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# Repair in arterial tissue

## Demonstration of fibrinogen/fibrin in the normal and healing rabbit thoracic aorta by the indirect immunoperoxidase technique

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**Summary.** The results of immunoperoxidase staining for fibrinogen/fibrin in ethanol- and formaldehyde-fixed, normal and healing arterial tissue are presented. Fibrinogen/fibrin was not observed in the normal aortic wall. The thoracic aorta damaged by a balloon catheter contained fibrinogen/fibrin in all layers of the wall. In the healing aortic wall there was a strong positive reaction in neo-intima, whereas the reaction in media was weak or absent. The staining reaction for fibrinogen/fibrin in formaldehyde-fixed neo-intima covered with aortic smooth muscle cells was strong and almost independent of proteolytic digestion, while such treatment increased the staining intensity for fibrinogen/fibrin in neo-intima covered with endothelium. Our results indicate that an extracellular matrix of fibrin and fibronectin may play a role in migration and proliferation of aortic smooth muscle cells.

**Key words:** Experimental arteriosclerosis – Fibrinogen – Fibrin – Immunoperoxidase staining – Neo-intima

In human aortas albumin and lipoproteins have been demonstrated by immunofluorescence techniques at all ages, while fibrinogen has not been observed in the human aortic wall under normal conditions (Haust 1971). However, immunoelectrophoresis has shown the presence of fibrinogen in normal intima, while an intact internal elastic lamella (IEL) reduces the infiltration of plasma fibrinogen into media (Smith and Staples 1980).

Fibrin is absent from early fatty lesions in man, while in raised fatty dots and streaks, gray gelatinous elevations and fibrous plaques variable amounts of fibrin have been demonstrated (Haust 1964; Wyllie et al. 1964; Kao and Wissler 1965; Woolf and Carstairs 1967; Walton and Williamson 1968; Haust 1971; Woolf 1978; Smith et al. 1979).

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Rokitansky (1852) suggested that atherosclerotic plaques were formed from blood constituents deposited on the endothelium and subsequently incorporated into intima. Virchow's (1856) theory is based on a passage of blood elements through the vessel wall with a subsequent proliferation of cells and hypertrophy of the vessel wall. Duguid (1946, 1948, 1976), and Astrup (1956) suggested that the balance between deposition of fibrin on the vessel wall (mural thrombosis) and fibrinolytic activity of the vascular intima may play a role in the atherogenic process. In the last few years new aspects of platelets in arteriosclerosis have been discovered, viz. the platelet-derived growth factor (Ross and Glomset 1976) and the balance between aggregation and non-aggregation of platelets (Moncada and Vane 1979).

The aim of the present study was to examine the distribution of fibrinogen/fibrin and their degradation products in the normal and healing arterial tissue by the indirect immunoperoxidase technique.

## Materials and methods

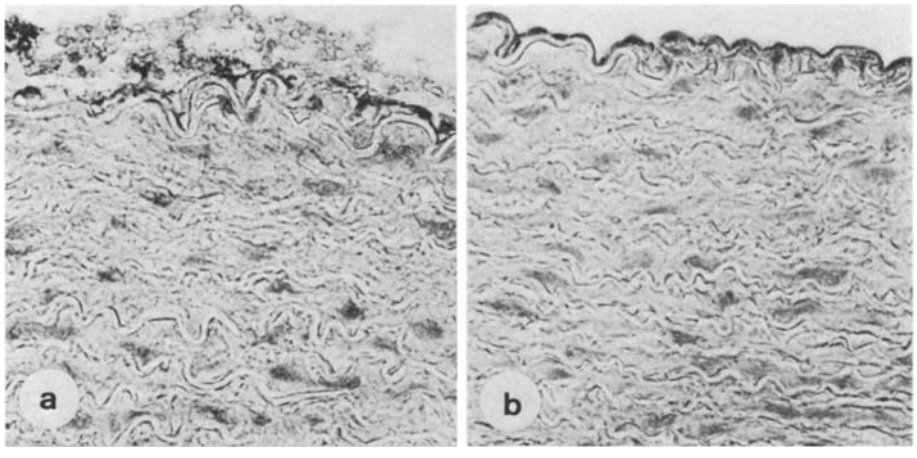
*Animals and tissue preparation.* Nine male albino rabbits of the Danish country strain each weighing about 3 kg were studied; 6 were submitted to a single dilatation trauma of the thoracic aorta by an embolectomy catheter according to a method previously described (Collatz Christensen and Garbarsch 1973). The normal thoracic aorta from 3 animals served as controls. In general anesthesia by Nembutal® 5 animals (2 normal rabbits, 1 rabbit, 2 days, and 2 rabbits, 21 days after the lesion) were perfusion-fixed with ethanol +1% acetic acid. The selected specimens were immersionfixed in the same fixative for 4 h at 4° C. Four animals (1 normal rabbit, 1 rabbit, 2 days, and 2 rabbits, 21 days after the lesion) were perfusion-fixed with 4% formaldehyde and the selected specimens were immersion-fixed for 4 h at 4° C. All the specimens were rinsed in phosphate buffered saline (PBS) for 24 h at 4° C before dehydration in ethanol and xylene, then they were embedded in Paraplast® at 56° C. All sections (5 μm) were placed on ethanol-cleaned glasses.

*Selection of tissue for examination.* The excision of specimens has previously been described in details by Collatz Christensen et al. (1979a). Forty-three specimens were examined, 10 from normal thoracic aortas between the intercostal arteries (IA), 8 from thoracic aortas 2 days after lesion (8 between IA), and 25 from thoracic aortas 21 days after lesion (16 excised close to and 9 between IA). The specimens from normal thoracic aortas and aortas, 21 days after lesion have previously been examined for the presence of fibronectin (Chemnitz and Collatz Christensen 1983). Consecutive sections from these specimens have been used in this study and thus the localization of fibrinogen/fibrin and fibronectin can be compared.

*Pre-treatment of sections with proteolytic enzymes.* Deparaffinized sections of specimens fixed in paraformaldehyde were incubated for 10, 15, 20, and 25 min at 37° C in the following proteolytic solutions: 0.01–0.1% (w/v) trypsin (DIFCO 1:250) in 0.1% (w/v) $CaCl_2$ adjusted to pH 7.8 with 0.1 M NaOH and 0.4% (w/v) pepsin (SIGMA, P 7012) in 0.01 M HCl.

*Antisera and control reagents.* Sheep anti-rabbit fibrinogen (IgG fraction) was purchased from Microtiter®, Dynatech (Cappel Laboratories, Inc.). The protein concentration of the IgG fraction was 50 mg/ml. The antibody was used in various dilutions from 1:200 to 1:1,000.

Rabbit anti-sheep IgG, peroxidase conjugated (DAKOPATT's, Copenhagen, P 163) was used i dilution 1:50. The control reagent was the Ig-fraction of serum from non-immunized goats (1:50) dissolved in 3% bovine serum albumin (BSA) or PBS. Furthermore the sheep anti-rabbit fibrinogen was tested on an in vitro system of aortic smooth muscle cells (ASMCs a system without the presence of fibrinogen, fibrin or degradations products of fibrin. No staining was observed in the ASMCs in vitro.




**Figs. 1a, b.** Normal rabbit thoracic aorta. ×400. **a** Ethanol fixation. **b** Formaldehyde fixation with proteolytic treatment. No staining reaction for fibrinogen/fibrin is seen in intima and media in **a, b.** Note the staining of coagulated blood on the surface of the endothelium in **a**

*Immunoperoxidase staining*. The previously described diaminobenzidine procedure for the demonstration of fibronectin was used (Chemnitz and Collatz Christensen 1983).

*Evaluation of immunoperoxidase staining*. The "positive" reaction product formed by the peroxidase reaction with diaminobenzidine, recognized as a brown to dark brown colour, was semiquantitated using the following scoring system. Staining intensity: 0: none, +: slight, ++: moderate, and +++: strong (Chemnitz and Collatz Christensen 1983). The evaluation was performed as a blind study by one of the authors (BCC).

## Results

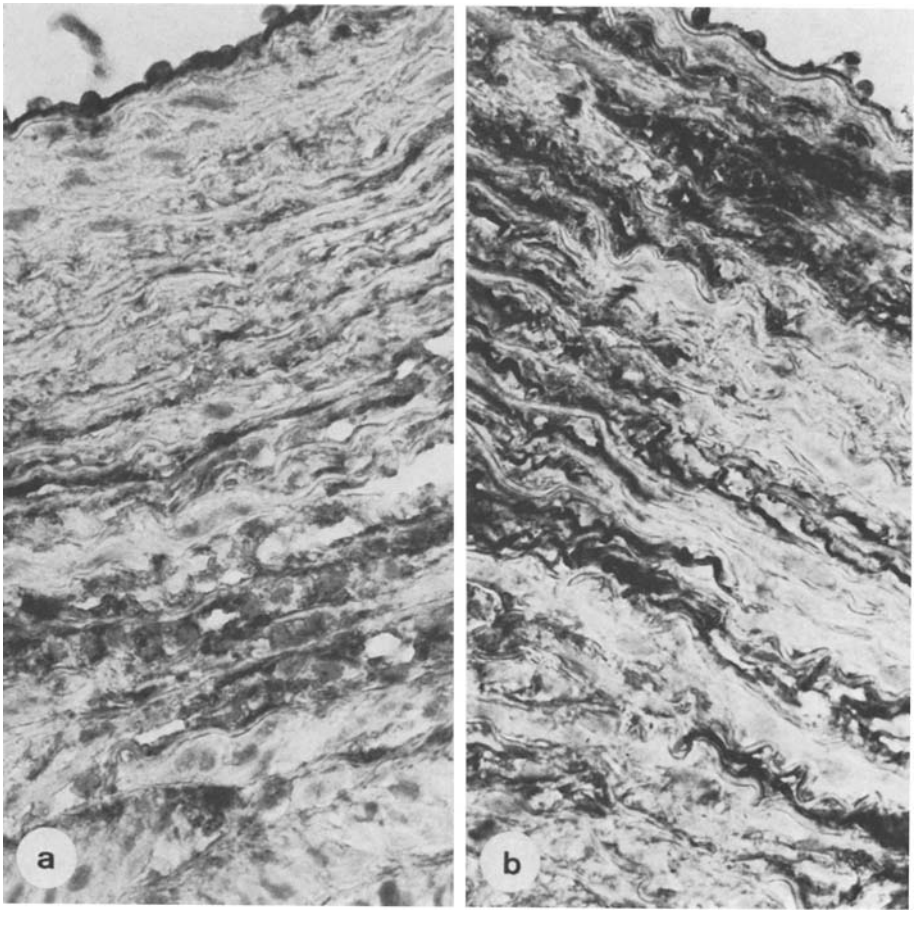
### *Fibrinogen/fibrin in the normal thoracic aorta*

Ethanol-acetic acid and formaldehyde-fixed tissue either non-treated or treated with proteolytic enzymes. Fibrinogen/fibrin was not observed in intima and media (Fig. 1a and b). Coagulated blood on the surface of the endothelium was stained (Fig. 1a). No staining was observed in sections incubated in control serum.

### *Fibrinogen/fibrin in the injured aortic wall two days after lesion*

*Formaldehyde-fixed tissue without proteolytic digestion.* The denuded intimal surface showed a strong staining intensity for fibrinogen/fibrin. In the media central areas with injured and disintegrated ASMCSs showed a moderate to strong staining intensity for fibrinogen/fibrin upon and between the elastic lamellae. Areas with "vital" ASMCs showed a negative or slight staining reaction. Fibrinogen/fibrin was not found within the ASMCs (Fig. 2a).

*Formaldehyde-fixed tissue treated with proteolytic enzymes.* Proteolytic digestion increased the staining intensity for fibrinogen/fibrin in media. In lumi-




**Figs. 2a, b.** Injured aortic wall two days after lesion. ×400. **a** Formaldehyde fixation. No proteolytic treatment. Positive staining for fibrinogen/fibrin of the denuded intimal surface. Areas in media with injured and disintegrated ASMCs showed a moderate staining intensity. No staining is seen within areas with vital ASMCs. **b** Formaldehyde fixation. Proteolytic treatment. The staining intensity for fibrinogen/fibrin is increased and extended to the luminal and adventitial zones of media

nal and adventitial areas with "vital" ASMCs a moderate to strong reaction was seen, however, no intracellular fibrinogen/fibrin was observed. Areas with injured and disintegrated ASMCs showed a strong positive reaction. The dark brown reaction product was observed on the denuded luminal surface, around "vital" ASMCs and between the elastic lamellae in injured areas (Fig. 2b). No staining was observed in sections incubated in control serum.

*Ethanol-fixed tissue.* The same staining intensity and localization of fibrinogen/fibrin as seen in formaldehyde-fixed tissue treated with proteolytic enzymes was obtained (data not shown).

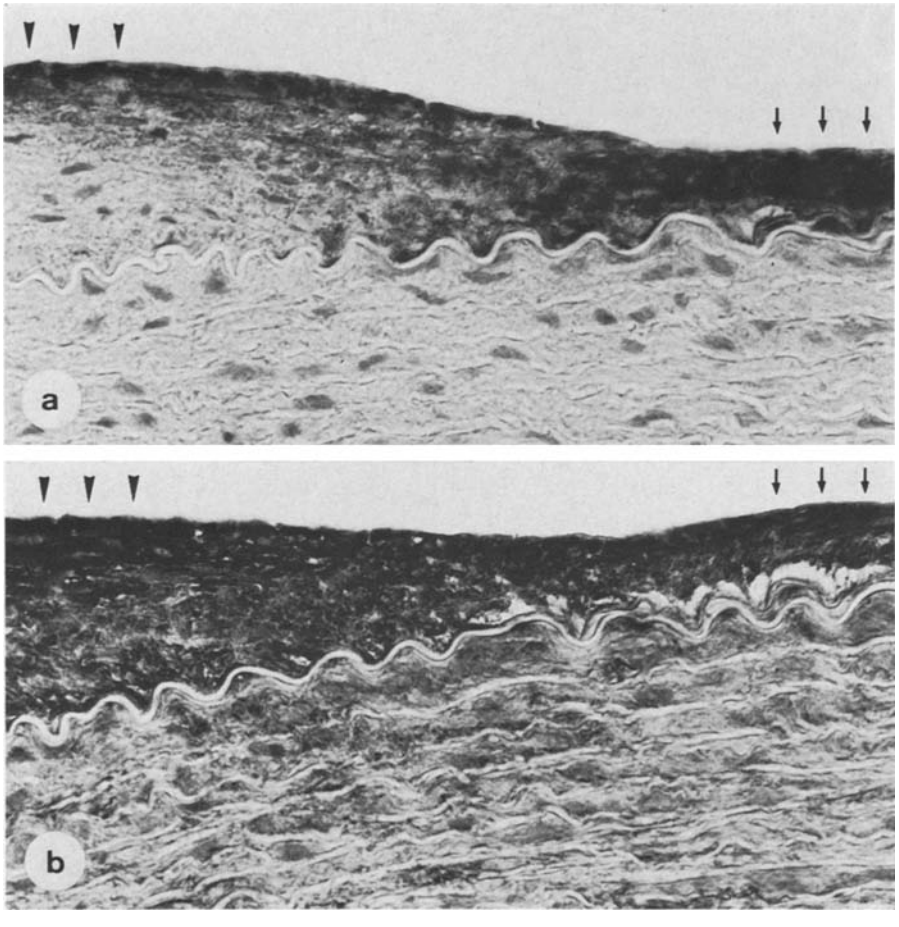



**Fig. 3a, b.** Re-endothelialized (*arrowheads*) and non-re-endothelialized (*arrows*) neo-intima 21 days after the lesion. ×400. **a** Formaldehyde fixation. No proteolytic treatment. EC-covered neo-intima, a minimal to moderate reaction for fibrinogen/fibrin is shown, however, the luminal part of neo-intima is heavily stained. ASMC-covered neo-intima, strongly positive reaction for fibrinogen/fibrin. No staining of media is seen. **b** Formaldehyde fixation. Proteolytic treatment. EC- and ASMC-covered neointima. Strongly positive reaction for fibrinogen/fibrin. Dispersed stained areas in media

*Fibrinogen/fibrin in the healing aortic wall, 21 days after the lesion*

*Formaldehyde-fixed tissue without proteolytic digestion.* A strong staining reaction was observed in ASMC-covered neo-intima compared to EC-covered neo-intima, which showed a minimal to moderate staining intensity. The superficial layer of the EC-covered neo-intima showed a strong staining intensity (Fig. 3a). No staining reaction was observed in media except in necrotic areas, where the reaction was strongly positive.

*Formaldehyde-fixed tissue treated with proteolytic enzymes.* Proteolytic digestion increased the staining intensity for fibrinogen/fibrin in re-endothelia-

lized neo-intima (Fig. 3b), whereas the staining reaction in ASMC-covered neointima remained strong. No staining reaction, except dispersed stained areas, was observed in the media. Areas of necrosis were heavily stained. No staining reaction was observed in sections incubated in control serum.

*Ethanol-fixed tissue.* Almost the same staining intensity and localization of fibrinogen/fibrin as seen in formaldehyde-fixed tissue treated with proteolytic enzymes were obtained with ethanol fixed materal.

## Discussion

Fibrinogen/fibrin was not present in the normal arterial wall. In the damaged and healing aortic wall fibrinogen/fibrin or their degradation products were present in considerable amounts giving a chromogenic reaction much like that described for fibronectin (Chemnitz and Collatz Christensen 1983; Jensen et al. 1983). However, the distribution of fibrinogen/fibrin differed from that of fibronectin, generally in accordance with the fact that fibrinogen penetrated the damaged aortic wall from the lumen, whereas the occurrence of fibronectin depended on both penetration and secretory activity.

In formaldehyde-fixed damaged arterial tissue with a high amount of proteoglycans the antigenic sites for anti-fibrinogen were masked. However, proteolytic digestion eliminated this masking effect and immunoperoxidase staining gave a strong positive reaction identical to results obtained on sections of ethanol-fixed arterial tissue. This masking of antigenic sites for anti-fibrinogen in formaldehyde-fixed tissue may be correlated with the content of proteoglycans as previously discussed (Chemnitz and Collatz Christensen 1983).

Fibrinogen/fibrin was not observed in media of normal thoracic aortas. Similar results have been found for the human aortic wall under normal conditions (Haust 1971). Immunohistochemical detection of fibrinogen in normal intima with ECs apposed closely to the internal elastic lamellae is difficult. However, it has been shown that plasma $^{131}$I-fibrinogen penetrated the normal pig aorta endothelium (Bell et al. 1974). Immunoelectrophoresis has also shown the presence of fibrinogen in normal intima, while the IEL probably provides a bariier against penetration of plasma fibrinogen into the media (Smith 1982). By contrast, fibronectin is a genuine ECM component of the normal aortic media (Stenman and Vaheri 1978; Natali et al. 1981; Chemnitz and Collatz Christensen 1983; Jensen et al. 1983).

The presence of fibrinogen/fibrin in the aortic wall, 2 days after initial injury, indicated that fibrinogen had free access to the injured media and accumulated in devitalized areas. Accordingly, in areas with "vital" ASMCs the amount of fibrinogen/fibrin was minimal. By contrast fibronectin was present in large amounts both intracellularly and extracellularly in areas with "vital" ASMCs indicating activated secretory cells (Chemnitz and Collatz Christensen, in preparation).

The content of fibrinogen/fibrin and their degradation products in the healing aortic wall 21 days after initial injury probably depends on the

current and previous quality of the neo-intimal barrier, on the distribution of fibrinolytic activities and the strength of binding forces between fibrin/fibrinogen, their degradation products and the connective tissue matrix. The occurrence of fibronectin, however, is probably a balance between penetration from plasma and the secretory activity of ECs and ASMCs.

Our observations indicated a coincidental and almost similar occurrence of fibrinogen/fibrin and fibronectin in the neo-intima with a similar unmasking effect by proteolytic enzymes on formaldehyde-fixed tissue in EC-covered neo-intima (Chemnitz and Collatz Christensen 1983). In the media, however, fibrinogen/fibrin and degradation products were only seen in dispersed areas, whereas fibronectin showed a diffuse distribution. A common feature was the unmasking effect by proteolytic enzymes and accumulation in devitalized and necrotic tissue. The high content of fibrinogen/fibrin in EC-covered as well as in ASMC-covered neo-intima may be interpretated as penetration of plasma fibrinogen into neo-intima due to defect barrier function and/or a slow fibrinolytic activity. The permeability of EC-covered neo-intima to albumin is normalized after 60 days (Helin et al. 1971). Studies on the permeability of Evans blue protein-complex indicated establishment of an intimal barrier between 21 and 60 days after initial injury coincident with the re-endothelialization and formation of well organized lamellated myoelastic tissue (Collatz Christensen et al. 1979a and b; Chemnitz and Collatz Christensen 1981). The interactions between proteoglycans and fibrous proteins may be of importance for ECM organization, which in turn affects the permeability of macromolecules across the arterial wall (Richardson et al. 1980; Radhekrishnamurthy et al. 1982). ASMC-covered neo-intima showed disorganized connective tissue and missing barrier against Evans blue protein-complex (Collatz Christensen et al. 1979a). Our present observations could indicate that the neo-intimal barrier, 21 days after injury, is far from effective against penetration of plasma fibrinogen.

The minimal content of fibrinogen/fibrin and/or their degradation products in the media, conspicuously reduced when compared with the 2 day lesion, indicated fibrinolytic removal. Proliferating ASMCs and reparative ECs (Levin and Loskutoff 1979) release plasminogen activator which converts plasminogen to plasmin. The accumulation of fibrinogen/fibrin in acellular necrotic areas may be due to minimal fibrinolytic activity in these areas. By contrast, fibronectin is a normal constituent of vascular connective tissue (Chemnitz and Collatz Christensen 1983; Jensen et al. 1983) and present in high amounts in the media. In our studies fibronectin was localized intracellularly in ASMCs and in the matrix as well, indicating secretory activity (Chemnitz and Collatz Christensen 1983), whereas fibrinogen/fibrin always was localized extracellularly.

Fibrin probably plays a role in atherogenesis. Evidence for the implication of fibrinogen and low density lipoproteins in the pathogenesis of atherosclerosis in the aorta has been presented (Smith et al. 1979). Ishida and Tanaka (1982) found that fibrin enhanced the growth of ASMCs in culture and fibrinogen degradation products inhibited such growth. Apart from fibrinogen/fibrin, other elements in the coagulatory mechanism may be im-

plicated in the reparatory response of arterial wall and in the atherogenesis. It is generally accepted that a key event in atherogenesis is proliferation of ASMCs (Ross and Glomset 1973). Plateletderived growth factor stimulates the proliferation of ASMCs in vitro (Ross et al. 1974), however, in vivo experiments are contradictory (Clowes and Karnovsky 1977).

This study has demonstrated that plasma fibrinogen is available to ASMCs following injury. Furthermore it is suggested that an ECM network formed by fibrin and fibronectin may be a substantial factor in ASMC migration and proliferation. It has been emphasized that fibrin may be involved in the progression of the early gelatinous lesion into the typical fibrous plaque (Smith 1982) and in addition human fibrous plaques contain an increased amount of fibronectin (Stenman et al. 1980). Our model mimics events that might occur during this progression and further investigations on the fate of fibrinogen/fibrin, fibronectin and fibrinolytic activity may be relevant.

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