal area after about 100 min of incubation and, just as on the arteries, no fibrin was left on the adventitia after that incubation period (Fig. 4). After a longer incubation period, the fibrin on the medial/intimal area was lysed in most instances, indicating that the inhibitory power of these veins was less pronounced than that of arteries of the same caliber. Large arteries also showed some inhibition above the medial/intimal area, but the effect was not as powerful as in the blood vessels of medium caliber.

Only slight inhibition could be detected in the vena cava but, surprisingly, an inhibitory effect in these walls was found above the adventitial area

comprising most of the vessel wall. No, or occasionally slight, inhibition of plasmin was observed in the internal jugular veins.

The described pattern of inhibition of the different vessels is a consistent one and was found in all cases studied.

FIG. 4 Cross-section of the human great saphenous vein tested for plasmin inhibition. After 100 min of incubation. a dark fibrin strand can be observed in relation to the medial/intimal area of the vessel, while on the adventitia and in the lumen no fibrin is left. Harris' alum haematoxylin. x 30.

By juxtaposition of the inhibition patterns and those of parallel tissue sections stained for elastic fibers, collagen, and smooth muscle cells, a strong relationship appeared to exist between the potency and area of the plasmin inhibition and the number and localization of the smooth muscle cells present.

The media of arteries of medium caliber (muscular arteries) consists almost exclusively of smooth muscle cells, correlating well with the intense plasmin inhibition at that site. In the media of muscular veins of medium caliber, especially the saphenous veins, more connective tissue is found between the smooth muscle cells, which correlates with a less powerful inhibition in the media of these veins as compared with the arteries of the same caliber. Large (elastic) arteries have many elastic fibers besides smooth muscle cells in their media and this correlates with the slight plasmin inhibition observed in that area. The adventitia of these three types of blood vessels, which possessed no inhibition capacity towards plasmin, contains no or only few smooth muscle cells and consists mainly of loose connective tissue. Large veins such as the vena cava, on the contrary, have large longitudinal bundles of smooth muscles in the adventitia simultaneously present with the plasmin inhibition observed at that site. Jugular veins have only a few smooth muscle cells in their entire wall which is in good accord with the

reported absence of fibrinolytic inhibition in this type of vessel.

In conclusion, these observations strongly indicate a relationship between the presence of smooth muscle cells and plasmin inhibition in the human vascular wall. To obtain more evidence on this relationship, three types of human tissues entirely different from the vascular wall, but with a considerable content of smooth muscle cells, were investigated with respect to their inhibitory capacity towards plasmin. The external muscular layer of the human gastrointestinal tract, which consists of two fairly substantial layers of smooth muscle fibers, was first examined. With the sandwich technique, a clear and intense inhibition of the plasmin-induced fibrinolysis was observed on top of this muscular wall, while no inhibition was seen above the adjacent submucosa which consists of loose connective tissue without smooth muscle cells (Fig. 5).

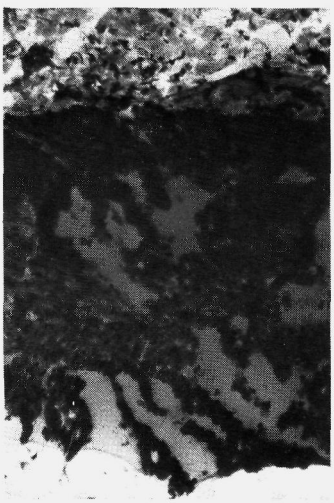

FIG. 5 Cross-section of the outer part of the human gastrointestinal tract. After 120 min of incubation, a fibrin strand covers the muscular layer which is located in the middle, while the submucosa (on top) apparently was not able to prevent lysis above its area. Harris' alum haematoxylin. x 100.

Secondly, the myometrium of the human uterus was tested for plasmin inhibition. The many smooth muscle fibers of this muscular layer are arranged in bundles separated from one another by interstitial connective tissue. A fibrin layer remained on top of the myometrium throughout a long incubation period, indicating the occurrence of plasmin inhibition in that entire area. However, the inhibition capacity was not as strong as in tissue structures consisting almost exclusively of smooth muscles such as the media of muscular arteries.

Thirdly, the human vas deferens was investigated for plasmin inhibition. This excretory duct of the male genital system is constructed mainly of a 1 mm thick layer of smooth muscles on the inner side bordered by a very small

lamina propria of connective tissue lined by columnar epithelium, while on the periphery there is an adventitial coat of connective tissue. The inhibition pattern of cross-sections of this duct resembled that of medium-size blood vessels. The fibrin on the adventitial area was lysed first, followed by the fibrin on the luminal area and the lamina propria, while the fibrin on the muscular coat was lysed last. Figure 6 demonstrates the inhibition pattern of a cross-section of the vas deferens after 120 min of incubation when most of the fibrin is still present on top of the muscle layer and the lumen, while no fibrin is left on the adventitia. As in human blood vessels,

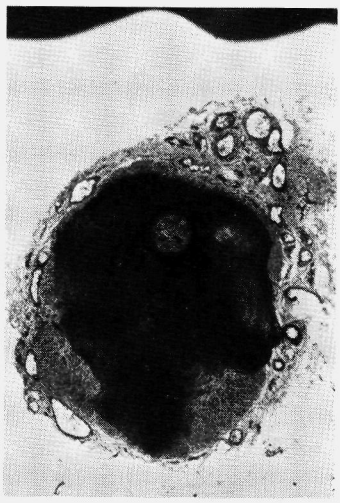

FIG. 6 Cross-section of the human vas deferens. After incubation for 120 min, most of the fibrin on top of the thick muscular wall is still present, while above the adventitia on the periphery all fibrin is lysed. Harris' alum haematoxylin. x 40.

the inhibitory effect of these three types of tissue towards plasmin could be abolished by heating the tissue sections prior to coverage with fibrin. These results with the fibrin slide sandwich technique further demonstrate a relationship between smooth muscle cells and plasmin inhibition and support the conclusion that the smooth muscle cell is responsible for the inhibition of plasmin-induced fibrinolysis by the wall of the human vascular system.

DISCUSSION

The demonstrated relationship between plasmin inhibition and the smooth muscle cells in the wall of the human vascular system evidently means that this inhibition is not limited to the media of the investigated blood vessels, but extends to the intimal area of most of these vessels, since smooth muscle cells, according to our own observations and those of others (Ross and Glomset 1973) are also present in varying numbers in that layer.

Taking this into account, our results are in good agreement with those of Neri Serneri et al. (1965) who found the plasmin inhibition in the intima/media complex of human arteries and not in the adventitia.

Benzer et al. (1966) also recorded the greatest plasmin inhibition in the intimal/medial area of human arteries, but they also found a greater inhibition in the intima when vascular occlusion had occurred. This is in agreement with the reported (Ross and Glomset 1973) increase in the number of intimal smooth muscle cells in such cases.

The most probable explanation for the inhibition of fibrinolysis in the luminal region of a cross-sectioned blood vessel is that the inhibitor converges towards the lumen of the vessel and diverges towards the adventitial side during diffusion. Converging means a greater increase in the concentration of the inhibitor than does diverging and, consequently, a greater inhibition is observed in the luminal area.

The inhibition of plasminogen activation in addition to plasmin inhibition cannot be excluded from these experiments, because plasmin inhibition masks possible inhibition of earlier phases in the fibrinolytic system in the fibrin slide sandwich technique.

Further studies are required in order to determine the chemical nature of the inhibiting compound(s) and to find out whether the smooth muscle cells liberate their inhibiting compound(s) in vivo during their entire lifespan or only during cell death. An interesting fact in this respect might be the finding of Mishchenko et al. (1972) of the release of a plasmin inhibitor from intact canine arteries and veins into a perfused salt solution.

Another manuscript is in preparation which deals with the arteriovenous differences in endothelial fibrinolytic activity on fibrin slides (Todd 1959). These differences may be due to inhibition from the surrounding tissue rather than to variability in the activity of the endothelium itself.

ACKNOWLEDGEMENTS

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Chapter III

HISTOCHEMICAL DEMONSTRATION OF INHIBITION OF FIBRINOLYSIS IN HUMAN TISSUES BY MEANS OF THE FIBRIN SLIDE SANDWICH TECHNIQUE

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INTRODUCTION

The processes of coagulation and fibrinolysis are of fundamental importance in the formation and dissolution of fibrin in maintaining the integrity of the organism. Fibrin formation at a site of injury is an essential part of tissue repair, and the amount of fibrin remaining at that site is a determining factor in the amount of reparative connective tissue formed. Since the fate of a fibrin clot formed in response to tissue injury is partly determined by the local release of fibrinolytic agents from the injured cells of the particular tissue (Astrup 1966), it is of great importance to know the nature as well as the localization of fibrinolytic enzymes in these tissues.

The nature of the fibrinolytic enzymes was discovered about 25 yr ago by Astrup and Permin (1947), who demonstrated that the fibrinolytic activity of tissues is due to an activator of plasminogen. The localization of this plasminogen activator in tissues was first made possible 15 yr ago when Todd (1958, 1959, 1960) developed a histochemical method in which frozen tissue sections are covered by a thin layer of plasminogen-rich fibrin. During incubation at 37°C structures containing the plasminogen activator cause lysis, which appears as clear zones in the subsequently stained fibrin. By means of this 'fibrin slide technique' it was established that vascular endothelium is the main structure containing the plasminogen activator (Kwaan and Astrup 1963; 1967; Pandolfi 1967; Todd 1959, 1960; Warren 1963, 1964).

In the literature, however, much incomprehensibility and confusion has arisen as to distribution of the plasminogen activator along the human

vascular system, since great local differences in endothelial fibrinolytic activity are displayed when different human tissues are examined on fibrin slides. A very high fibrinolytic activity was found, for instance, in relation to small vessels present in loose connective tissue areas such as the adventitia of many blood vessels (Pandolfi et al. 1968; Todd 1960) and connective scar tissue (Kwaan and Astrup 1964a; Kwaan and Astrup 1964b; Peterson et al. 1969). On the other hand, liver and spleen almost completely fail to show fibrinolytic activity (Denk et al. 1970; Todd 1960) despite their intense vascularization. These differences in endothelial fibrinolysis have puzzled many investigators, and many suggestions have been made as to their physiologic implications.

In the fibrin slide technique frozen sections of tissue were examined for activator activity only, and the influence of tissue compounds which exert an inhibitory effect on fibrinolysis was not ruled out in one way or another. The conspicuous differences in endothelial fibrinolytic activity on fibrin slides might very well be due to inhibitory influences from the surrounding tissue rather than to a true variability in activator content.

Therefore if we want to investigate human tissues for localization of the plasminogen activator by means of the fibrin slide technique, we must also examine these tissues for their ability to inhibit the fibrinolytic process. For the latter purpose, we devised a histochemical technique - the fibrin slide sandwich technique (Noordhoek Hegt and Brakman 1973; Noordhoek Hegt and Brakman 1974a and 1974b) - which is a modification of Todd's fibrin slide technique.

FIBRIN SLIDE SANDWICH TECHNIQUE

Small blocks of tissues to be examined for fibrinolysis inhibition are taken from autopsy or biopsy material and immediately placed in isopentane cooled with liquid nitrogen. After freezing, the specimens are stored in aluminium air-tight jars at a temperature below -20°C until use. Eight frozen sections 16 µm thick are cut from each tissue with a cryostat microtome and covered by a plasminogen-free fibrin film. The film is spread over the tissue in the same manner and over the same area as described by Todd (1959) for his fibrin slide technique.

To obtain a clear-cut inhibition pattern, the fibrin film must be 120 µm thick. This is accomplished by spreading a mixture of 100 µl of a solution of 0.7% bovine plasminogen-free fibrinogen (Poviet, Amsterdam) in a phosphate

buffer (pH 7.75, ionic strength 0.15) and 20 µl of a solution of bovine thrombin (Leo Pharmaceuticals, Copenhagen) in saline (20 NIH units/ml) over an area 2.5 x 4 cm.

After clotting the preparations are left for 1 h (unless stated otherwise) in a moist refrigerator to allow diffusion of inhibiting compounds from the tissue section into the overlying fibrin. A section (approximately 10 µm thick) of a previously frozen plasmin solution is then placed on top of each fibrin film. We use human plasmin (Sgouris et al. 1960) from the State of Michigan Department of Public Health (United States) diluted to 1.5 CU/ml in saline with 15% gelatin.

The general construction of the sandwich so formed is illustrated in Fig. 1a. Finally a series of preparations is incubated in a moist environment at 37°C for varying periods of time ranging from 40 to 180 min with intervals of 20 min, rinsed in running tap water for another 15 min, and stained in Harris' alum haematoxylin stain for 12 h. During incubation the top layer thaws, and subsequently the liberated plasmin digests the underlying fibrin. This fibrin digestion can be inhibited, however, at sites where material from the tissue section has previously diffused into the fibrin layer.

As a result of this inhibition, a fibrin isle remains at those sites. Such inhibition patterns are shown in Figs. 1b and 2. Adjacent tissue sections which were heated at 100°C in a dry oven for 12 h prior to coverage with fibrin were used as controls. In addition, adjacent sections were stained histologically with Lendrum's picro-Mallory stain (Lendrum et al. 1962) in order to correlate the inhibition patterns with certain tissue structures and to detect contaminating fibrin-like material containing inhibiting substances in the tissues studied.

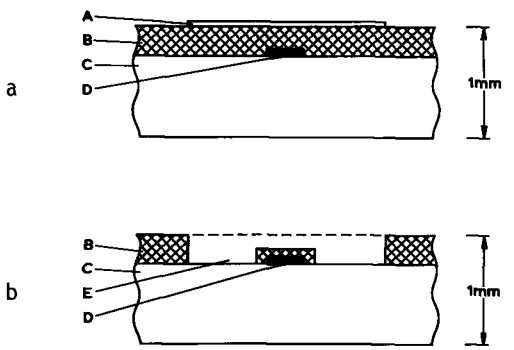

FIG. 1 Fibrin slide sandwich technique on cross-section before (a) and after (b) incubation. A, fibrinolytically active top layer, B, fibrin film. C, microscope slide. D, tissue to be examined for inhibition of fibrinolysis. E, lysed film.

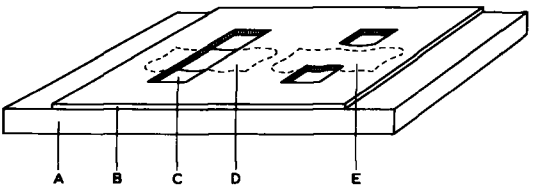

FIG. 2 Top view of the fibrin slide sandwich technique after incubation. A, microscope slide. B, fibrin film. C, lysed fibrin caused by active top layer. D, tissue without inhibition. E, tissue with inhibition. Note that all fibrin above the left tissue has been lysed, indicating the absence of inhibition. On the other hand, a fibrin strand has remained on top of the right tissue, indicating inhibition of the plasmin-induced fibrinolysis at that site.

By means of this fibrin slide sandwich technique, frozen sections of human liver, loose connective tissue, vas deferens, and the walls of the vascular system and the gastrointestinal tract, among other tissues, were investigated for their inhibitory activity towards plasmin. The same human tissues were also examined for localization of the plasminogen activator using the usual fibrin slide technique. The results obtained with both techniques were compared in order to find out whether a correlation exists between the presence of inhibition and the absence of activity and vice versa.

RESULTS

When sections of human liver were examined for inhibition of fibrinolysis by means of the sandwich technique, a fibrin isle persisted above the entire section, even after 3 h of incubation (Fig. 3), indicating inhibition of fibrinolysis at that site.

Adjacent sections of the same liver exhibited very little fibrinolytic activity when examined on plasminogen-rich fibrin slides. Incubation for 3 h resulted in only a few small lysed zones confined to vessels in the connective tissue of some portal areas (Fig. 4).

In contrast to the liver, vascularized loose connective tissue already showed a great number of lysed zones all over the section in relation to small vessels present after 5 min of incubation (Fig. 5). After incubation for 20 min, all fibrin in contact with the tissue was lysed, indicating the extremely high fibrinolytic activity of this type of tissue.

FIG. 3 Section of human liver assayed on plasminogen-free fibrin with the fibrin slide sandwich technique using plasmin as the active compound in the top layer. After incubation for 180 min a dark-stained fibrin isle has remained on top of the entire section, indicating inhibition of the plasmin--induced lysis by the liver tissue. Harris' alum haematoxylin. x 8.

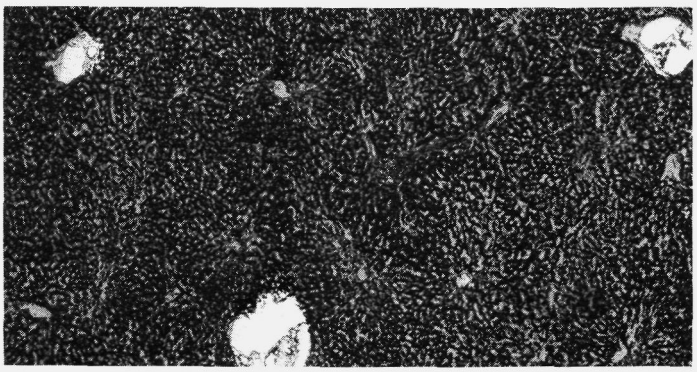

FIG 4 Lysis produced by a section of liver tissue examined for fibrinolytic activity on a usual plasminogen-rich fibrin slide incubated for 3 h. Note the small number of lysed areas in such a highly vascularized organ. Harris' alum haematoxylin. x 26.

When an adjacent section of this connective tissue was examined for inhibition of fibrinolysis by means of the sandwich technique, it was observed that the plasmin from the top layer had already lysed all of the fibrin it contacted after a short incubation period; and, in contrast to the liver, no fibrin was left on top of the tissue (Fig. 6). This indicates that loose connective tissue showed no inhibitory effect on fibrinolysis when examined by the sandwich technique.

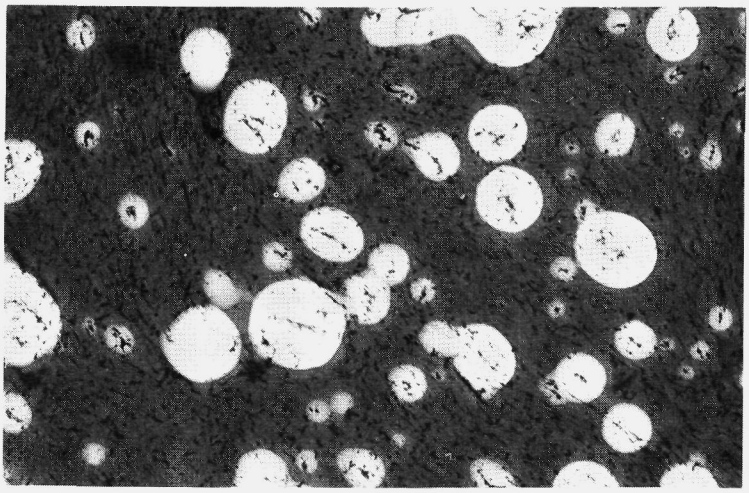

FIG. 5 Lysis produced by a section of vascularized loose connective tissue examined for fibrinolytic activity on a fibrin slide incubated for only 5 min. Small vessels all over the section already show activity after this short incubation period, indicating the extremely high activity of this tissue. Harris' alum haematoxylin. x 26.

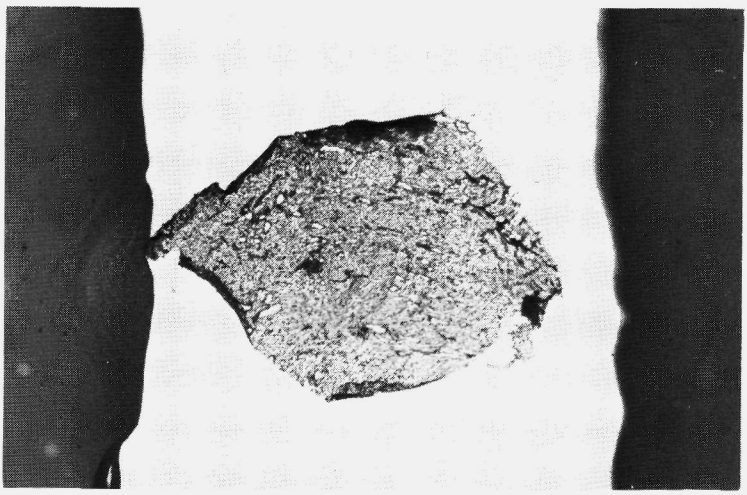

FIG. 6 Section of vascularized loose connective tissue examined for inhibition of fibrinolysis with the fibrin slide sandwich technique. After incubation for 100 min the plasmin from the top layer has lysed all the fibrin it contacted, and no fibrin was left on top of the tissue, indicating the absence of inhibition at that site. Harris' alum haematoxylin. x 14.

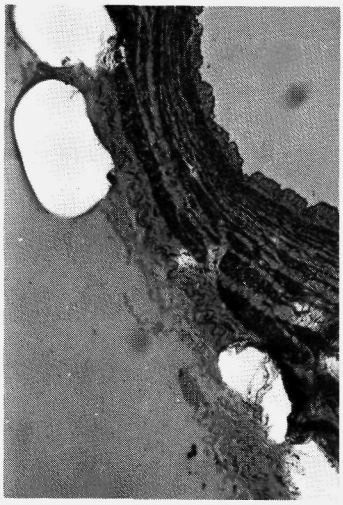

FIG. 7 Part of a cross-sectioned human great saphenous vein examined for fibrinolytic activity on a plasminogen-rich fibrin slide incubated for 30 min. Note the differences between the diameters of the lysed zones above the adventitia and those above the media of the vessel wall. Harris' alum haematoxylin. x 50.

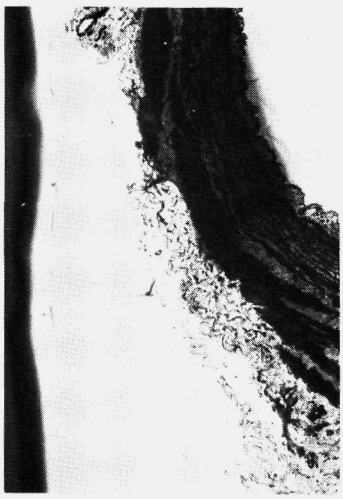

FIG. 8 Part of a cross-section of the great saphenous vein examined for inhibition of fibrinolysis with the sandwich technique. After 100 min of incubation a dark-stained fibrin strand has persisted above the medial/intimal area of the vessel wall, while in contrast no fibrin remains on top of the adventitia. Harris' alum haematoxylin. x 50.

The next tissue investigated was the wall of the human vascular system in which the local differences in endothelial fibrinolytic activity are so extremely impressive. Figure 7 shows a part of a section of a human great saphenous vein examined for fibrinolytic activity with the fibrin slide technique. Confirming the extensive studies of Pandolfi and co-workers (1967, 1968), a nearly complete absence of lysis was observed in the intimal and medial area of the vessel; while, in contrast, the vasa vasorum in the

adventitia were highly active. This indicates that the vasa vasorum apparently lose lysing ability when penetrating from the adventitia deeply into the media of the vessel.

When an adjacent section of the same saphenous vein was examined for inhibition of fibrinolysis with the sandwich technique, it was observed that the medial/intimal area of the vessel wall had inhibited the plasmin-induced lysis; this is evidenced by the persisting presence of dark-stained fibrin on top of that area after 100 min of indubation (Fig. 8). No fibrin was left, however, above the adventitia of the vessel, indicating the absence of such inhibition at that site.

In the human vas deferens examined for fibrinolytic activity, lysed areas were related to small vessels in the connective tissue surrounding the duct (Fig. 9), confirming the literature on this subject (Liedholm and Astedt 1974). Just as in the saphenous vein the adventitial vessels lose their lysis capacity when penetrating the muscular wall of the duct. When the vas deferens was examined with the fibrin slide sandwich technique, inhibition of the plasmin-induced fibrinolysis was found above the thick muscular layers and not above the adventitia on the periphery of the duct wall (Fig. 10).

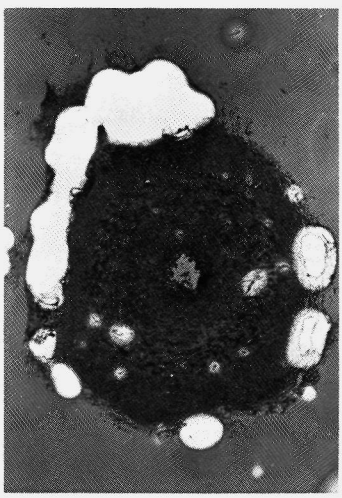

FIG. 9 Cross-section of the human vas deferens examined for fibrinolytic activity after 30 min of incubation. Note that the small vessels in the adventitia produce much more lysis than those in the thick muscular wall. Harris' alum haematoxylin. x 20.

In the wall of the human gastrointestinal tract examined for fibrinolytic activity, plasminogen activator activity was consistently found in relation to small veins and capillaries in the connective tissue of the submucosa and serosa; these vessels in the muscularis externa showed less activity, and the majority of the vessels in the mucosa remained almost completely inactive (Fig. 11). These results substantiate the literature on this subject (Eras et

al. 1970; Kwaan et al. 1969).

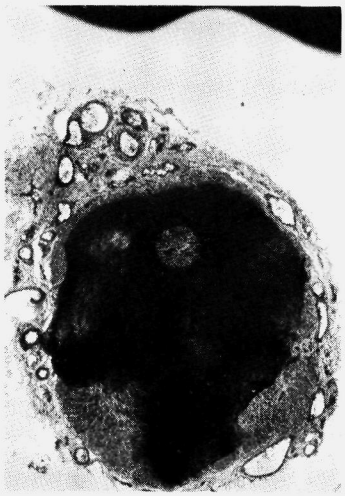

FIG. 10 Cross-section of the human vas deferens examined for plasmin inhibition. After incubation for 120 min, most of the fibrin on top of the thick muscular wall is still present, while all fibrin is lysed above the adventitia on the periphery. Harris' alum haematoxylin. x 20.

FIG. 11 Cross-section of the human duodenum examined for fibrinolytic activity after incubation for 30 min. From top to bottom, four regions can be seen: inactive mucosa, very active submucosa, inactive muscularis externa, and active serosa. Note that extension of lysis occurred above the loose connective tissue of the submucosa. Harris' alum haematoxylin. x 80.

Inhibition of plasmin-induced lysis was found above the mucosa and muscularis externa of the gastrointestinal tract, while the connective tissue of the submucosa and serosa revealed no such inhibitory effect (Fig. 12).

The inhibitory effect of the types of tissue just described was abolished in all instances by heating the tissue sections prior to the coverage with fibrin. Many other human tissues were examined in the same way, and in most

instances the localization of activity appeared to be inversely proportional to the site of inhibition.

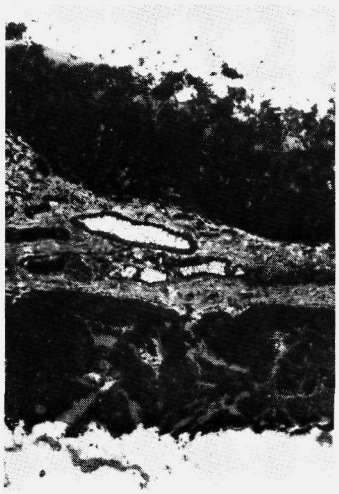

FIG. 12 Cross-section of the human duodenum examined for inhibition of fibrinolysis. After 120 min of incubation fibrin strands remain above the mucosa (top) and the muscularis externa (bottom), while no fibrin is left in the middle above the submucosa. Harris' alum haematoxylin. x 50.

DISCUSSION

From the data presented it appears that much of the variation in endothelial fibrinolytic activity on fibrin slides seems to be due to inhibitory effects from the surrounding tissues rather than to variability in the activator content of the endothelium itself. Because of these inhibitory effects we feel that the fibrin slide technique can no longer be regarded as a sound method for the detection and exact localization of the plasminogen activator in human tissues. It seems, moreover, that the unrestricted transfer of the results obtained on fibrin slides to the in vivo situation is not permissible, since it is not known at this time whether the inhibitory effects are also found in the healthy organism. On the other hand, the results obtained on fibrin slides might very well be comparable with the situation in the human body after tissue injury and cell death, since the slide technique reveals the net result of the action of compounds that activate and inhibit fibrinolysis, both compounds being present in a tissue section in which the majority of cells is damaged.

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Chapter IV

DISTRIBUTION AND VARIATION OF FIBRINOLYTIC ACTIVITY IN THE WALLS OF HUMAN ARTERIES AND VEINS

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Key Words. Arteries - Veins - Endothelial cells - Fibrinolysis - Plasminogen activation - Fibrin slide technique

Abstract. A systematic study on the location and intensity of the fibrinolytic activity in more than 500 samples of human arteries and veins from 50 routine necropsies and 35 blood vessel biopsies was performed. Data were obtained for an overall comparison of the fibrinolytic activity along and across the walls of human blood vessels by the use of a standardized fibrin slide technique.

Arteries generally showed little or no fibrinolytic activity in the intima and media but strong activity in the adventitia. Veins showed a comparable strong fibrinolytic activity in the external layer of loose connective tissue. Fibrinolytic activity in the venous intima, media and adventitia was generally weaker but varied greatly according to position in the body.

Veins situated in the lower parts of the body had less fibrinolytic activity than the veins at the upper levels. Fibrinolytic activity was found to be related to the endothelium of the vasa vasorum and/or of the main lumen of the vascular wall.

Increased fibrinolytic activity was observed in arteries and veins in cases of sudden death, vasogenic shock, cerebral hemorrhage and cirrhosis. Decreased fibrinolytic activity was encountered in blood vessels in cases of endotoxin shock, hyaline membrane disease and a case of Waterhouse-Friderichsen syndrome.

INTRODUCTION

The fibrinolytic activity of tissues is usually initiated by an activator of plasminogen (Astrup and Permin 1947). Todd (1958, 1959) devised a histochemical fibrin slide technique by which he was able to identify the cellular source of the tissue plasminogen activator. He found the activator to be associated with vascular endothelial cells, in particular those of the veins and venules, an observation confirmed by many subsequent investigators (reviews by Astrup (1966) and Pandolfi (1972)).

A survey of the current literature on the distribution of endothelial plasminogen activator activity in the walls of the human vascular system reveals many discrepancies. Thus, Todd(1959, 1960, 1964) found little or no activator activity in the intima and media of human arteries, while other investigators reported a high activity in the inner arterial layers (Constantini et al. 1972; Onoyama and Tanaka 1969). In the vena cava, strong intimal activator activity was found by Bleyl (1969), moderate activity by Todd (1959) and no activity by Glas-Greenwalt (1972). These, and other observations made it likely that the plasminogen activator activity varies between blood vessels from different individuals. However, the reported variations in fibrinolytic activity might be caused in part by differences in the materials and methods used by the different investigators.

To provide an overall comparison of the activator activity along and across the human vessel wall, the present report summarizes data obtained in a systematic study of the activator activity in more than 500 samples of human arteries and veins from necropsies and biopsies employing a standardized fibrin slide technique with a standardized substrate.

MATERIALS AND METHODS

Source of blood vessels

Samples of human blood vessels were obtained from 50 routine necropsies and 35 vessel biopsies. The small blood vessels were studied in specimens of different organs and tissues, while the medium-sized and large vessels were obtained without adjacent tissues. Since rapid freezing is important (Pandolfi et al. 1972), all specimens were immediately frozen in isopentane, cooled with liquid nitrogen or dry ice, and then stored at -20 C in air-tight jars.

Preparation of fibrin slides

Fibrin films were prepared on microscope slides precleaned with ethanol. A set of six fibrin slides was prepared for each specimen. Five slides were covered with plasminogen-rich fibrin to determine activator activity and one slide was covered with plasminogen-free fibrin to detect possible activity caused by a direct proteolytic action upon fibrin. The fibrin films were made by spreading over an area of 2.5 x 4 cm a mixture of 60 µl of a solution of bovine plasminogen-rich fibrinogen (0.7%; w/v) prepared according to Brakman (1967) in phosphate buffer, pH 7.8 and ionic strength 0.15, and 10 µl of a solution of bovine thrombin (Leo Pharmaceuticals, Ballerup, Denmark) 20 NIH U/ml in saline. The plasminogen-free fibrin films were prepared with a solution of bovine fibrinogen without plasminogen (from Poviet, Organon-Teknika, Oss, The Netherlands) with a final fibrinogen concentration of 0.7% (w/v) in a phosphate buffer, pH 7.8, adjusted to an ionic strength of 0.15. For clotting, the slides were left in a moist chamber at room temperature for 30 min. Six frozen sections (8 µm thick) were cut from each specimen on a cryostat microtome and individually placed on a fibrin slide.

Incubation

These slides were incubated at 37°C in a moist chamber. The five plasminogen-rich slides were incubated for 10, 20, 40, 60 and 120 min, respectively. The slide with the plasminogen-free fibrin was incubated for 120 min.

Staining methods

After fixation in 4% (v/v) formaldehyde for 15 min, the slides were rinsed, stained with Harris' haematoxylin or Lawson's elastic tissue stain (both for 12 h) and mounted with glycerin jelly. Lawson's (1936) stain provided a better differentiation between arteries and veins than did the haematoxylin stain.

Grading

The activator activity of a section on the plasminogen-rich fibrin slide was evaluated according to 4 grades: grade I, punctate areas of lysis; grade II, small lysed zones, often of irregular outline; grade III, large, confluent lysed zones; grade IV, digestion of all the fibrin in contact with the tissue. To grades I, II, III and IV were allotted 1, 2, 3 and 4 points, respectively. The sum of the points scored by each set of 5 slides was taken as an arbitrary measure of the activity of that particular specimen.

Correlation between fibrinolytic activity and histologically identifiable structures

Adjacent sections were studied with specific stains to relate fibrinolytic activity to histologically identifiable structures. For that purpose, three different trichrome staining techniques were used: the MSB method of Lendrum (1962), Lendrum's (1962) picro-Mallory stain, and Ladewig's (1938) modification of the Mallory-Heidenhain method.

On plasminogen-rich fibrin slides, plasminogen activator activity was occasionally found to be related to thrombi or clots in the vascular lumen detected by Lendrum's and Ladewig's staining techniques. Such thrombi-related fibrinolysis was also described by Todd (1964, 1967, 1969). All specimens showing such fibrin-related activity were excluded from the present survey.

Distinction between layers of the vessel walls

An attempt was made to evaluate the three principal layers of the vessel wall in both arteries and veins. By means of Lendrum's picro-Mallory stain the intima, media and adventitia were easy to distinguish in the wall of the arteries. An intimal layer, a medial/adventitial layer, and an external layer of loose connective tissue could be distinguished in the venous wall. It was difficult to differentiate between media and adventitia in most veins, because bundles of smooth muscle cells cross both layers. Structurally, these layers resembled the smooth muscle cell-rich arterial media. The external layer of loose connective tissue of the veins generally did not contain many smooth muscle cells.

RESULTS

None of the sections of human blood vessels showed any liquefaction on slides prepared with plasminogen-free fibrin, indicating the absence of non-specific protease activity. On slides prepared with plasminogen-rich fibrin, varying degrees of plasminogen activator activity were observed in relation to vascular endothelial cells. No other vascular elements showed activity, even after long periods of incubation. Detached endothelial cells usually showed higher activity than those remaining at the endothelial lining.

Results of the calculations of the values for the mean endothelial plasminogen activator activities in the walls of large and medium-sized human arteries and veins are presented in tables I and II. The localization of the activator activity along and across the walls of the human vascular system

Table I. Fibrinolytic activity in human arteries

	Number of cases	Mean activator activity arbitrary units		
		I	M	A
Large arteries				
Aorta	40	1	0	12
Innominate artery	9	0	0	9
Subclavian artery	9	1	0	13
Common carotid artery	18	1	0	11
Pulmonary artery	37	1	0	12
Weighted mean activator activity		1	0	12
Medium-sized arteries				
Femoral artery	21	1	0	12
Popliteal artery	6	0	0	10
Posterior tibial artery	6	2	0	14
Hepatic artery	6	0	0	10
Splenic artery	18	1	0	9
Renal artery	22	1	0	11
Superior mesenteric artery	6	0	0	7
Coronary arteries	33	1	0	12
Internal mammary artery	21	1	0	13
Inferior thyroid artery	1	0	0	6
Vertebral artery	3	3	0	15
Basilar artery	2	4	0	10
Weighted mean activator activity		1	0	11

I = Intima; M = Media; A = Adventitia

Table II. Fibrinolytic activity in human veins

	Number of cases	Mean activator activity arbitrary units		
		I	M/A	E
Large veins				
Vena cava	42	3	7	12
Internal jugular vein	16	11	11	12
Subclavian vein	11	8	9	13
Portal vein	14	2	6	8
Pulmonary vein	31	5	8	12
Weighted mean activator activity		5	8	12
Medium-sized veins				
Femoral vein	14	2	6	12
Popliteal vein	6	1	4	10
Saphenous vein	36	2	4	12
Hepatic vein	7	1	6	8
Splenic vein	15	3	7	9
Renal vein	16	3	8	12
Superior mesenteric vein	2	2	4	7
Coronary sinus	19	8	9	12
Internal mammary vein	19	12	11	12
Azygos vein	2	13	14	14
Superior sagittal sinus	3	15	15	15
Meningeal veins	8	13	13	13
Weighted mean activator activity		5	7	11

I = Intima; M/A = Medial/Adventitial layer; E = External layer of loose connective tissue.

was distributed as follows:

Endothelial activator activity in arteries

Intima. There was little or no activator activity in the intimal endothelium of most large, medium-sized, and small arteries. However, the intimal activity was often more pronounced at sites of arterial branchings (Fig. 1). Enhanced intimal activity was also encountered in five biopsy specimens of aorta obtained from patients undergoing surgery for coarctation of the aorta (Fig. 2). Nine aorta biopsies obtained at aorta-to-coronary-bypass grafting revealed no such intimal activity. Intense intimal activity was regularly found in individuals who had suffered from cerebral haemorrhage, vasogenic shock, cirrhosis or had died suddenly independently of the cause of death (Fig. 3).

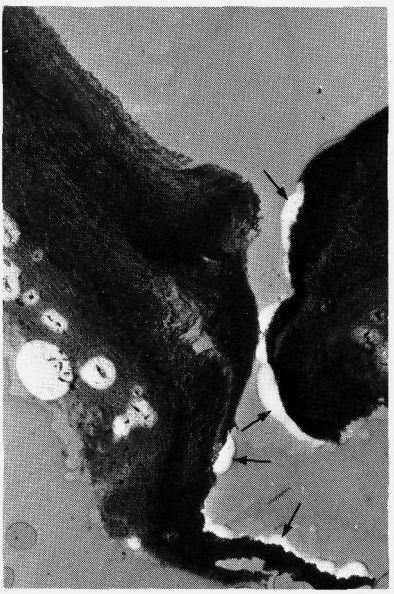

FIG. 1. Human aorta with intercostal branch, incubated for 40 min on a plasminogen-rich fibrin slide. Clear zones of lysis extend from the vasa vasorum in the aortic adventitia and from the intima of the intercostal branch (arrows). No lysis can be detected in the aortic intima and media. Harris' alum haematoxylin. x 50.

Media. The endothelium lining the vasa vasorum in the outer part of the media of all normal appearing arteries revealed no activator activity. However, in some specimens from coarcted aorta vasa vasorum in the fibrous, altered areas of the media produced foci of lysis (Fig. 2). Lysis was also occasionally observed in relation to newly formed vessels in specimens with severely calcified arteriosclerotic plaques which extended into the media.

Table III. Variations in fibrinolytic activity among 10 autopsy cases

	Activator activity in the outer vascular layers[1], arbitrary units									
	393/72[2]	401/72	411/72	466/72	493/72	504/72	507/72	55/73	71/73	110/73
Aorta	6	13	16	0	10	3	8	16	4	12
Coronary artery	6	12	17	0	9	2	9	17	6	13
Internal mammary artery	5	14	18	0	12	3	10	19	7	11
Vena cava	6	14	17	0	11	4	8	17	5	13
Coronary sinus	7	12	16	0	10	4	10	18	7	14
Internal mammary vein	5	14	18	0	12	3	11	19	7	12

[1]Endothelial plasminogen activator activity of the vasa vasorum in the arterial adventitia and in the venous external layer of loose connective tissue.
[2]Autopsy number.

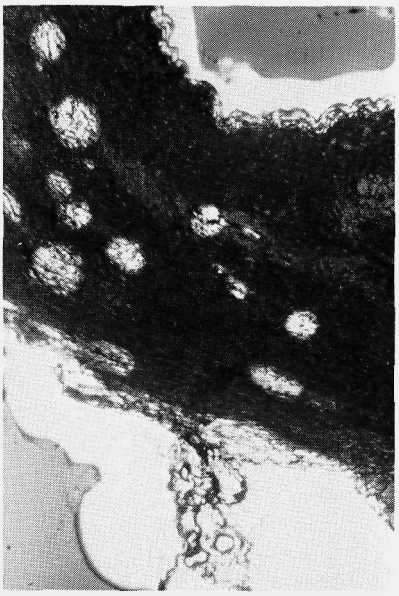

FIG. 2. Human aorta at a site of coarctation, incubated for 120 min on a fibrin slide. All fibrin under the adventitia (bottom) as well as the intima (top) is lysed. Foci of lysis in the media are related to the presence of small vessels. Harris' alum haematoxylin. x 60.

Adventitia. Activator activity related to the vasa vasorum was generally high in the adventitia of large and medium-sized arteries but low or absent in the adventitia of small arteries with a diameter less than 1 mm (Fig. 4). The adventitial activity of the larger arteries appeared to be quite uniformly distributed among different arteries in the same individual, but it varied greatly from one individual to another (table III).

Endothelial activator activity in veins

Intima. Activator activity was generally more pronounced in the intimal endothelium of veins than in that of arteries. When specimens from large veins and medium-sized veins were compared there was no gross difference in mean intimal activity. Small veins (Fig. 4) usually exhibited a stronger intimal activity than larger veins. In contrast to arteries, the veins showed a great diversity in intimal activity when segments from different locations in the same individual were compared (see below).

Media/adventitia. Activator activity related to the endothelium lining the vasa vasorum abundantly present in both layers, was generally stronger than in the intimal endothelium. Large and medium-sized veins showed similar degrees of medial/adventitial activity but the small veins, like the small arteries, generally failed to exhibit activity in these layers. The activator

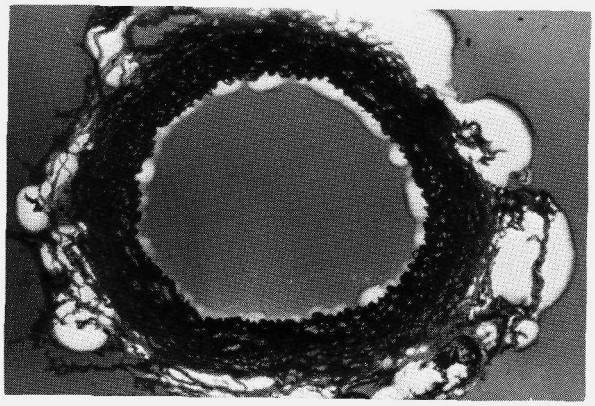

FIG. 3. Hepatic artery obtained after sudden cardiac death and assayed on a fibrin slide. After 20 min of incubation, extending areas of lysis are present under the adventitia and the intima. Harris' alum haematoxylin. x 40.

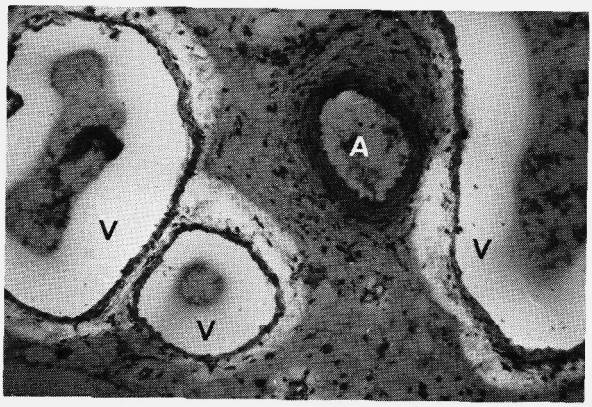

FIG. 4. Small artery (A) and small veins (V) incubated for 10 min on a fibrin slide. Lysis is generated by the veins but not by the artery. Harris' alum haematoxylin. x 200.

activity in the intima, media and adventitia varied greatly in venous segments from different body levels in the same individual. For instance, among specimens from one autopsy, the great saphenous vein below the knee showed little or no intimal and medial activity (Fig. 5a), while the vena cava taken at the level of the liver showed clear activator activity in comparable layers (Fig. 5b). The subclavian vein revealed a stronger activity than the vena cava (Fig. 5c) and the internal jugular vein exhibited the highest activity of these four veins (Fig. 5d). When comparing the mean endothelial activator activity of all veins studied, it appeared that veins from the upper parts of the body, such as the meningeal veins, the superior sagittal sinus, the internal jugular vein, the subclavian vein, the mammary vein, the coronary sinus and the azygos vein, exhibited a much higher mean activator activity in their intimal, medial and adventitial layers than veins from the lower parts of the body (Fig. 6).

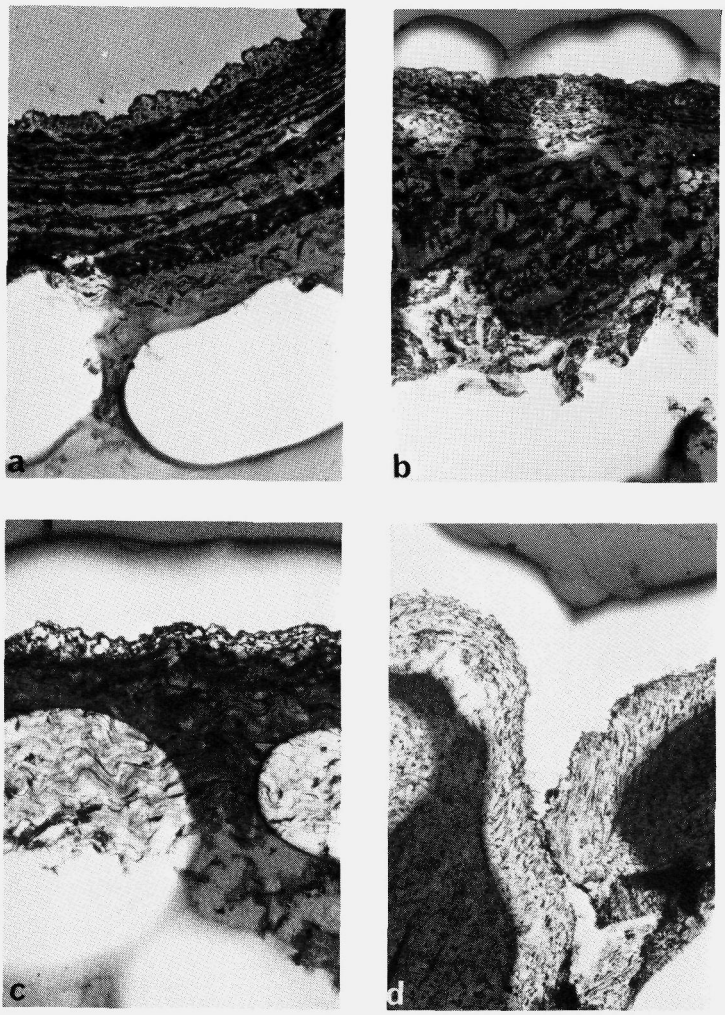

FIG. 5. Fibrinolytic activity in veins from different body levels incubated for 40 min on fibrin slides and stained with Harris' alum haematoxylin. *a* Great saphenous vein with high activity in the external connective tissue layer but no activity in the inner layers. x 50. *b* Vena cava with high activity in the external connective tissue layer and some activity in the inner layers. x 50. *c* Subclavian vein with high activity in all layers. x 50. *d* Jugular vein with very high activity in all layers. x 75.

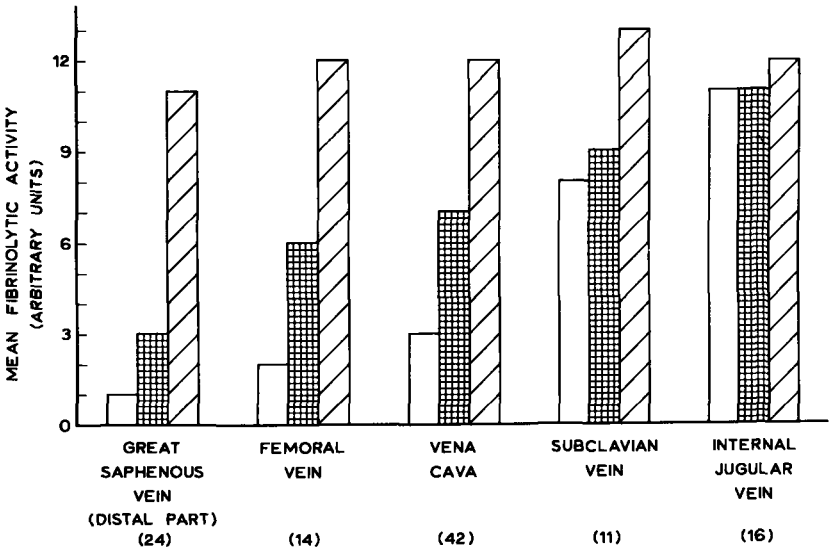

FIG. 6. Mean capacities of fibrinolytic activity in the intimal layer (open columns), the medial/adventitial layer (cross-hatched columns), and the external layer of loose connective tissue (hatched columns) of human veins from different locations in the body.

External layer of loose connective tissue. This outer layer of the veins, which is devoid of smooth muscle cells, showed a higher activator activity than other parts of the venous wall. The activity was related to the endothelium of the vasa vasorum, and equalled the activity of the arterial adventitia. Like the adventitial activity of arteries, the activity in the venous external layer of loose connective tissue was quite uniformly distributed among different medium-sized and large veins in one and the same individual and it did not vary according to the position of the vein in the body (table III). However, the activator activity in this connective tissue layer, as in other venous layers and in the arterial adventitia, varied from one individual to another (table III).

Altered vascular fibrinolytic activity. Extremely strong activator activity (15 arbitrary units or more) was seen in eleven autopsies related to sudden death, vasogenic shock, cerebral haemorrhage and cirrhosis, among other causes (table IV). In these cases, lysis occurred around the arterial intima

Errata proefschrift V. Noordhoek Hegt

→ Pag. 52 : Tabel IV eerste geval moet zijn :
malignant hepatoma in post-hepatitic cirrhosis

Pag. 63 : Dertiende regel van onderen moet zijn :
They were then heated at 100° C .....

Pag. 65 en 66 : Tabel 1 en 2 horen op pag. 81 en 82

Pag. 76 : Derde regel van boven moet zijn :
..... and/or inhibition .....

Pag. 77 : Derde regel van onderen moet zijn :
..... in which the vasa vasorum were situated ...

Pag. 79 : Vijfde regel in Fig. 2A moet zijn :
Right: fibrin present above the inner vascular layers but not above the external connective tissue layer.

Pag. 81 en 82 : Tabel I en II horen op pag. 65 en 66

Pag. 86 : Eerste regel in Fig. 5A moet zijn :
..... incubated for 60 min.
Eerste regel in Fig. 5B moet zijn :
..... incubated for 120 min.

Table IV. Cases with extremely high fibrinolytic activity

Autopsy No.	Sex	Age	Cause of death	Mean activator activity[1] arbitrary units
428/71	M	67	malignant hepatoma in post-hepatic cirrhosis	17
411/72	M	71	sudden death after myocardial infarction	17
446/72	M	72	vasogenic shock after bronchoscopy	16
456/72	F	58	shock due to mesenterial embolism and ischemic enteritis	16
489/72	F	6	cerebral intraventricular hemorrhage after trauma	15
509/72	F	52	sudden death after lung puncture	17
21/73	F	30	cerebral intraventricular hemorrhage in eclampsia	15
55/73	M	88	cardiac failure in pulmonary emphysema	18
65/73	M	72	progressive pulmonary insufficiency in bronchogenic carcinoma	16
89/73	F	65	bronchopneumonia, renal adenocarcinoma and alcoholic cirrhosis	16
98/73	M	17	cerebral hemorrhage in Pfeiffer's disease	15

These figures represent the mean values observed in at least 6 different large and medium-sized blood vessels (3 arteries and 3 veins).

[1] Mean endothelial plasminogen activator activity of the vasa vasorum in the arterial adventitia and in the venous external layer of loose connective tissue.

Table V. Cases with extremely low fibrinolytic activity

Autopsy No.	Sex	Age	Cause of death	Mean activator activity[1] arbitrary units
393/72	F	79	endotoxin shock due to faecal peritonitis	6
398/72	M	40 days	endotoxin shock (Escherichia coli)	2
403/72	F	1½ days	hyaline membrane disease	5
426/72	M	32	endotoxin shock (E. coli)	8
451/72	M	61	progressive pulmonary insufficiency	8
460/72	M	57	endotoxin shock (E. coli)	4
466/72	M	½ day	Waterhouse-Friderichsen syndrome (Hemophilus influenzae)	0
504/72	F	2 days	hyaline membrane disease	3
525/72	F	47	septic shock (Candida albicans)	4
71/73	F	71	endotoxin shock (Pseudomonas aeruginosa)	6

These figures represent the mean values observed in at least 6 different large and medium-sized blood vessels (3 arteries and 3 veins).

[1] Mean endothelial plasminogen activator activity of the vasa vasorum in the arterial adventitia and in the venous external layer of loose connective tissue.

and adventitia as well as around all venous layers after a very short incubation period (Fig. 3).

In contrast, low activator activity (8 arbitrary units or less) was found in ten cases related to endotoxin shock caused by gram-negative bacteria, to hyaline membrane disease and to a case of Waterhouse-Friderichsen syndrome (table V). In such cases, lysis occurred only after 60 or 120 min of incubation (Fig. 7) or remained totally absent even after incubation for 120 min (Fig. 8).

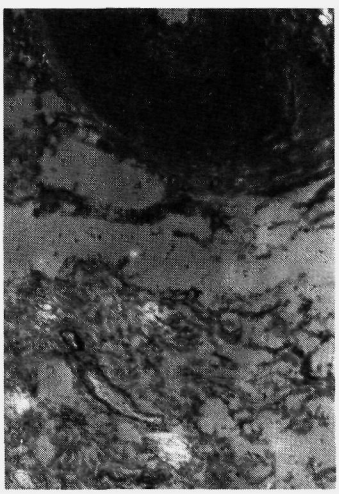

FIG. 7. Medium-sized artery from a case of endotoxin shock incubated for 60 min on a fibrin slide. Only very small lysed areas are present under the venules in the adventitia. Harris' alum haematoxylin. x 75.

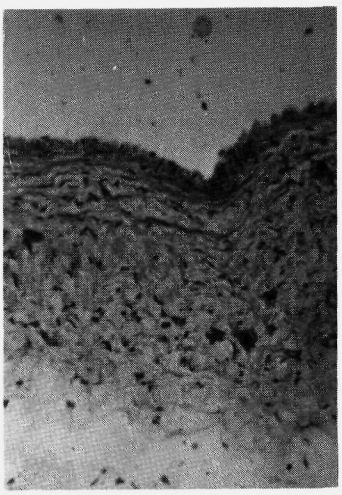

FIG. 8. Jugular vein from a case of endotoxin shock incubated for 120 min on a fibrin slide. No lysis can be detected in the vascular wall. Harris' alum haematoxylin. x 60.

No gross differences in the overall activator activity were found when comparing biopsy and autopsy material, neither between specimens obtained within a short (2 h) or a long (48 h) period after death nor among specimens obtained from patients of different ages (0-88 years).

DISCUSSION

Nilsson and Isacson (1973) reported a correlation between low endothelial plasminogen activator activity and the incidence of thrombosis. Because of the clinical relevance of this finding it is important to obtain insight into the distribution of the activator activity in human vessel walls.

The present study was undertaken to localize endothelial plasminogen activator activity along and across the human vascular system. Using the fibrin slide technique (Todd 1959), plasminogen activator activity was generally restricted to vascular endothelial cells, which is in agreement with results of others (reviews by Astrup 1966, and Pandolfi 1972). Detached endothelial cells from arteries and veins generally showed higher activity than those remaining at the endothelial lining in accordance with the literature (Bleyl 1969; Glas-Greenwalt 1972; Pandolfi et al. 1968). Endothelial plasminogen activator activity was low or absent in the intima and media of most arteries as described in most other fibrinolysis studies on human material (Bleyl 1969; Donner and Safrankova 1975; Fischer 1970; Glas-Greenwalt 1972; Kwaan and Astrup 1967; Pandolfi 1969; Todd 1959, 1960, 1964). This low activity was not due to post-mortem changes, since nine biopsy specimens of human aorta, freshly obtained at coronary-bypass grafting and immediately processed on fibrin slides, revealed a similar pattern of fibrinolysis. Interestingly, high intimal activator activity was encountered locally in biopsy specimens of coarcted aorta and at sites of arterial branching, the latter being briefly mentioned in another report (Glas-Greenwalt 1972). The increased endothelial cell turnover at such sites (Wright 1970) may be related to the high activity, since desquamating cells release plasminogen activator (Astrup 1975). Activator activity along the intima of many arteries was frequently encountered in cases of sudden death, vasogenic shock, cerebral haemorrhage and cirrhosis. The latter confirms an observation in the aorta of one case of alcoholic cirrhosis (Glas-Greenwalt 1972). Release of large amounts of plasminogen activator from the endothelium into the blood may explain the hyperfibrinolysis found in the blood of some of such patients (Brakman et al. 1974; Harms 1971; Pises et al. 1973). Strong intimal activity in arteries was also reported to occur in a limb amputated under bloodless conditions (Todd 1960) as well as in the aortic intima in a case of aspergillosis (Pelczar et al. 1972).

The activator activity in the adventitia of the large and medium-sized arteries was much higher than that of the intima and media. The adventitia of small arteries however, showed little or no activity, agreeing well with the

fact that these vessels possess little or no vasa vasorum.

In the venous wall, endothelial activator activity in intima, media and adventitia varied greatly according to the position of the vein in the body. Veins from upper parts usually showed higher activity in their inner layers than veins from the lower parts. This finding corresponds well with the higher activity reported to be present in superficial veins from the arms than in those from the legs (Dodman et al. 1973; Franz et al. 1975; Pandolfi et al. 1968; Pandolfi et al. 1967).

In contrast to the inner venous layers, the venous external layer of loose connective tissue, like the arterial adventitia, usually showed a remarkably uniformly distributed high activator activity of the vasa vasorum throughout the body of the same individual. Only small veins, like small arteries, showed no adventitial activity because of the lack of vasa vasorum.

These findings substantiate those of Almēr et al. (1975) who found no differences in the activity of the vasa vasorum of human temporal arteries and the superficial arm veins taken by biopsy. Since the outer layers of loose connective tissue of the arterial and venous wall generally showed a pronounced and constant activity, these layers were suitable for studying variations in endothelial activator activity among individuals. Comparing activity in these layers small variations in the mean fibrinolysis values could not be associated with causes of death or basic illness. However, like the high intimal activator activity described above, high activity was also found in the outer layers of loose connective tissue of arteries and veins in cases of sudden death, vasogenic shock, cerebral haemorrhage and cirrhosis. Very low endothelial activator activity was encountered in all layers, but was most clearly expressed by the outer layers of loose connective tissue of arteries and veins in cases of endotoxin shock, hyaline membrane disease, and a case of Waterhouse-Friderichsen syndrome. A possibly related decrease in endothelial activator activity has been described for lung tissue in cases of hyaline membrane disease (Ekelund et al. 1973) and in post-traumatic cases with long survival times (Rammer and Saldeen 1970). In addition, increased inhibition of fibrinolysis was reported to occur in the lungs of the post--traumatic cases (Rammer and Saldeen 1970). A similar increase in inhibition of fibrinolysis was found in our laboratory in autopsy cases showing decreased endothelial activator activity (Noordhoek Hegt 1977).

From a comparative study of both the activation and the inhibition of fibrinolysis in the walls of human blood vessels, it appears that many of the differences and variations in endothelial activator activity as described in

the present paper are likely to be governed by local or systemic differences in inhibition of the fibrinolytic process (Noordhoek Hegt 1977).

ACKNOWLEDGEMENT

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Chapter V

LOCALIZATION AND DISTRIBUTION OF FIBRINOLYSIS INHIBITION IN THE WALLS OF HUMAN ARTERIES AND VEINS

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Abstract. A systematic study on the location and intensity of fibrinolysis inhibition in human arteries and veins by the use of a standardized fibrin slide sandwich technique, yielded data for an overall comparison of fibrinolysis inhibition along and across the human vessel wall. Arteries generally showed marked plasmin inhibition in the intimal and medial layers, but little inhibition in the adventitia. The external layer of loose connective tissue in the veins generally showed no plasmin inhibition. Plasmin inhibition was usually present in the combined intimal/medial/adventitial layer of the veins, but inhibition in these layers varied greatly according to the location of the vein in the body. Veins situated in the lower parts of the body exhibited more plasmin inhibition than veins at the upper levels. Inhibition of plasmin was found to be related to the presence of smooth muscle cells. However, in endotoxin shock, hyaline membrane disease and a case of Waterhouse-Friderichsen syndrome, additional plasmin inhibition was observed in vascular areas of loose connective tissue free from smooth muscle cells. Selective inhibition of urokinase-induced fibrinolysis was observed only in the connective tissue around the vessels of the human umbilical cord.

INTRODUCTION

Sixty years ago, Fleischer and Loeb (1915) demonstrated an inhibitory effect of suspensions of animal liver on lysis of a plasma clot. The liver tissue apparently had a special inhibitory effect, since serum of the same animal produced less or no inhibition.

Nowadays, proteinase inhibitors are known to be widely distributed within

animal tissues and body fluids (Vogel et al. 1968), but our knowledge of fibrinolytic inhibitors in human tissues, particularly the blood vessels, is still scanty. MacFarlane and Biggs (1948) found fibrinolysis inhibiting material in saline extracts of a number of organs and tissues, but the vessel wall was not included in their study. Presence of fibrinolysis inhibitors in the walls of human vessels was first reported by Isidori et al. (1963). They found that homogenates of human arteries inhibited the proteolytic effect of plasmin. Different types of human blood vessels were studied by Neri Serneri et al. (1965). They reported that plasmin was more strongly inhibited by extracts of arterial tissue than those of venous tissue. Moreover, the intimal/medial layers of the arterial wall appeared to contain much more inhibiting material than did the adventitia. Similar results for extracts of human blood vessels were obtained by other investigators (Benzer et al. 1966).

Recently, a low molecular weight proteinase inhibitor with affinity for plasmin was isolated from bovine aorta and partially characterized (Sorgente et al. 1976). Comparative studies of inhibition of fibrinolysis in explants of human blood vessels and human organs (Bernik and Kwaan 1971) revealed by far the strongest plasmin inhibition in the blood vessels. The latter observation suggest an important role for blood vessels in fibrinolysis inhibition.

With the development of the fibrin slide sandwich technique (Noordhoek Hegt and Brakman 1973, 1974a, 1974b, 1974c, 1974d, 1975; Fornasari et al. 1976), it has become possible to detect and localize fibrinolysis inhibiting material in tissue sections at a microscopical level. In this method, tissue sections are incubated under a fibrin layer onto which a fibrinolytic enzyme is applied. During incubation, the top layer digests the underlying fibrin but digestion is prevented at sites where fibrinolysis inhibiting material from the tissue section has diffused into the fibrin. The remaining fibrin, which can be localized microscopically, is indicative for the site of inhibition. By this sandwich technique, it has been demonstrated that plasmin fibrinolysis inhibition in human blood vessels is related to the presence of smooth muscle cells (Noordhoek Hegt and Brakman 1974c, 1974d).

In the present study, human arteries and veins were studied with the fibrin slide sandwich technique in order to determine the location of and the capacity for inhibition of plasmin fibrinolysis as well as of activator-induced fibrinolysis along and across the human vascular wall.

MATERIALS AND METHODS

Vascular specimens were obtained from 20 routine autopsies and 15 biopsies. In addition, specimens of aorta, vena cava and coronary artery were obtained from a number of autopsy cases (numbers in parenthesis) which showed unusual patterns of fibrinolytic activity in a previous study (Noordhoek Hegt 1976). Cases of endotoxin shock (393/72; 460/72), hyaline membrane disease (403/72) and a Waterhouse-Friderichsen syndrome (466/72) showed extremely low fibrinolytic activity and cases of sudden death (411/72), vasogenic shock (446/72), cerebral hemorrhage (21/73) and cirrhosis (89/73) exhibited extremely high fibrinolytic activity. All specimens were collected without the use of a fixative. Contaminating blood was removed with saline and fatty parts were separated by dissection. The specimens were immediately frozen in isopentane, cooled with liquid nitrogen or dry ice and then kept in air-tight jars at -20°C. From each blood vessel, five 16 µm thick sections were serially cut on a cryostat microtome and individually placed on five cleaned microscope slides. To each slide were separately applied: 20 µl of a solution of bovine thrombin (Leo Pharmaceuticals, Ballerup, Denmark) in saline (20 NIH units/ml) and 100 µl of a 0.7% (w/v) bovine fibrinogen solution (plasminogen-free fibrinogen from Poviet, Organon-Teknika, Oss, The Netherlands) or plasminogen-rich fibrinogen prepared according to Brakman (1967), both dissolved in phosphate buffer, pH 7.8 and ionic strength 0.15. The solutions were quickly mixed and evenly spread over an area of 2.5 x 4 cm covering the tissue section. The slides were left for 1 h in a moist chamber at 4°C to solidify the fibrin film and to allow diffusion of inhibiting material from the tissue sections into the overlying fibrin. Five 10 µm thick sections were then cut from a frozen plasmin or urokinase solution as previously described (Noordhoek Hegt and Brakman 1974a, 1974d, 1975) and individually placed on top of the fibrin slide above the tissue section. Top layers of plasmin were prepared from human plasmin (from the State of Michigan Department of Public Health, USA) prepared according to Sgouris et al. (1960) diluted to 1.5 CU/ml in saline containing 15% (w/v) gelatin and placed on top of plasminogen-free fibrin films. Activator containing top layers were prepared with human urokinase (Leo Pharmaceuticals, Ballerup, Denmark) dissolved to 7 Ploug units/ml in saline containing 15% (w/v) gelatin and placed on top of plasminogen-rich fibrin films. The five sandwich slides of each set were then incubated at 37°C in a moist chamber for 100, 120, 140, 160 and 180 min, respectively, and finally fixed in a 4% (v/v) formaldehyde solution for 15 min and stained in

Errata proefschrift V. Noordhoek Hegt

Pag. 52 : Tabel IV eerste geval moet zijn :
malignant hepatoma in post-hepatitic cirrhosis

→ Pag. 63 : Dertiende regel van onderen moet zijn :
They were then heated at 100° C .....

Pag. 65 en 66 : Tabel 1 en 2 horen op pag. 81 en 82

Pag. 76 : Derde regel van boven moet zijn :
..... and/or inhibition .....

Pag. 77 : Derde regel van onderen moet zijn :
..... in which the vasa vasorum were situated ...

Pag. 79 : Vijfde regel in Fig. 2A moet zijn :
Right: fibrin present above the inner vascular layers but not above the external connective tissue layer.

Pag. 81 en 82 : Tabel I en II horen op pag. 65 en 66

Pag. 86 : Eerste regel in Fig. 5A moet zijn :
..... incubated for 60 min.
Eerste regel in Fig. 5B moet zijn :
..... incubated for 120 min.

Harris' hematoxylin for 12 hrs. The concentrations of the active agents in the top layers were adjusted so that they would digest the underlying fibrin within a period of exactly 100 min when there was no underlying tissue section. On sandwich slides with tissue sections the fibrin digestion was delayed at sites where inhibiting material was present. Histological staining with the picro-Mallory stain (Lendrum et al. 1962) slightly modified for this purpose (Noordhoek Hegt and Brakman 1974d) was done on parallel frozen tissue sections in order to be able to relate fibrinolysis inhibition to the morphology of the tissues. The inhibitory capacity of each specimen was graded according to the following scheme: Grade I, nearly all the fibrin in contact with the underlying tissue has been digested by the active top layer; grade II, three-fourths of the area of the fibrin covering the tissue is lysed; grade III, one-half of the area of the fibrin covering the tissue is lysed; grade IV, less than one-fourth of the fibrin film covering the specimen is lysed by the active top layer. To grades I, II, III and IV were allotted 1, 2, 3 and 4 points, respectively. The sum of the points scored by each set of five slides was taken as a measure of the inhibitory capacity of that specimen. Although only semiquantitative this grading system provides a useful method by which the inhibitory capacity of different tissues can be compared. However, care must be taken that the thickness of the used preparations is kept constant since differences in thickness may cause different susceptibility to lysis and disturb the quantitation system. To determine the heat stability of the inhibiting material, 5 sections were serially cut from a specimen and placed on glass slides. There were then heated at 100°C in a dry oven for 12 hrs and finally processed in the same manner as were the unheated sections Two sheets of the vessel wall were separately evaluated: In arteries, an intimal/medial layer and the adventitia; in veins, an intimal/medial/adventitial layer and the external layer of loose connective tissue. The intimal/medial layer of the arteries structurally resembles the intimal/medial/adventitial layer of the veins; both usually contain many smooth muscle cells. The arterial adventitia and the venous external layer of loose connective tissue both consist of ordinary loose connective tissue, usually without smooth muscle cells.

RESULTS

The determinations of the mean inhibitory capacities of sections of large and medium-sized human arteries and veins, as obtained by means of the fibrin slide sandwich technique using a top layer of plasmin on a plasminogen-free

fibrin layer, are presented in Tables I and II. The localization and distribution of sites of plasmin inhibition along and across the walls of human blood vessels will now be described in detail.

Plasmin inhibition in arteries

Intima/media: All arteries showed plasmin inhibition in the intimal/medial layer (Fig. 1). When the media was separated from the intima, with only small amounts of medial tissue remaining in the latter, inhibition was observed in each layer separately (Fig. 2). The capacity for inhibition was more pronounced in the medium-sized arteries than in the large arteries. The fibromuscular thickened intimal lesions of five large arteriosclerotic arteries showed intense plasmin inhibition (Fig. 3).

Adventitia: Inhibition of plasmin in the outer layer of the arterial wall was generally weak or absent in large as well as in medium-sized arteries. However, strong inhibition of plasmin-induced fibrinolysis was observed in the arterial adventitia in cases of endotoxin shock, hyaline membrane disease and a Waterhouse-Friderichsen syndrome (Fig. 4).

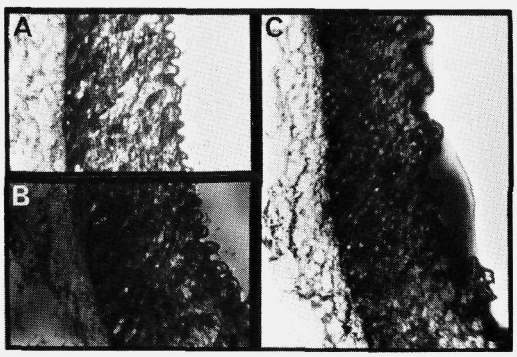

FIG. 1. Human renal artery studied by the fibrin slide sandwich technique. Harris' alum hematoxylin (x 50). A: Section before coverage with fibrin. B: Section after coverage with fibrin. C: Section after incubation for 160 min with plasmin on top of the fibrin film. Fibrin remains above the medial/intimal area indicating plasmin inhibition at that site. No fibrin was left above the adventitia or above areas beyond the section.

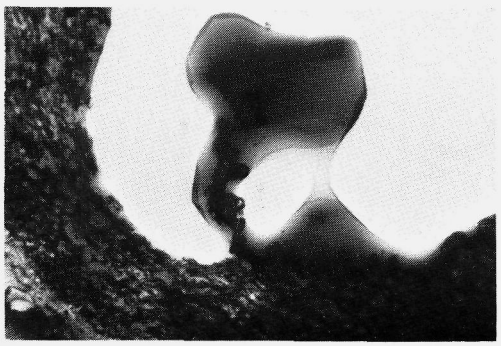

FIG. 2. Human renal artery from which the intima was partially separated from the media. After incubation for 160 min with plasmin on top the dark stained fibrin is seen to be present above the media as well as around and above the loosened intima, while complete dissolution has occurred at areas outside the section. Harris' alum hematoxylin (x 150).

Table 1

Fibrinolytic activity and inhibition in human arteries

	Number of Cases	Mean activator activity[a] (arbitrary units)		Mean inhibitory capacity[b] (arbitrary units)	
		I/M	A	I/M	A
Large arteries					
Aorta	10	1	12	10	1
Innominate artery	4	0	11	11	0
Subclavian artery	3	1	14	12	3
Common carotid artery	5	1	11	10	2
Weighted mean		1	12	10	1
Medium-sized arteries					
Popliteal artery	5	0	11	14	2
Hepatic artery	4	0	11	17	1
Splenic artery	7	1	10	16	1
Renal artery	5	1	12	16	3
Right coronary artery	6	1	12	18	1
Weighted mean		1	11	16	2
Cases with low fibrinolytic activity ※)					
Aorta	4	0	4	15	15
Right coronary artery	4	0	4	17	17
Cases with high fibrinolytic activity +)					
Aorta	4	6	16	11	1
Right coronary artery	4	7	15	17	2

a) Endothelial plasminogen activator activity;

b) Plasmin fibrinolysis inhibition capacity;
I = intima; M = media; A = adventitia.

※) Two cases of endotoxin shock, a case of hyaline membrane disease and a case of a Waterhouse-Friderichsen syndrome;

+) One case of sudden death, a case of vasogenic shock, a case of cerebral hemorrhage and a case of cirrhosis.

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..... incubated for 60 min.
Eerste regel in Fig. 5B moet zijn :
..... incubated for 120 min.

Table 2

Fibrinolytic activity and inhibition in human veins

	Number of Cases	Mean activator activity[a] (arbitrary units)		Mean inhibitory capacity[b] (arbitrary units)	
		I/M/A	E	I/M/A	E
Large veins					
Vena cava	10	5	12	10	1
Internal jugular vein	10	11	12	3	2
Subclavian vein	4	9	13	6	2
Weighted mean		8	12	6	2
Medium-sized veins					
Femoral vein	5	5	12	11	2
Great saphenous vein	10	4	12	12	1
Superior sagittal sinus	3	15	15	2	2
Weighted mean		6	12	10	1
Cases with low fibrinolytic activity ⁎)					
Vena cava	4	1	3	16	16
Cases with high fibrinolytic activity +)					
Vena cava	4	10	16	9	1

a) Endothelial plasminogen activator activity;

b) Plasmin fibrinolysis inhibition capacity;
I = intima; M = media; A = adventitia; E = external layer of loose connective tissue.

⁎) Two cases of endotoxin shock, a case of hyaline membrane disease and a case of a Waterhouse-Friderichsen syndrome;

+) One case of sudden death, a case of vasogenic shock, a case of cerebral hemorrhage and a case of cirrhosis.

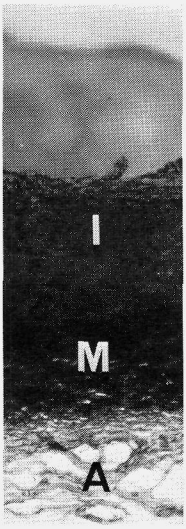

FIG. 3

FIG. 4

FIG. 3. Arteriosclerotic subclavian artery with a fibro-muscular thickened intima incubated for 180 min under a fibrin film with plasmin on top. Fibrin is present above and around the intima (I) and above the media (M) but not above the adventitia (A). Harris' alum hematoxylin (x 80).

FIG. 4. Medium-sized artery from a case of endotoxin shock incubated for 160 min on a fibrin 'sandwich' slide with plasmin in the active top layer. Fibrin is still present above the entire section including the adventitia, while complete dissolution has occurred at areas outside the section. Harris' alum hematoxylin (x 75).

The inhibition of plasmin in sections of the arterial wall was closely associated with the presence of smooth muscle cells demonstrable by the picro-Mallory staining. In the inner layers of medium-sized arteries, which revealed a strong inhibitory capacity, smooth muscle cells were abundantly present, while fewer smooth muscle cells were present in large arteries with less inhibition. The adventitia of arteries which usually had no inhibitory potency consisted mainly of loose connective tissue without smooth muscle cells.

In general, this pattern of smooth-muscle-cell-related plasmin inhibition was quite uniform among different arteries in an individual as well as among different individuals.

Plasmin inhibition in veins

Intima/media/adventitia: Plasmin inhibition in this part of the venous wall was generally lower than in the corresponding inner arterial layers, although some exceptions were observed (Tables I and II). In contrast to the arteries, veins showed a great diversity in plasmin inhibition when segments from different locations in an individual were compared. For instance, of the specimens from the same autopsy case, a sample of the great saphenous vein below the knee showed very strong inhibition, a sample of the vena cava taken at the level of the liver showed somewhat less inhibition, the subclavian vein revealed only a small zone of inhibition near the main lumen and the internal jugular vein showed scarcely any inhibitory capacity towards plasmin fibrino-

lysis. When comparing the mean plasmin inhibitory capacities of the veins studied, it appeared that veins which were situated in the upper parts of the body, such as the superior sagittal sinus, the internal jugular vein, and the subclavian vein, had much lower inhibitory capacity in their inner layers than veins from the lower parts of the body and the umbilical vein. The decrease in inhibition in veins from the feet to the head is illustrated in Fig. 5.

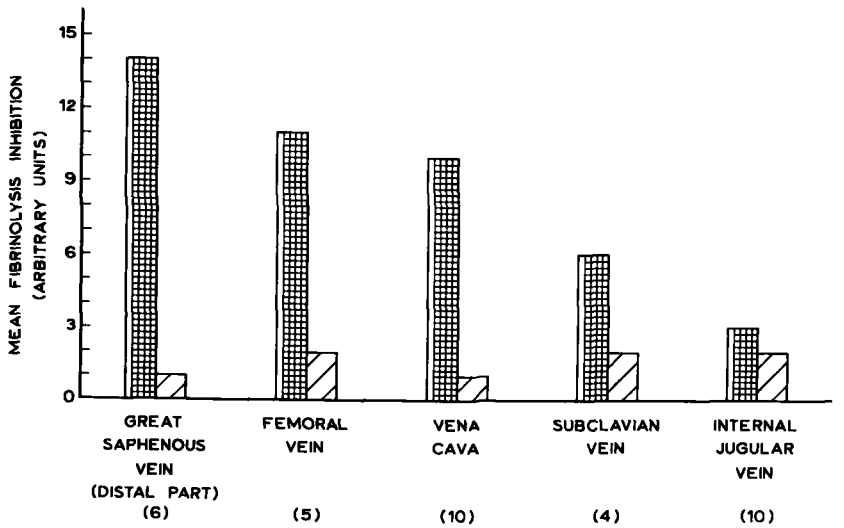

FIG. 5. Mean capacities of inhibition of fibrinolysis in the intimal/medial/adventitial layer (combined open and cross-hatched columns), and the external layer of loose connective tissue (hatched columns) of human veins from different locations in the body.

External layer of loose connective tissue

Plasmin inhibition in this outer layer of the venous wall was generally weak or absent in the large as well as in the medium-sized veins; it also did not vary according to the position of the vein in the body. These results correspond well to those obtained in samples of the arterial adventitia. As in arteries, unusually strong plasmin inhibition was observed in the loose connective tissue layers of veins in cases of endotoxin shock, hyaline membrane disease and a Waterhouse-Friderichsen syndrome.

Inhibition of plasmin in sections of the venous wall was closely associated with the presence of smooth muscle cells. Veins from the lower parts of the body which were rich in muscle cells (e.g., the inferior vena cava, the

femoral vein, and the great saphenous vein) showed greater plasmin inhibition than did veins from the upper parts of the body which were less rich in muscle cells (e.g., the superior sagittal sinus, the internal jugular vein, and the subclavian vein). In addition, the umbilical vein, which has a thick muscular wall, possessed a very strong inhibitory capacity. The external layer of loose connective tissue of most veins did not contain smooth muscle cells and it was usually devoid of inhibition. As in the arteries, the pattern of smooth-muscle--cell-related plasmin inhibition in the veins was quite uniformly distributed among different individuals. In cases of sudden death, vasogenic shock, cerebral hemorrhage and cirrhosis, the plasmin inhibition was also related to smooth muscle cells and it did not differ in strength from the plasmin inhibition in blood vessels obtained by routine autopsy.

The pattern of plasmin inhibition related to the smooth muscle cells in arteries and veins showed no gross difference between biopsy and autopsy material, nor were differences seen between specimens obtained within a short (2 hrs) or long (48 hrs) period after death or among specimens obtained from patients of different ages (0-88 years).

In confirmation of our previous studies (Noordhoek Hegt and Brakman 1974a, 1974d) the capacity to inhibit plasmin was abolished when tissue sections of various types of blood vessels were heated at 100°C in a dry oven for 12 hrs prior to preparation of the fibrin slides.

Urokinase-induced fibrinolysis inhibition

When the fibrin slides were prepared with urokinase in the active top layer covering a layer of plasminogen-rich fibrin, the pattern of inhibition was usually similar to that described for the plasmin inhibition. Therefore, inhibition of urokinase-induced fibrinolysis, if present, could not be distinguished from the plasmin inhibition, because the latter masks the possible inhibition of the activation step in the fibrin slide sandwich technique.

An exception to this finding was observed in the human umbilical cord. The umbilical arteries and veins both showed regular plasmin inhibition in their inner layers, where smooth muscle cells are abundantly present (Fig. 6). The connective tissue of the umbilical cord (Wharton's jelly) which surrounds the blood vessels hardly revealed any plasmin inhibition, resembling in this respect other areas of loose connective tissue. On the other hand, the urokinase--induced fibrinolysis was strongly inhibited by Wharton's jelly (Fig. 7). This embryonic mucoid connective tissue, in contrast to ordinary loose connective tissue, apparently contains material which is able to specifically

inhibit the activation of plasminogen by urokinase. The umbilical blood vessels themselves did not produce lysis on the plasminogen-rich fibrin. Interestingly, in contrast to plasmin inhibition, the inhibition of urokinase-induced fibrinolysis was still clearly demonstrable in the dry-heated sections of the umbilical cord.

FIG. 6

FIG. 7

FIG. 6. Umbilical cord of a human fetus at term studied by the fibrin slide sandwich technique using plasminogen--free fibrin covered by a layer of plasmin. After 100 min of incubation plasmin has lysed the fibrin except for areas above and around the blood vessels. Harris' alum hematoxylin (x 20).

FIG. 7. Adjacent section of the same umbilical cord examined for inhibition of activator-induced fibrinolysis under a plasminogen-rich fibrin layer to which urokinase was applied. After incubation for 180 min, urokinase-induced fibrinolysis has removed the fibrin outside the section but lysis was inhibited above and around the umbilical cord. Harris' alum hematoxylin (x 20).

DISCUSSION

The results of the systematic studies presented here support and extent our previous reports (Noordhoek Hegt and Brakman 1974c, 1974d) of a correlation between inhibition of plasmin fibrinolysis in sections of human blood vessels and the presence of histologically detectable smooth muscle cells. Areas of ordinary loose connective tissue, such as the arterial adventitia or the venous external layer of connective tissue, usually did not exhibit inhibitory activity towards plasmin. Plasmin inhibition is also reported to be absent in bovine (cartilage) connective tissue (Sorgente et al. 1976).

The inhibition of plasmin fibrinolysis observed in connective tissue areas in cases of endotoxin shock, hyaline membrane disease and a Waterhouse-Friderichsen syndrome may be caused by inhibiting material from plasma, which is known to escape into the interstitial space in certain cases of shock where the vascular bed has lost its integrity and has become permeable to large

molecules (Aust et al. 1957; Chien et al. 1964). The relationship between the decrease in fibrinolytic activity and the increase of inhibition in these autopsy cases will be reported later (Noordhoek Hegt, submitted for publication).

The enhanced intimal plasmin inhibition observed in some fibromuscular ateriosclerotic lesions confirm a similar finding by Benzer et al. (1966), who used an extraction method. Our observations, as well as those of others (review by Ross and Glomset (1973), show that proliferating smooth muscle cells are present in great numbers in these fibromuscular arteriosclerotic lesions. Next to the inhibitory material derived from these cells, diffusion of plasma protease inhibitors, such as alpha-2-macroglobulin, through the injured endothelium into the arteriosclerotic plaque (Patricot and Perrin 1973) may have caused additional inhibition.

In the present study a selective inhibition of activator-induced fibrinolysis was observed only in the connective tissue of the human umbilical cord. The presence of this particular material in the umbilical cord seems to be a new observation. Interestingly, this inhibitory material appeared to be extremely resistent to heat, in contrast to the plasmin inhibiting material found in the tissues. The difference in heat stability might be useful in a further discrimination between inhibition of plasmin and of activator-induced fibrinolysis in human and animal tissues. The possible relationship of the umbilical cord inhibitor to the urokinase inhibitor present in the human placenta (Kawano et al. 1968; Uszynski and Abildgaard 1971) remains to be established.

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Chapter VI

RELATIONS BETWEEN ACTIVATION AND INHIBITION OF FIBRINOLYSIS IN THE WALLS OF HUMAN ARTERIES AND VEINS

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Summary. From systematic studies of both the activation and the inhibition of fibrinolysis in human blood vessels, data which provided insight into the fibrinolysis antagonism along and across the human vessel wall were obtained.

The capacity for fibrinolysis initiated by plasminogen activator in sections of human arteries and veins as demonstrated by the fibrin slide technique differed greatly along and across the vessel walls. Inhibition of plasmin fibrinolysis in these blood vessels as detected by the fibrin slide sandwich technique was present at sites of low fibrinolytic activity, while inhibition was absent in areas showing strong fibrinolysis. Fibrinolysis was related to endothelial cells, while inhibition of fibrinolysis was brought about by smooth muscle cells. The results indicate that differences in endothelial fibrinolysis may result from differences in inhibition of fibrinolysis caused by variations in the number of smooth muscle cells present locally. A systemic decrease in endothelial fibrinolysis observed in endotoxin shock, hyaline membrane disease and a Waterhouse-Friderichsen syndrome appeared to be associated with a systemic increase in inhibition of fibrinolysis not related to smooth muscle cells.

INTRODUCTION

By means of the 'fibrin slide technique', in which frozen tissue sections are incubated in contact with a fibrin film rich in plasminogen, Todd (1959) was able to identify the vascular endothelium as a source of plasminogen activator. The activity of the endothelial plasminogen activator, however, appeared to differ greatly along the vascular wall (Todd, 1959, 1960). This finding

was confirmed in a detailed survey of the overall localization of the endothelial plasminogen activator activity along and across human arteries and veins (Noordhoek Hegt 1976). Recently, a 'fibrin slide sandwich technique', in which tissue sections are incubated under a fibrin layer on top of which a fibrinolytic enzyme is applied, was devised for the detection and localization of fibrinolysis inhibiting material in tissue sections (Noordhoek Hegt and Brakman 1974a). With this method, Noordhoek Hegt and Brakman (1974b, 1974c) were able to identify the smooth muscle cell as the source of fibrinolysis inhibiting material in the human vessel wall.

The localization and distribution of areas of inhibition of plasmin fibrinolysis along and across human arteries and veins was described in a previous report (Noordhoek Hegt 1977).

Since the plasminogen activator activity is determined on fibrin slides by measuring the size of the areas lysed by activated plasminogen, viz plasmin, it is conceivable that the plasmin inhibiting material from vascular smooth muscle cells is responsible, in part, for the low endothelial plasminogen activator activity observed in many blood vessels. In that case the inhibition of plasmin fibrinolysis in sections of blood vessels would be most pronounced at sites exhibiting low plasminogen activator activity, and vice versa. Since the inhibition of plasmin in the fibrin slide sandwich technique is determined on plasminogen-free fibrin slides, where lysis caused by activation of plasminogen is absent, the areas of plasmin inhibition can be identified independently of the presence or absence of plasminogen activator. Therefore, in order to determine whether a correlation exists between areas of low activator activity and sites of high inhibitory capacity and vice versa in the walls of human arteries and veins, a number of selected specimens were studied simultaneously for activator activity on plasminogen-rich fibrin slides and for plasmin inhibitory capacity by the fibrin slide sandwich technique. In addition, the inhibition of activator-induced fibrinolysis was directly demonstrated by the distortion of the circular shape of lysis zones on the plasminogen-rich fibrin slides.

MATERIALS AND METHODS

Specimens of human blood vessels were quickly frozen in isopentane, cooled with liquid nitrogen or dry ice, and stored in air-tight jars at -20°C. Specimens from large and medium-sized blood vessels were selected from 15 routine autopsies which had been used in previous studies (Noordhoek Hegt 1976, 1977).

In addition, an aorta, a vena cava and a coronary artery were selected from a number of autopsy cases (numbers in parenthesis) which formerly showed an altered fibrinolytic activity (Noordhoek Hegt 1976) an/or inhibition (Noordhoek Hegt 1977): endotoxin shock (393/72; 460/72), hyaline membrane disease (403/72), a Waterhouse-Friderichsen syndrome (466/72), sudden death (411/72), vasogenic shock (446/72), cerebral hemorrhage (21/73) and cirrhosis (89/73).

Fibrin Slide Technique

The materials and technique were those previously described (Noordhoek Hegt 1976). Briefly, this technique consists of incubating for various periods of time a series of frozen sections of a specimen placed in contact with a thin film of fibrin-rich plasminogen. During incubation, structures containing plasminogen activator may cause lysis which appear as clear zones in the subsequently stained fibrin. The size of the lysis zone is taken as an arbitrary measure of the strength of the activator-induced fibrinolytic activity.

Fibrin Slide Sandwich Technique

The materials and technique for the localization of areas exhibiting inhibition of fibrinolysis by plasmin were as previously described (Noordhoek Hegt 1977). Briefly, a series of frozen sections of a specimen covered by a thin film of plasminogen-free fibrin on top of which a slice of a frozen plasmin--gelatin solution is placed is incubated for various periods of time. During incubation, the plasmin digests the underlying film of fibrin, except at sites where inhibiting material from the tissue section is present. The area of the fibrin remaining above the tissue section is taken as an arbitrary measure of the capacity for plasmin fibrinolysis inhibition.

Histological staining with the picro-Mallory stain (Lendrum et al. 1962), slightly modified for this purpose (Noordhoek Hegt 1974c), was done on parallel frozen tissue sections in order to correlate the activation and inhibition patterns with tissue structures present in the sections.

Two sheets of the vessel wall were separately evaluated: an intimal/medial layer and the adventitia in the wall of arteries and an intimal/medial/adventitial layer and the external layer of loose connective tissue in the venous wall. Structurally, the intimal/medial layer of the arteries resembles the intimal/medial/adventitial layer of the veins, in that both usually contain many smooth muscle cells. The arterial adventitia and the venous external

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Pag. 79 : Vijfde regel in Fig. 2A moet zijn :
Right: fibrin present above the inner vascular layers but not above the external connective tissue layer.

Pag. 81 en 82 : Tabel I en II horen op pag. 65 en 66

Pag. 86 : Eerste regel in Fig. 5A moet zijn :
..... incubated for 60 min.
Eerste regel in Fig. 5B moet zijn :
..... incubated for 120 min.

layer of loose connective tissue both consist of ordinary loose connective tissue, usually without smooth muscle cells.

RESULTS

The calculated mean of simultaneously determined endothelial plasminogen activator activities and plasmin inhibition capacities of the selected specimens are presented in Tables 1 and 2. The localization of the activator-induced fibrinolysis and the inhibition of plasmin fibrinolysis along and across the wall of human blood vessels was distributed as follows:

Activation and Inhibition of Fibrinolysis in Arteries

Intima/media: Specimens from arteries showed little or no endothelial fibrinolytic activity but a strong inhibition of plasmin fibrinolysis in these layers (Tables 1 and 2; Fig. 1). As before, the plasmin inhibition was related to the high number of smooth muscle cells randomly present in the media and intima as determined by the picro-Mallory stain. The medium-sized arteries showed stronger plasmin inhibition than the large arteries, closely reflecting the smaller number of smooth muscle cells present in the latter.

Adventitia: This layer of loose connective tissue, which usually does not contain smooth muscle cells, generally showed a very pronounced activator activity related to the vasa vasorum, while plasmin inhibition was low or absent (Tables 1 and 2; Fig. 1). In contrast, in samples from cases of endotoxin shock, hyaline membrane disease and a Waterhouse-Friderichsen syndrome, this layer showed weak activator activity (Noordhoek Hegt 1976) and strong plasmin inhibition (Noordhoek Hegt 1977) (Tables 1 and 2).

Activation and Inhibition of Fibrinolysis in Veins

Intima/media/adventitia: Activator activity related to the vasa vasorum in this part of the venous wall was generally more pronounced and inhibition of plasmin was usually less than in comparable inner arterial layers. The smooth muscle cells were not randomly distributed as in the arterial media, but they were arranged in circular and longitudinal muscle bundles (with inhibitory capacity) separated by varying amounts of loose connective tissue (without inhibitory capacity) in which the vasa vasorum was situated. Veins from the lower parts of the body contained more numerous and more closely arranged muscle bundles leaving less connective tissue interspaced than did the veins

FIG. 1A

FIG. 1B

FIG. 1A. Human carotid artery incubated for 40 min on a plasminogen-rich fibrin slide. Clear zones of lysis are seen under and around the vasa vasorum in the adventitia and around some detached intimal endothelial cells in the lumen. Harris' alum hematoxylin (x 75).

FIG. 1B. Adjacent section of the same carotid artery studied by the fibrin slide sandwich technique using plasminogen-free fibrin with plasmin in the active top layer. After 140 min of incubation fibrin is still present above the media and intima but is lysed above the adventitia and areas beyond the section. Harris' alum hematoxylin (x 75).

from upper levels. Therefore, the activator activity as well as plasmin inhibition in the venous wall differed greatly in segments from different locations in one individual. For instance, sections from the great saphenous vein below the knee showed low activator activity and strong plasmin inhibition in the inner layers (Fig. 2A); the vena cava taken at the level of the liver showed somewhat more fibrinolytic activity and less inhibition (Fig. 2B); the subclavian vein showed still more fibrinolytic activity and low inhibition (Fig. 2C) and the jugular vein exhibited extremely strong fibrinolytic activity and scarcely any inhibitory capacity (Fig. 2D).

Thus, increasing mean activator activity involves decreasing mean inhibition capacity in veins from ascending body levels as illustrated in Fig. 3.

External layer of Loose Connective Tissue

The activator activity related to the vasa vasorum in this venous connective tissue layer (corresponding to the arterial adventitia) was generally strong, while inhibition was weak or absent. Neither activity nor inhibition in this layer varied according to the location of the vein in the body. In endotoxin shock, hyaline membrane disease, and a Waterhouse-Friderichsen syndrome, an extremely low activator activity (Noordhoek Hegt 1976) and an unusually strong inhibition (Noordhoek Hegt 1977) was observed in the venous external layer (Tables 1 and 2). The endothelial activator activity along and

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..... incubated for 120 min.

FIG. 2. Adjacent sections of veins from different body levels investigated for fibrinolytic activity on plasminogen-rich fibrin slides incubated for 40 or 60 min (left sides), and for inhibition of fibrinolysis on plasminogen-free 'sandwich' slides covered by plasmin incubated for 100 min (right sides). Harris' alum hematoxylin (approximately x 50).

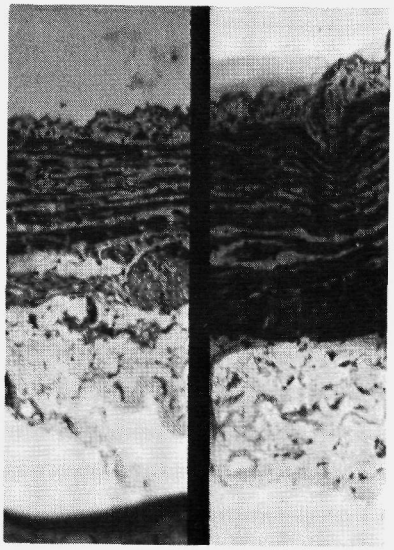

FIG. 2A.
Left: great saphenous vein showing lysis which extends under the external connective tissue layer but not under the inner layers; Right: fibrin present above the external connective tissue layer.

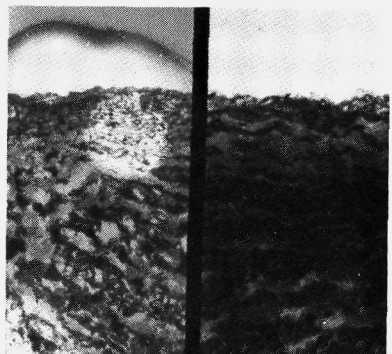

FIG. 2B.
Left: inferior vena cava with lysis extending under the area of the external connective tissue layer and extending away from the intima; extension of lysis was inhibited under the inner vascular layers; Right: intact fibrin above the inner layers but not above the external connective tissue layer.

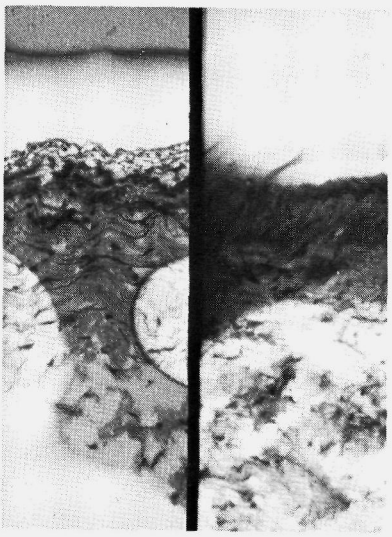

FIG. 2C.
Left: subclavian vein in which intimal lysis was strong but hampered in the extension near the subintimal region;
Right: a rim of fibrin remains above and around the subintimal region.

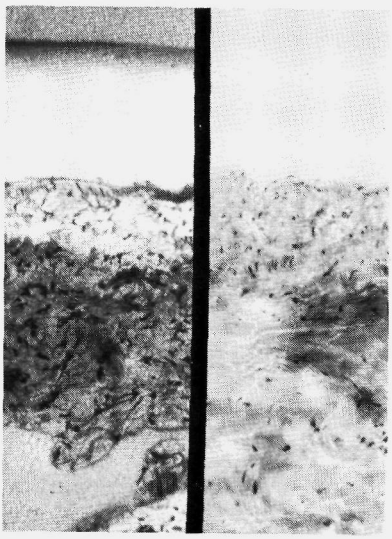

FIG. 2D.
Left: jugular vein in which nearly no inhibition of the large lysis zones was detected;
Right: no fibrin is present above the section.

across the wall of arteries and veins was strongly increased in cases of sudden death, vasogenic shock, cerebral hemorrhage and cirrhosis (Noordhoek Hegt 1976). However, the capacity for plasmin inhibition had not changed and equalled that of the vessels taken at routine autopsy (Noordhoek Hegt 1977) (Tables 1 and 2).

Table 1

Plasmin inhibition in human arteries

	Number of cases	Mean inhibitory capacity[a] (arbitrary units)	
		I/M	A
Large arteries			
Aorta	15	13	1
Innominate artery	4	11	0
Subclavian artery	3	12	3
Common carotid artery	5	10	2
Weighted mean inhibitory capacity		12	1
Medium-sized arteries			
Popliteal artery	5	14	2
Hepatic artery	4	17	1
Splenic artery	7	16	1
Renal artery	5	16	3
Right coronary artery	6	18	1
Umbilical artery	5	15	3
Weighted mean inhibitory capacity		16	2
Cases with low fibrinolytic activity[**]			
Aorta	4	15	15
Right coronary artery	4	17	17
Cases with high fibrinolytic activity[+]			
Aorta	4	11	1
Right coronary artery	4	17	2

a) Mean plasmin fibrinolysis inhibition capacity calculated as described; I = intima; M = media; A = adventitia.

**) Two cases of endotoxin shock, a case of hyaline membrane disease and a case of a Waterhouse-Friderichsen syndrome;

+) One case of sudden death, a case of vasogenic shock, a case of cerebral hemorrhage and a case of cirrhosis.

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Pag. 86 : Eerste regel in Fig. 5A moet zijn :
..... incubated for 60 min.
Eerste regel in Fig. 5B moet zijn :
..... incubated for 120 min.

Table II

Plasmin inhibition in human veins

	Number of cases	Mean inhibitory capacity[a] (arbitrary units) I/M/A	E
Large veins			
Vena cava	10	10	1
Internal jugular vein	10	3	2
Subclavian vein	4	6	2
Weighted mean inhibitory capacity		6	2
Medium-sized veins			
Femoral vein	5	11	2
Great saphenous vein	10	12	1
Superior sagittal sinus	3	2	2
Umbilical vein	5	11	3
Weighted mean inhibitory capacity		10	2
Cases with low fibrinolytic activity[*]			
Vena cava	4	16	16
Cases with high fibrinolytic activity[+]			
Vena cava	4	9	1

[a] Mean plasmin fibrinolysis inhibition capacity calculated as described; I = intima; M = media; A = adventitia; E = external layer of loose connective tissue.

[*] Two cases of endotoxin shock, a case of hyaline membrane disease and a case of a Waterhouse-Friderichsen syndrome;

[+] One case of sudden death, a case of vasogenic shock, a case of cerebral hemorrhage and a case of cirrhosis.

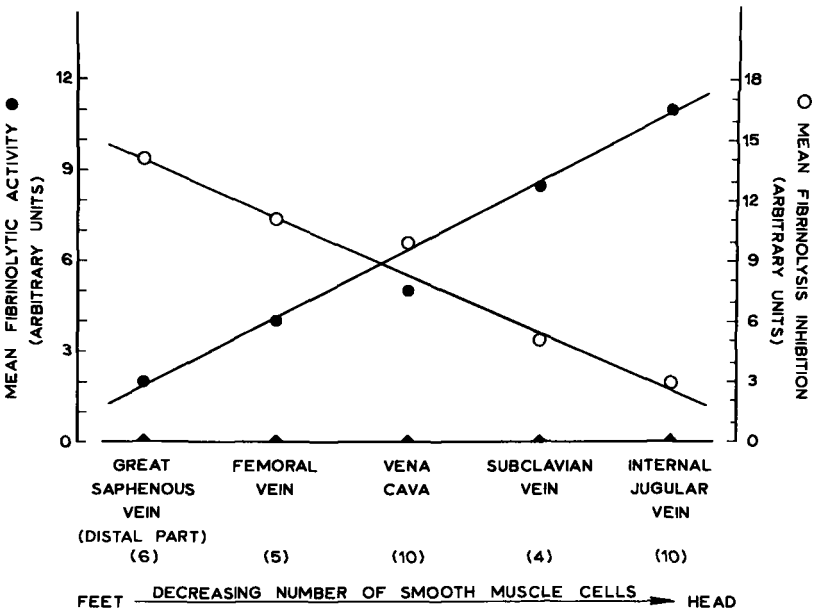

FIG. 3. The inverse relationship between the fibrinolytic activity (closed circles) and the fibrinolysis inhibition (open circles) in veins from ascending body levels.

Theoretically, a decrease in fibrinolytic (activator) activity can be caused by either a decreased level of plasminogen activator and an increased level of fibrinolytic inhibitors. However, comparison of the present data shows that, under these in vitro conditions, the vascular activator activity usually follows a pattern of decrease when the local plasmin inhibition increases. It seems, therefore, reasonable to assume that differences in endothelial activator activity are mainly governed by differences in inhibition, the latter being dependent on the number of smooth muscle cells present locally. The systemic decrease in endothelial activator activity observed in endotoxin shock, hyaline membrane disease, and a Waterhouse-Friderichsen syndrome seems to be due to the systemic increase in inhibition of fibrinolysis found in these cases.

Distortion of the Circular Shape of Lysis Zones in the Fibrin Slide Technique as an Indication for Fibrinolysis Inhibition

In view of the interrelationships described above between activator activity and plasmin inhibition, the development of zones of fibrinolysis in the neighbourhood of smooth muscle cells were studied on plasminogen-rich fibrin slides in sections from arteries and veins.

Arteries

Areas of lysis resulting from plasminogen activator present in the vasa vasorum of the adventitia were generally round-shaped after short incubation periods (Fig. 4A), but most often these zones flattened towards the site of the media while extending during prolonged incubation (Fig. 4B). Lysis generated by the intimal endothelium appeared to be arrested by the vascular wall (Figs. 4A and 4B). Since the tissue section was placed upon an intact fibrin layer, a uniform increase in size of the zone of lysis would have been anticipated. The distortion in the medial/intimal area must therefore be a result of interference with local lysis by the inhibiting material present in the smooth muscle cells of the media and intima.

Veins

In veins, a distortion of the zones of lysis was also detectable in areas rich in smooth muscle cells, areas which showed strong inhibition of plasmin by the sandwich technique. Fig. 5 illustrates the distortion above the bundles of smooth muscle cells in the superior vena cava. Veins with a thick muscular coat, such as those in the lower parts of the body, showed more lysis distortion than the less muscular veins from the upper levels (compare Figs. 2A, 2B, 2C and 2D).

From these observations, it can be concluded that inhibition of fibrinolysis as revealed by distortion of lysis zones on plasminogen-rich fibrin slides is completely in agreement with the results obtained by the fibrin slide sandwich technique.

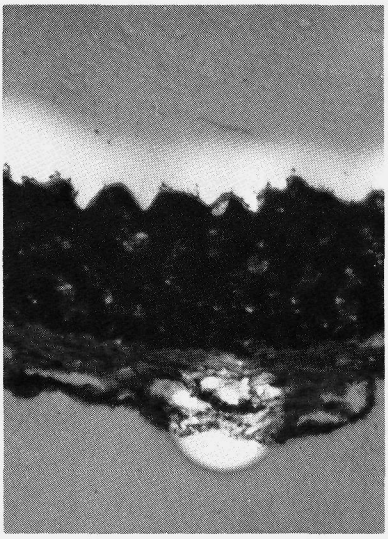

FIG. 4A. Hepatic artery from a case of sudden death assayed on plasminogen-rich fibrin after 10 min of incubation. A clear round-shaped zone of lysis is present in the adventitia related to a vasa vasorum. Lysis is also generated by the intima. Harris' alum hematoxylin (x 100).

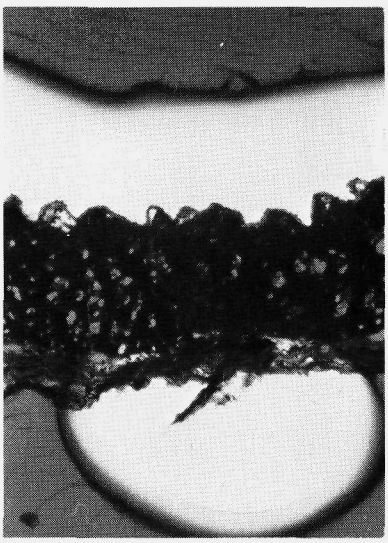

FIG. 4B. Same artery incubated for 40 min on a fibrin slide. The extension of lysis from intima and adventitia was arrested under the media. Harris' alum hematoxylin (x 100).

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..... incubated for 120 min.

FIG. 5. Inner part of a superior vena cava.

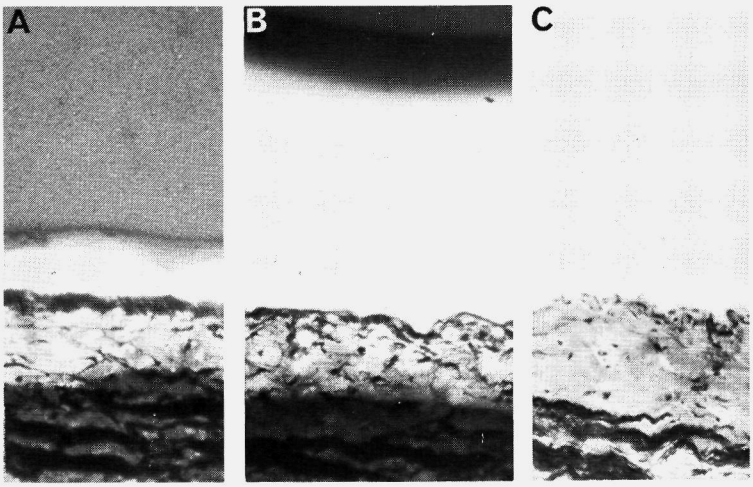

FIG. 5A FIG. 5B FIG. 5C

FIG. 5A. Section assayed under plasminogen-rich fibrin incubated for 120 min. Extension of lysis from the intimal endothelium was not arrested. Harris' alum hematoxylin (x 150).

FIG. 5B. Section assayed under plasminogen-rich fibrin incubated for 60 min. Extension of lysis from the intimal endothelium was arrested under the subintimal region. Harris' alum hematoxylin (x 150).

FIG. 5C. Histologically stained section showing dark bundles of smooth muscle cells in the subintimal region where lysis was arrested. Lendrum's picro-Mallory stain (x 150).

DISCUSSION

Confirming a preliminary report (Noordhoek Hegt 1975) these data on the location and intensity of plasminogen activator activity and plasmin inhibition in sections of the human vascular wall show a pattern of inverse relationship. Differences in fibrinolytic activity observed on fibrin slides along and across the vascular system may therefore be due to differences in inhibitory capacity of the surrounding tissues, rather than to variations in the plasminogen activator content of the endothelium itself.

Ruling out the influence of inhibiting compounds, it is not surprising that, with immunologic methods, endothelial plasminogen activator antigen

appears to be much more widely distributed than activator activity measured by the fibrin slide technique (Todd and Hargreaves 1975). Lysis observed in the fibrin slide technique seems to depend mainly on the relative amounts of the activating and inhibiting materials present and the distance between the activator containing endothelium and the inhibitory smooth muscle cells in the vascular wall. Variations in distance between the endothelium and the smooth muscle cells caused by structural alterations in the vessel wall, or differences in the amounts of activating or inhibitory material from tissue cells or the blood, will therefore change the fibrinolytic pattern on fibrin slides. In terms of the latter possibility, the strong fibrinolytic activity generated by arterial and venous walls in cases of sudden death, vasogenic shock, cerebral hemorrhage and cirrhosis seems to be due to an increase in the amounts of available plasminogen activator released from the endothelial cells, since there was no decrease in plasmin inhibition in these cases. On the other hand, the systemic decrease in fibrinolytic activity observed in samples of vessels in endotoxin shock, hyaline membrane disease and a Waterhouse-Friderichsen syndrome seems to be related to the systemic increase in plasmin inhibition observed in these cases, probably due to diffusion of plasma proteinase inhibitors into the interstitial space (Lindeman 1976; Noordhoek Hegt 1977). An example of changed fibrinolysis due to alterations in the structure of the vessel wall is found in lesions of hereditary haemorrhagic telangiectasia, in which the small arteries showed an unusually strong endothelial fibrinolytic activity associated with severe atrophy of the (inhibitory) muscular layers (Kwaan and Silverman 1973). A comparable situation seems to be present in some varicose saphenous veins which showed strong intimal fibrinolytic activity adjacent to areas of atrophied muscular elements (Barchewitz et al. 1973, 1975). Moreover, endothelial cells detached from the inhibitory elements of the vascular wall have been repeatedly found to exert a stronger fibrinolytic activity than those remaining at the endothelial lining; this applies to veins (Todd 1959,1960; Pandolfi et al. 1968; Glas-Greenwalt 1972; and Noordhoek Hegt 1976) as well as to arteries (Bleyl 1969; Glas-Greenwalt 1972; and Noordhoek Hegt 1976). The normally inactive medial vasa vasorum of large arteries showed marked fibrinolytic activity when the (inhibitory) medial smooth muscle cells had been replaced by collagenous tissue, such as occurs in coarctation of the aorta or in arteriosclerotic calcified plaques (Noordhoek Hegt 1976).

As to the synthesis and release of the fibrinolysis activating and inhibi-

ting materials it is known that explants of blood vessels synthesize and release plasminogen activator (Pandolfi 1970; Åstedt and Pandolfi 1975) as well as plasmin inhibitor (Bernik and Kwaan 1971).

Moreover, blood vessels perfused in situ appear to release plasminogen activator (von Kaulla and Wasantapruek 1969; Aoki and von Kaulla 1971; Ishizu et al. 1972; Aoki 1974; Gurewich et al. 1975; Markwardt and Klöcking 1976) as well as plasmin inhibitor (Mishchenko et al. 1972) into the perfused saline. These findings suggest that both compounds are released from living cells.

Nevertheless, the immediate extrapolation of the results obtained in vitro on fibrin slides to the in vivo situation is as yet unjustified, since little is known about the mechanism of release of these compounds from the cells. Furthermore, the stimuli for the release of activator from endothelial cells and of inhibitor from smooth muscle cells may differ. However, the conditions on the fibrin slides might well represent the situation after tissue injury and cell death since the thin sections of frozen tissue which are used in the slide technique contain a majority of damaged cells.

The inhibition of fibrinolysis by vascular smooth muscle cells may, for instance, be of importance when fibrin is formed in or on the walls of an injured blood vessel. Under such conditions, whether the released inhibiting material will be able to prevent the fibrin from lysis by the fibrinolytic compounds from endothelial cells or from the blood may depend upon the number of damaged smooth muscle cells locally present. Considerable amounts of unlysed fibrin have, in fact, been demonstrated in and around degenerating smooth muscle cells of arteries during temporary hypoxia (Kerényi and Jellinek 1972) and in malignant hypertension (Hüttner et al. 1968 and Ooneda et al. 1973), ultimately leading to the fibrinoid transformation of arteries observed in atherosclerosis and arteriolosclerosis. Moreover, it is generally known that thrombosis is more frequent in the veins of the lower body levels than in those of the upper levels. This difference in thrombosis tendency may be due to the fact that the lower body veins contain more smooth muscle cells than the veins situated in the upper parts of the body, so that resolution of fibrin deposits is slower.

The data presented in this study may be helpful in the further evaluation of the role of fibrinolysis activation and inhibition in the pathogenesis of thrombosis and occlusive vascular disease.

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SUMMARY

The balance between fibrin formation and fibrinolysis regulates normal haemostasis. Deficiencies in the blood clotting system as well as excess activity of the fibrinolytic system can produce a haemorrhagic diathesis. On the other hand, increased activity of the clotting system or decreased activity of the fibrinolytic system may lead to extensive fibrin deposition or thrombosis.

In order to elucidate the role of tissues in the vessel wall in the resolution of fibrin, it was decided to undertake a systematic study of the fibrinolytic activity in human arteries and veins employing a standardized histochemical 'fibrin slide technique'.

During this investigation, however, it was discovered that the observed fibrinolytic activity was influenced by fibrinolysis inhibiting material present in some human tissues. To determine the localization and extent of this inhibition of fibrinolysis in tissue sections, a 'fibrin slide sandwich technique' was devised. This technique is as described in detail in the Appendix.

In Chapter I, it is reported how by means of this new histochemical technique, fibrinolysis inhibiting material could be detected in the media of human arteries but not in the adventitia. The inhibiting material appeared to be able to diffuse out of the vessel wall into an adjacent fibrin layer. The inhibitory effect could be abolished by heating of the vascular tissue.

Chapter II describes the demonstration of a relationship between fibrinolysis inhibition and the smooth muscle cells present in the wall of the human vascular system. The inhibitory effect of these cells was directed to plasmin.

In Chapter III it is shown that the inhibiting material from tissue cells may exert a strong inhibitory effect on the fibrinolytic activity of the tissues. It was concluded from this study that the fibrin slide technique reveals the net result of the action of compounds that activate and inhibit fibrinolysis.

Chapter IV provides an overall view on the distribution and variation in fibrinolytic activity in the walls of human arteries and veins examined by the 'fibrin slide technique'.

Chapter V deals with the distribution and variation in fibrinolysis inhibition in the walls of human arteries and veins examined by the 'fibrin slide sandwich technique'.

The relationships between fibrinolysis activation and inhibition in the

human vascular wall are discussed in Chapter VI. From this study, it appeared that inhibition of fibrinolysis is strong at sites of low fibrinolytic activity and vice versa. Fibrinolysis promoting activity is related to endothelial cells, while fibrinolysis inhibition is generally brought about by smooth muscle cells. It was concluded that differences in endothelial fibrinolytic activity along and across the human vascular wall may result from differences in fibrinolysis inhibition caused by variations in the number of smooth muscle cells present locally.

The local balance between fibrinolysis activation and inhibition is supposed to be of importance when considering the fate of fibrin deposited on an injured blood vessel.

SAMENVATTING

Het evenwicht tussen fibrinevorming en fibrinolyse is van groot belang voor een normaal verlopende haemostase. Deficienties in het bloedstollingssysteem of een buitengewoon sterke fibrinolytische aktiviteit kunnen een haemorrhagische diathese teweeg brengen. Toegenomen aktiviteit van het stollingssysteem of een afgenomen aktiviteit van het fibrinolytische systeem zouden uitgebreide fibrine-afzettingen of thrombose kunnen veroorzaken.

Om na te gaan wat de rol van de weefsels in de vaatwand is bij het oplossen van fibrine, werd besloten om een systematisch onderzoek op te zetten naar de fibrinolytische aktiviteit van menselijke arteriën en venen met behulp van een gestandaardiseerde histochemische 'fibrin slide technique'.

Gedurende dit onderzoek echter, werd ontdekt dat de waargenomen fibrinolytische aktiviteit beïnvloed was door fibrinolyseremmend materiaal, aanwezig in sommige menselijke weefsels. Om de lokalisatie en de uitgebreidheid van deze fibrinolyseremming in weefselcoupes te bepalen werd de 'fibrin slide sandwich technique' ontwikkeld. Deze nieuwe histochemische techniek staat nauwkeurig beschreven in de Appendix.

In Hoofdstuk I wordt beschreven hoe met behulp van deze 'fibrin slide sandwich technique' een fibrinolyseremmende faktor kon worden opgespoord in de media van menselijke arteriën, maar niet in de adventitia. Het remmende materiaal bleek uit de vaatwand te diffunderen in een daarmee in kontakt gebrachte fibrinelaag. De remmende invloed kon worden uitgeschakeld door verhitting van de vaatwand.

Hoofdstuk II beschrijft hoe er een duidelijke relatie werd gevonden tussen fibrinolyseremming en de gladde spiercellen welke aanwezig zijn in de wand van het vaatstelsel. De remmende invloed van deze spiercellen bleek gericht te zijn op plasmine.

In Hoofdstuk III wordt aangetoond dat fibrinolyseremmend materiaal uit een weefsel een duidelijke invloed kan uitoefenen op de fibrinolytische aktiviteit van dat weefsel. Uit dit onderzoek wordt gekonkludeerd dat de 'fibrin slide technique' het netto resultaat weergeeft van de werking van fibrinolyse-aktiverende en -remmende stoffen.

Hoofdstuk IV verschaft inzicht in de verdeling en variatie van de fibrinolytische aktiviteit van menselijke arteriën en venen die werden onderzocht met behulp van de 'fibrin slide technique'. Arteriën toonden in het algemeen weinig of geen fibrinolytische aktiviteit in de intima en media maar sterke aktiviteit in de adventitia. Venen toonden eenzelfde sterke fibrinolytische

aktiviteit in de buitenste bindweefsellaag; de fibrinolytische aktiviteit in de veneuze intima, media en adventitia was meestal zwakker maar verschilde aanzienlijk van vene tot vene, afhankelijk van hun lokalisatie in het menselijk lichaam. Venen uit gebieden onder het diafragma bleken in het algemeen een duidelijk zwakkere fibrinolytische aktiviteit te vertonen dan venen uit gebieden boven het diafragma. De fibrinolytische aktiviteit kon worden gerelateerd aan het endotheel van de vasa vasorum en/of aan het endotheel dat het lumen van het bloedvat bekleed. Toegenomen fibrinolytische aktiviteit werd waargenomen in arteriën en venen in gevallen van plotselinge dood, vasogene shock, hersenbloeding en levercirrhose. Afgenomen fibrinolytische aktiviteit werd gezien in bloedvaten in gevallen van endotoxinen shock, hyaline membraan ziekte en in een geval van een Waterhouse-Friderichsen syndroom.

Hoofdstuk V geeft de resultaten van het onderzoek naar de verdeling en variatie van de fibrinolyseremming in menselijke arteriën en venen die werden onderzocht met behulp van de 'fibrin slide sandwich technique'. Arteriën vertoonden in het algemeen sterke fibrinolyseremming in de intima en media en slechts zwakke remming in de adventitia. De buitenste bindweefsellaag in de venen toonde eveneens weinig of geen fibrinolyseremming. Fibrinolyseremming was meestal wel aantoonbaar in het intima/media/adventitia gebied van de venen maar deze verschilde sterk van vene tot vene. Venen uit gebieden onder het diafragma bleken een duidelijk sterkere fibrinolyseremming te vertonen dan venen uit gebieden boven het diafragma. De fibrinolyseremming kon worden gerelateerd aan de gladde spiercellen in de vaatwand. In gevallen van endotoxinen shock, hyaline membraan ziekte en in een geval van een Waterhouse-Friderichsen syndroom werd remming van de fibrinolyse ook nog waargenomen in gebieden van losmazig bindweefsel waarin zich nauwelijks of geen gladde spiercellen bevonden. Naast de zojuist beschreven remming van de plasmine-fibrinolyse werd selektieve remming van aktivator-geïnduceerde fibrinolyse uitsluitend gevonden in het bindweefsel om de bloedvaten van de menselijke navelstreng.

De relaties tussen fibrinolyse-aktivering en -remming in de menselijke vaatwand worden beschreven in Hoofdstuk VI. Uit deze studie komt duidelijk naar voren dat fibrinolyseremming sterk is op plaatsen met een zwakke fibrinolytische aktiviteit en vice versa. Er wordt geconcludeerd dat verschillen in endotheliale fibrinolytische aktiviteit langs de menselijke vaatwand het resultaat kunnen zijn van verschillen in fibrinolyseremming veroorzaakt door een van plaats tot plaats variërend aantal gladde spiercellen.

De lokale balans tussen fibrinolyse-aktivering en -remming wordt verondersteld om het uiteindelijke lot van een fibrineneerslag op een beschadigde vaatwand te bepalen.

Appendix

FIBRIN SLIDE SANDWICH TECHNIQUE: DETECTION OF INHIBITION OF FIBRINOLYSIS IN TISSUE SECTIONS

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INTRODUCTION

The fibrin slide sandwich technique was recently devised (Noordhoek Hegt and Brakman 1974a, 1974b, 1975) to determine the distribution of the inhibition of fibrinolysis in tissue sections. The principle of this method is based on the well-known fibrin slide technique of Todd (1959) and consists of incubating a histological section under a thin layer of fibrin on top of which is placed another section of a fibrinolytically active compound. During incubation, the top layer digests the underlying fibrin, but digestion is prevented at sites where inhibiting material from the tissue section has diffused into the fibrin. The area of the remaining fibrin can be detected microscopically and is indicative for the site of inhibition.

MATERIALS

Tissue: Tissue samples, preferably not exceeding 0.5 cm^3 taken from biopsy or autopsy material are collected without the use of fixative. Contaminating blood is removed by saline, and fatty parts are cut off by scalpel. The specimens are placed in isopentane, cooled with liquid nitrogen or dry ice, and kept in air-tight jars to prevent evaporation. Storage takes place at a temperature of -20°C or lower. Tissues can be stored under these conditions for

several months without significant change in inhibition of fibrinolysis.

Phosphate buffer: $\mu = 0.15$; pH = 7.8.
The buffer contains 0.06 M KH_2PO_4 and is adjusted to pH 7.8 with a NaOH solution.

Fibrinogen: A stock solution of plasminogen-rich bovine fibrinogen (about 2% w/v) prepared according to Brakman (1967) in phosphate buffer pH 7.8, ionic strength 0.15, is stored below - 20°C in vials containing 0.5 ml. This stock solution is thawed at room temperature and diluted with saline (0.9% (w/v) NaCl) to a final solution of 0.7% (w/v) fibrinogen.

Bovine fibrinogen without plasminogen from Poviet (Organon-Teknika, Oss, The Netherlands) is dissolved in phosphate buffer pH 7.8 adjusted to an ionic strength of 0.15. The final fibrinogen solution (0.7% (w/v)) is stored in 0.5 ml aliquots at a temperature below -20°C.

Thrombin: Plasminogen-free bovine thrombin, 5,000 NIH units (Leo Pharmaceuticals, Ballerup, Denmark) is dissolved in saline to give a final concentration of 20 NIH units/ml.

Urokinase: Human urokinase (Leo Pharmaceuticals, Ballerup, Denmark) is used in a concentration of 7 Ploug units/ml saline containing 15% (w/v) gelatin.

Plasmin: Human plasmin, prepared according to Sgouris et al. (1960), was obtained from the State of Michigan Department of Public Health (USA) and is used in a concentration of 1.5 CU/ml saline containing 15% (w/v) gelatin.

It is absolutely necessary to add 15% gelatin to the urokinase or plasmin solution, since sectioning would otherwise be impossible after freezing.

Top layer: Urokinase or plasmin is added to gelatin dissolved in saline at 37°C. The solution is then poured into boxes of 0.5 x 1 x 2 cm and cooled immediately at 4°C. When solidified, the gels are taken out and stored in air-tight jars at -20°C until frozen sections are cut from them. Besides urokinase, frozen sections (6 µm thick) of normal human lung tissue having a very pronounced tissue plasminogen activator activity can also be used.

Harris' alum hematoxylin stain

Aluminium sodium sulfate (Merck 1047)	100 g
Hematoxylin (Merck 4305)	5 g
Mercuric oxide red (BDH 29168)	2.5 g

Ethanol 99% (v/v)	50	ml
Distilled water	1,000	ml

The aluminium sodium sulfate is dissolved in 1 liter distilled water in a 4-liter Erlenmeyer flask and is heated to 90°C. The hematoxylin is dissolved in the ethanol and is then carefully poured into the heated salt solution. Mercuric oxide is added to this mixture. After rapid cooling and filtering, the staining solution is ready for use and can be kept for several months.

Glycerin-gelatin mounting medium

Gelatin is dissolved by stirring 7 g in 42 ml distilled water for 2 hr at 56°C. Fifty ml of glycerin is then added, and the suspension is stirred at 56°C for 15 min. After filtering through glass wool, the suspension is stored in small vials at room temperature. The medium is liquified at 56°C before use.

METHOD

Tissue sections: Normally, five frozen 16 µm thick tissue sections are cut with a cryostat microtome and individually placed on five microscope slides cleaned with alcohol 96% (v/v).

Fibrin films: To each slide, 20 µl of the thrombin solution and 100 µl of a fibrinogen solution (with or without plasminogen) are applied separately with an Eppendorf microliter pipette. The solutions are then quickly mixed and evenly spread over the tissue section over an area of 2.5 x 4 cm with a thin glass rod followed by gentle tilting of the slide. The slide is then immediately placed on a horizontal level in a moist chamber at 4°C.

Slides are left for 1 hr in the moist refrigerator to solidify and to allow diffusion of inhibiting compounds from the tissue sections into the overlying fibrin.

Top layers: Five 10 µm thick sections are then cut from a frozen plasmin or urokinase solution or from frozen human lung tissue (6 µm thick sections), and each is individually placed on top of a fibrin slide where it covers a tissue section. Plasmin is used on plasminogen-free fibrin films, urokinase, or lung on plasminogen-rich films. The frozen top layer sections may be picked up either directly with the covered microscope slides or transferred with a fine brush from the microtome knife to the slides. The slides with the sec-

tions on top are immediately returned to the moist chamber to avoid drying of the fibrin.

Incubation: Fibrin slides are incubated at 37°C in a moist chamber. The first section in the series is incubated for 180 min, the following for 160, 140, 120, and 100 min, respectively.

Further treatment of the slides: After incubation, the slides are fixed in a 4% (v/v) formaldehyde solution for 15 min, rinsed in running tap water for another 15 min, and stained in Harris' hematoxylin stain for 12 hr. After staining, the slides are rinsed in water and left in water until they are finally covered with coverslips mounted with glycerin-gelatin mounting medium. Routine histological staining on adjacent parallel frozen tissue sections that are not covered by fibrin is usually done in order to correlate the inhibition patterns with certain tissue structures present in the sections.

Heating: To determine the heat stability of the inhibiting compound, a series of five adjacent sections is cut from the tissue to be investigated for inhibition and heated at 100°C in a dry oven for 12 hr prior to the coverage with fibrin.

Evaluation of the inhibitory capacity: The inhibitory capacity of each specimen can be graded according to the following scheme:
Grade I: Nearly all the fibrin in contact with the underlying tissue has been digested by the active top layer.
Grade II: Three-fourths of the fibrin covering the tissue is lysed.
Grade III: One-half of the fibrin covering specimen is lysed.
Grade IV: Less than one-fourth of the fibrin covering the specimen is lysed by the active top layer.
To grades I, II, III, and IV are allotted 1, 2, 3, and 4 points, respectively. The sum of the points scored on each set of five slides is taken as a measure of the inhibitory capacity of that specimen.

RESULTS

The general construction of the fibrin slide sandwich technique before and after incubation is schematically illustrated in Fig. 1.

The fibrinolytically active top layer containing plasmin or urokinase normally digests the underlying fibrin exactly within 100 min of incubation

at sites where no tissue is present underneath the film. However, fibrin digestion is prevented to a certain extent at sites where inhibiting compounds from the tissue section have previously diffused into the fibrin layer. Areas of remaining fibrin appear as dark blue stained islets on a clear background.

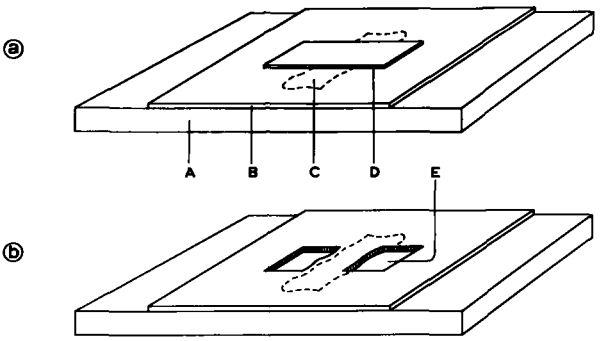

FIG. 1. Schematic top view of the fibrin slide sandwich technique before (a) and after (b) incubation. A, microscope slide; B, fibrin film; C, tissue section; D, fibrinolytically active top layer; E, lysed fibrin caused by active top layer. After incubation fibrin is still present on top of the tissue section, indicating complete inhibition of fibrinolysis at that site.

They are indicative for the site of inhibition and can be related to histologic structures in the underlying tissue section.

As an example, we illustrate a human great saphenous vein which was assayed for plasmin inhibition by the sandwich technique, using plasminogen-free fibrin covered by a plasmin top layer (Fig. 2). After incubation for 100 min, complete fibrin dissolution had occurred above the adventitia as well as outside the section. However, a fibrin strand remained on top of the intima and the media during 160 min of incubation, indicating the presence of plasmin-inhibiting material in the intimal/medial area which had diffused into the fibrin layer above it, thus preventing lysis at that site.

Plasmin inhibition was also observed when investigating human placenta with the sandwich technique (Fig. 3a), but the placenta also showed very pronounced urokinase-inhibiting activity, as assessed on plasminogen-rich fibrin covered with urokinase top layer (Fig. 3b).

In both vascular tissue and the placenta, plasmin inhibition was completely abolished when the tissue sections were heated prior to the coverage with fibrin. Inhibition of activator-induced fibrinolysis in sections of the

placenta was, however, still clearly demonstrable after the heating procedure.

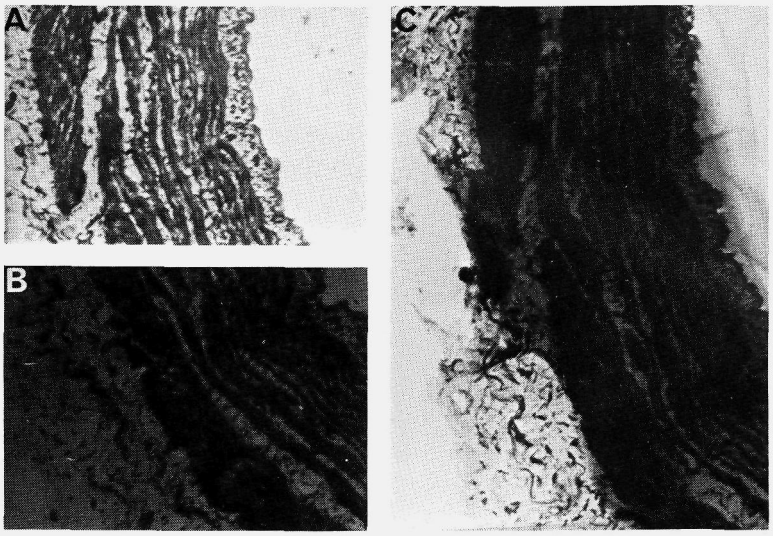

FIG. 2. Cross sections of the great saphenous vein studied by the fibrin slide sandwich technique. a: Section before coverage with fibrin; b: Section after coverage with fibrin; c: Section after incubation for 100 min with plasmin on top. Fibrin has remained above the intima and media, which are rich in dark bundles of smooth muscle cells, indicating plasmin inhibition at that site. Fibrin was dissolved above the adventitia and beyond the section. Harris' alum hematoxylin. x 100.

COMMENTS

Any tissue may be investigated by the sandwich technique for its ability to inhibit plasmin fibrinolysis, as plasminogen-free fibrin films which are insensitive to the action of the plasminogen activator from the tissue section are used. However, when investigating a tissue section for inhibition of activator-induced fibrinolysis using urokinase or lung tissue on top of plasminogen-rich fibrin, care must be taken that the underlying tissue itself has only little or no plasminogen activator activity; the latter would otherwise interfere with the inhibition pattern. Detection of plasminogen activator inhibition by this technique is thus confined to rather inactive tissues. The difference observed in heat stability of the urokinase-inhibiting compound and the material which causes the plasmin inhibition might help to differentiate between plasmin and plasminogen activator inhibition.

Tissues to be investigated for inhibition of fibrinolysis by the sandwich technique must be freed of fatty layers, since fat may repel the covering

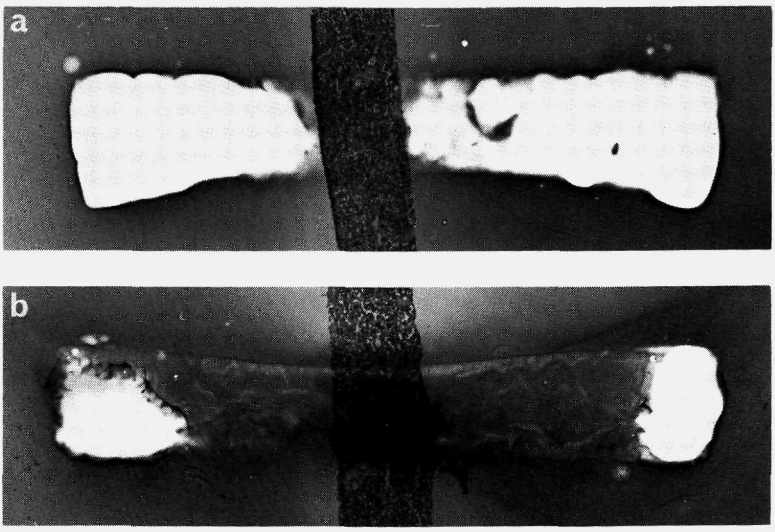

FIG. 3. Adjacent cross sections of human placenta studied by the fibrin slide sandwich technique. a: Plasminogen-free fibrin covered by a layer of plasmin and incubated for 100 min; b: Plasminogen-rich fibrin covered by a layer of urokinase and incubated for 180 min. Plasminogen-free fibrin is present above and slightly around the upper tissue section, indicating that the plasmin-inhibiting material has diffused out to that extent. In contrast, urokinase-induced fibrinolysis of plasminogen-free fibrin has occurred only after 180 min incubation at a great distance from the lower tissue, indicating that the urokinase inhibiting material has diffused over a much greater distance than the plasmin-inhibiting material. Note that the placenta tissue itself has no plasminogen activator activity. Harris' alum hematoxylin. x 5.

solution thus causing false negative reactions. Blood inhibits fibrinolysis and must therefore be carefully washed out from the tissue to avoid false-positive reactions. Moreover, care must be taken that fibrin films are not dried out before incubation.

Dry films are less susceptible to lysis.

Because the sandwich technique detects inhibitory material that has diffused out of the tissue into the overlying fibrin, exact localization of the source may be difficult when diffusion is extremely rapid. In such cases, a shorter diffusion period in the refrigerator may be required. When assessing the grade of inhibition of a tissue on the basis of the size of unlysed fibrin, care must be taken that the thickness of the sections used is kept constant.

APPLICATIONS

Up to now, the fibrin slide sandwich technique has been used successfully in the demonstration of plasmin inhibition in the human vascular wall (Noordhoek Hegt and Brakman 1974a) which appears to be related to the presence of smooth muscle cells (Noordhoek Hegt and Brakman 1974b). Moreover, the sandwich technique has been of great value for the critical evaluation of Todd's (Todd 1959) fibrin slide technique which is widely used for the detection of the plasminogen activator in tissue sections. It appears that fibrinolysis-inhibiting material derived from tissue cells and detectable with the sandwich technique greatly influences the size and shape of lysis zones produced by vascular endothelium on plasminogen-rich fibrin slides (Noordhoek Hegt and Brakman 1975).

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