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Repair in arterial tissue

Demonstration of fibronectin in the normal and healing rabbit thoracic aorta by the indirect immunoperoxidase technique

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Summary. Immunoperoxidase staining for fibronectin in ethanol- and formaldehyde-fixed, normal and healing arterial tissue is presented. Proteolytic digestion of sections fixed in formaldehyde increased the staining intensity for fibronectin in media from normal thoracic aorta and in neo-intima covered with endothelium, and had a similar effect on media from thoracic aortae with an embolectomy catheter lesion. The staining reaction for fibronectin in formaldehyde-fixed neo-intima covered with aortic smooth muscle cells was strong and almost independent of proteolytic digestion. In re-endothelialized neo-intima with a high content of proteoglycans a masking effect was observed, probably depending on poor access of immunoglobulins to fibronectin. The masking effect observed in non-re-endothelialized neo-intima with a low content of proteoglycans was much weaker. The correlation between the choice of fixative and unmasking of antigenic determinants by proteases is discussed.

Key words: Experimental arteriosclerosis – Fibronectin – Immunoperoxidase staining – Neointima

Introduction

Fibronectin is a high molecular weight multifunctional adhesive glycoprotein found on cell surfaces, in extracellular matrices (ECM), and in body fluids, e.g. as cold insoluble globulin in plasma (Ruoslahti 1981). Matrix fibronectin has a wide distribution, it is found associated with basement membranes, in the connective tissue matrix, and around individual cells, e.g. smooth muscle cells (Wartiovaara and Vaheri 1980; Hølund et al. 1981). Previous reports using the indirect immunofluorescence technique (Stenman and Vaheri 1978; Natali et al. 1981) have shown that fibronectin in the normal arterial wall is found to be associated consistently with both the inner and external aspects of the internal elastic membrane and that it is present around the smooth muscle cells.

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In inflammatory reactions fibronectin is deposited locally in the tissue (Kurkinen et al. 1980; Repesh et al. 1982; Hølund et al. 1982) suggesting that it may play a role in processes involving tissue damage and repair. The sequential morphological changes of neo-intima in the healing arterial wall following an embolectomy catheter lesion have previously been described (Helin et al. 1971; Collatz Christensen et al. 1979a and b; Chemnitz and Collatz Christensen 1981). Transmission electron microscopy (TEM) of neointima covered with endothelial cells (ECs) showed a well organized lamellated myolastic tissue with a decreased permeability to Evans blue. A complex lining layer of proteoglycans, elastin, microfibrils, anchoring fibers, and aortic smooth muscle cells (ASMCs) basal membrane was evident. Neo-intima covered with ASMCs showed disorganized connective tissue with reduced content of proteoglycans visible at the TEM level. No effective barrier against Evans blue developed within half a year. Stenman et al. (1980) have shown that the fibrous plaque of early human atherosclerotic lesions contains a large amount of fibronectin. The aim of the present immunohistochemical investigations was to examine the localization of fibronectin in normal and healing arterial tissue, especially in regenerating neo-intimal connective tissue. Furthermore the influence of fixation and tissue preparation on the demonstration of fibronectin by the indirect immunoperoxidase technique was examined. Difference in the staining intensity dependent on the ripeness of the ECM is discussed.

Materials and Methods

Animals and tissue preparation. Eight male albino rabbits of the Danish country strain each weighing about 3 kg were studied; 5 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 three animals served as controls. In general anesthesia by Nembutal® 4 animals (2 normal rabbits and 2, 21 days after the lesion) were perfusion-fixed with ethanol +1% acetic acid. The selected specimens were immersion-fixed in the same fixative for 4 h at 4° C. Three animals (1 normal rabbit and 2, 21 days after the lesion) were perfusion-fixed with 4% paraformaldehyde 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 the tissue was embedded in Paraplast® at 56° C. All sections (5 μm) were placed on ethanol-cleaned glasses. Unfixed specimens of one thoracic aorta, 21 days after the lesion, were immediately after removal frozen at −20° C. Frozen sections (5–10 μm) were cut and stained unfixed.

Selection of tissue for examination. The specimens were excised from the posterior aortic wall close to and between intercostal arteries (IA). The excision of specimens (previously described in details by Collatz Christensen et al. 1979a) made it possible to compare early re-endothelialized areas close to IA with later re-endothelialized or non-re-endothelialized areas between IA. Transitions between neo-intima covered by EC and ASMC were found in specimens excised close to IA. Thirty-five specimens were examined, 10 from normal thoracic aortae and 25 from thoracic aortae submitted to a single dilatation trauma (16 excised close to and 9 between IA).

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° in the following proteo-

Table 1. Semiquantitative evaluation of immunoperoxidase staining for fibronectin on paraffin embedded material fixed in the above mentioned fixatives at 4°C for 4 h. The number of animals in the control group and the group with embolectomy catheter lesion is indicated by brackets. *Scoring system, staining intensity: 0=none, +=slight, ++=moderate, and +++=strong*

	Non operated controls (3)		Embolectomy catheter lesion, 21 days survival (4)			
			Re-endothelialized lesion		Non-re-endothelialized lesion	
	Intima	Media	Neo-intima	Media	Neo-intima	Media
Ethanol+acetic acid	+++	++/+++	+++	+++	+++	+++
Formaldehyde:						
No enzymatic digestion	++/+++	0/+	+/++	0/+	+++	0/+
Trypsin	+++	+++	+++	+++	+++	+++
Pepsin	+++	+++	+++	+++	+++	+++

lytic solutions: 0.1% (w/v) trypsin (DIFCO 1:250) in 0.1% (w/v) $CaCl_2$ adjusted to pH 7.4 with 0.1 M NaOH and 0.4% (w/v) pepsin (SIGMA, P 7012) in 0.01 M HCl.

Antisera and control reagents. Goat antiserum against rabbit fibronectin was a gift from I. Clemmensen, Department of Clinical Chemistry, Hvidovre Hospital, Denmark. The antibody was used in various dilutions from 1:100 to 1:1,000. In addition, rabbit anti-sheep IgG, peroxidase conjugated (DAKO, P 163), was used in dilution 1:50. The control reagent (used in dilution 1:50) was the Ig-fraction of serum from non-immunized goats or PBS.

Immunoperoxidase staining. The indirect immunoperoxidase technique as previously described by Clausen and Thomsen (1978) was used. The staining procedure briefly comprises the following steps: 1) 0.1% periodic acid, 5 min, 2) PBS, pH 7.2, 3×5 min, 3) 3% (w/v) bovine serum albumin (BSA) (Sigma, St. Louis, MA, USA, B 4503) dissolved in PBS, 30 min, 37° C, 4) goat anti-rabbit fibronectin, variable dilution, 30 min, 37° C, 5) peroxidase-conjugated rabbit anti-sheep immunoglobulin, 1:50, 30 min, 37° C, 6) 0.06% (w/v) 3.3-diaminobenzidine-HCl (Sigma, St. Louis, MA, USA, D 8126), 0.03% (v/v) H_2O_2 in Tris buffer pH 7.6, room temperature, 7) counterstaining in Mayer's hemalun and mounting in Aquamount®. All antisera were diluted in PBS containing 3% BSA. When staining frozen sections step 1 was omitted. Control stainings were performed by replacing the primary antiserum (step 4) with either IgG-fraction from non-immunized goats dissolved in 3% BSA or PBS. Control stainings were negative.

Evaluation of immunoperoxidase staining (Table 1). The „positive" reaction product formed by the peroxidase reaction with diaminobenzidine (Graham and Karnovsky 1966) recognized as a brown to dark brown colour was semi-quantitated using the following scoring system. Staining intensity: 0: none, +: slight, ++: moderate, and +++: strong. The evaluation was performed as a blind study independently by the authors.

Acid glycosaminoglycan (AGAG) staining. Sections from specimens of normal thoracic aortae and aortae submitted to an embolectomy catheter lesion were stained for AGAGs with Alcian blue 8 GX, pH 1.0 and pH 3.0 (Pearse 1968). Alcian blue critical electrolyte concentration staining technique was also used (Scott et al. 1965) for a better differentiation of AGAGs.

Results

Light microscopy of the normal and healing arterial wall

Three control animals had macroscopically and microscopically normal aortae. Five experimental animals sacrificed 21 days after the lesion showed neo-intimal hyperplasia with cushion shaped thickenings in re-endothelialized areas close to ASMC covered neo-intima. Necroses occurred in the media, especially beneath early re-endothelialized neo-intima.

Light microscopic demonstration of AGAGs from 21 days survival after an embolectomy catheter lesion. The alcianophilia of re-endothelialized neo-intima was more intense than the staining of non-re-endothelialized neo-intima (Fig. 1a and b). Alcian blue staining using the critical electrolyte concentration technique showed a decreased alcianophilia between 0.8 M and 1.0 M $MgCl_2$. In media between the elastic membranes lacunae with an increased amount of AGAGs were observed.

Fibronectin in the normal thoracic aortae

Ethanol-fixed tissue. Staining for fibronectin was observed as dense deposits in the zone between ECs and the internal elastic lamina (IEL) (Fig. 2). Fibronectin in media was observed between elastic lamellae in the ECM around ASMCs. As seen in Table 1 the staining intensity was estimated as moderate and strong for the ethanol-fixed tissue.

Formaldehyde-fixed tissue without proteolytic digestion. Fibronectin was observed as dense deposits in the EC basement membrane zone similar to ethanol-fixed tissue. However, as shown in Table 1 the staining intensity of the media was minimal compared to ethanol-fixed tissue (Fig. 3).

Formaldehyde-fixed tissue treated with either trypsin or pepsin. Proteolytic digestion increased the staining intensity for fibronectin in media. The reaction product was located in the ECM between the elastic lamellae around the ASMCs (Fig. 4). No staining was observed in sections incubated in control serum (Fig. 5).

Figs. 1a, b. Re-endothelialized and non-re-endothelialized neo-intima, 21 days survival. Ethanol fixation. Alcian blue staining, pH 3. ×400. **a** EC-covered neo-intima. Intense alcianophilia around ASMCs. **b** Minimal alcianophilia in ASMC-covered neo-intima. *Asterisk* indicates original internal elastic membrane in **a, b**

Fig. 2. Normal rabbit thoracic aorta. Ethanol fixation. Positive reaction for fibronectin in the subendothelial layer and around ASMCs in the media. ×400

Fig. 3. Normal rabbit thoracic aorta. Formaldehyde fixation. No proteolytic treatment. Positive reaction in the subendothelial layer. Minimal reaction in media. ×400

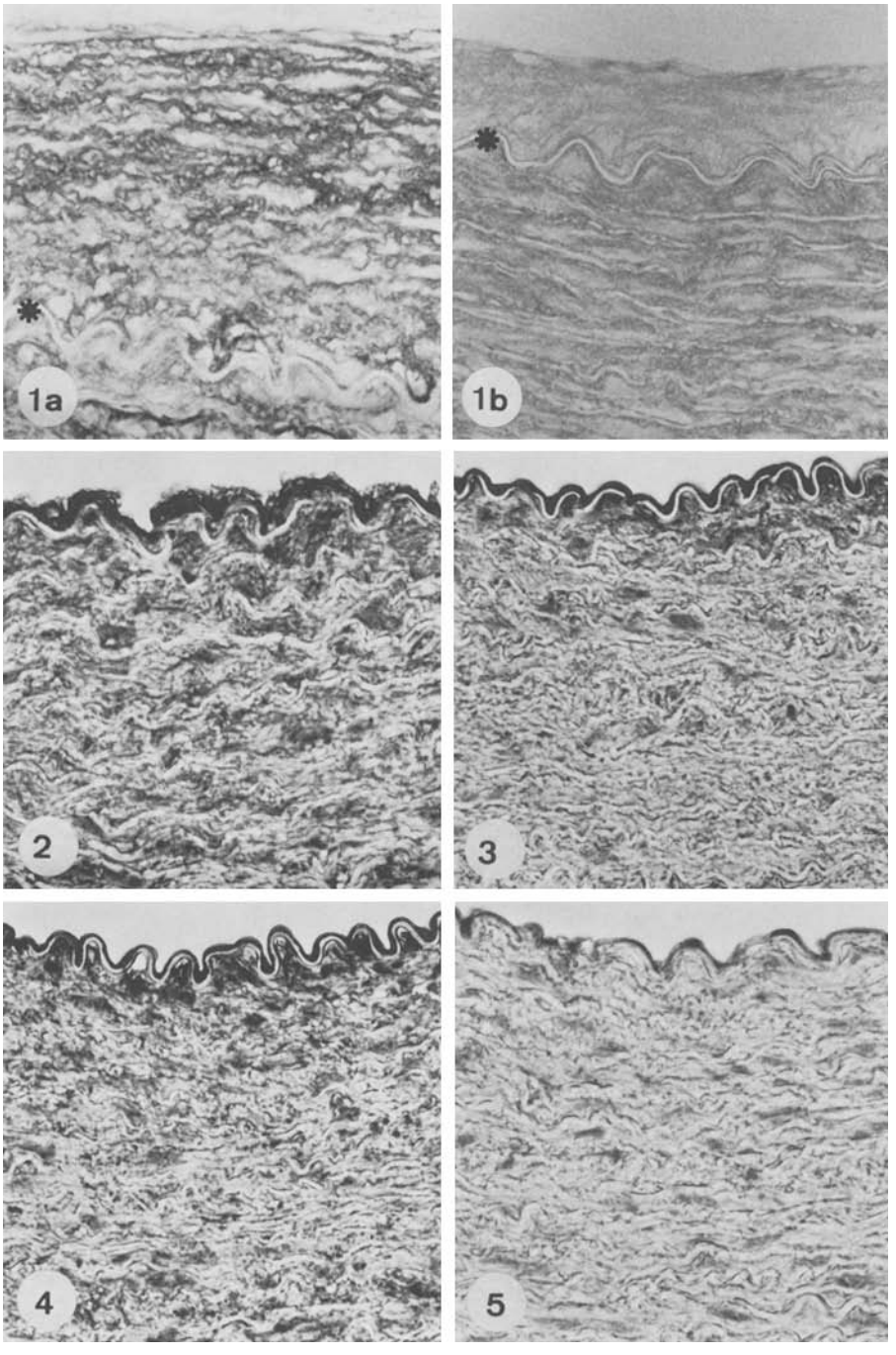
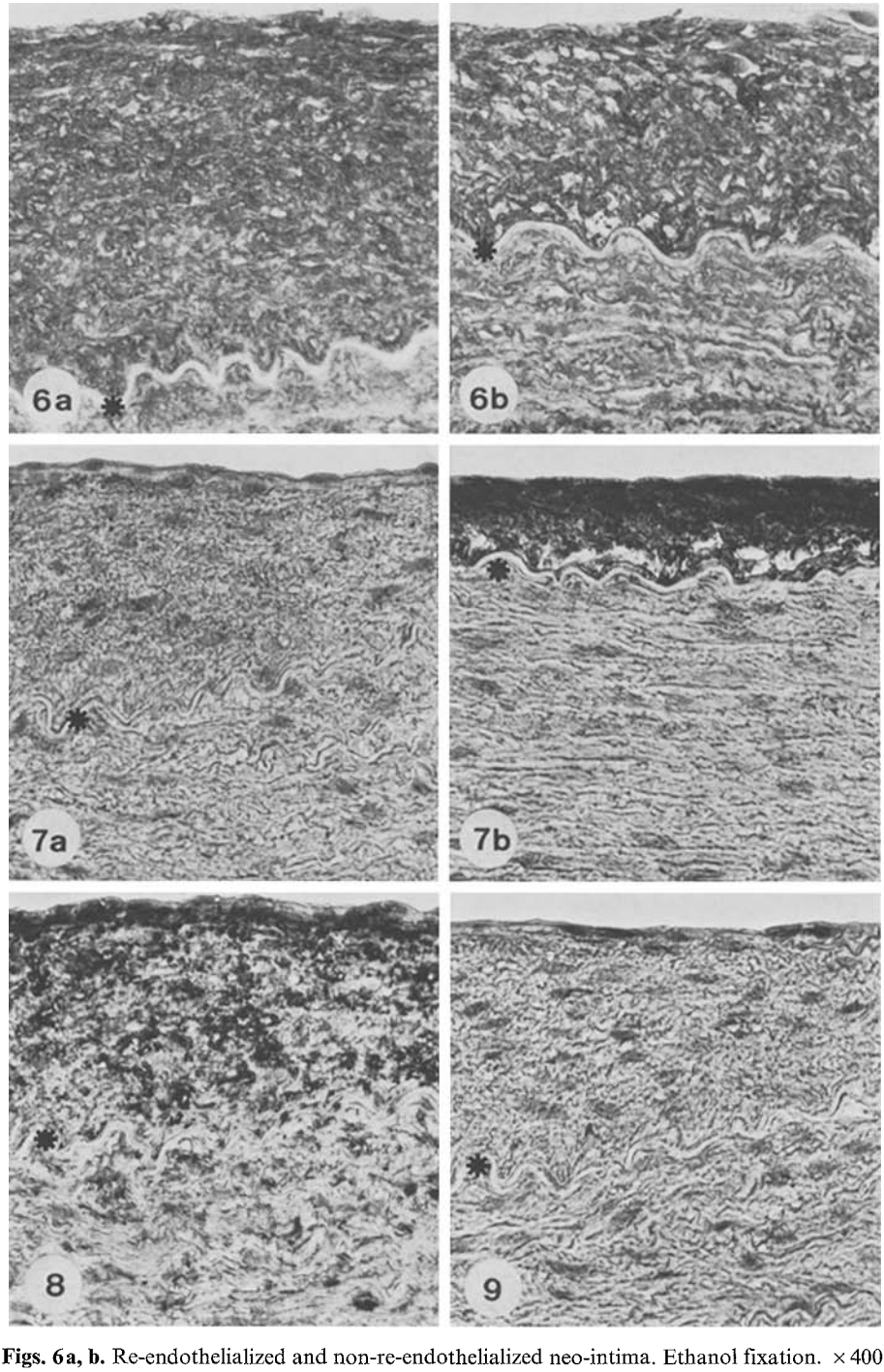


Fig. 4. Normal rabbit thoracic aorta. Formaldehyde fixation. Pepsin treatment. Positive reaction in the subendothelial layer. Positive reaction around ASMCs in the media. Comparison between Fig. 3 and 4 illustrates the unmasking effect of proteolytic treatment concerning the staining reaction for fibronectin in the media. × 400

Fig. 5. Normal rabbit thoracic aorta. Formaldehyde fixation. Pepsin treatment. Incubation with control serum. No staining reaction. × 400



Figs. 6a, b. Re-endothelialized and non-re-endothelialized neo-intima. Ethanol fixation. ×400. **a** EC-covered neo-intima strongly positive for fibronectin. **b** ASMC-covered neo-intima strongly positive for fibronectin

Fibronectin in the healing arterial wall

Fresh frozen and ethanol-fixed tissue. Table 1 summerizes the staining intensity in neo-intima and media beneath re-endothelialized and non-re-endothelialized lesions. The staining intensity for fibronectin was almost the same in neo-intima covered by ECs and ASMCs (Fig. 6a and b). Fibronectin was observed both intracellularly and extracellularly in neo-intimal tissue. In media a conspicuous observation was the presence of scattered areas with intense intracellular and extracellular staining. More exact localization of reaction product in terms of the components of the ECM was difficult, due to the lack of resolution.

Formaldehyde-fixed tissue without proteolytic digestion. There was a conspicuous dense reaction in ASMC-covered neo-intima, located in ASMCs and in the ECM, compared with EC-covered neo-intima, which showed a minimal staining intensity for fibronectin (Fig. 7a and b). In the media a faint reaction was visible in the laminar spaces, whereas necroses were heavily stained.

Formaldehyde-fixed tissue treated with trypsin or pepsin. Proteolytic digestion with trypsin or pepsin increased the staining intensity for fibronectin in re-endothelialized neo-intima and in the media. Reaction product was observed in ASMCs and in the ECM (Fig. 8). Intracellular staining was observed as small brown grains, in the ECM the staining occurred as threadlike and rodlike distinct bands parallel to elastic grains and membranes. In media fibronectin was observed in the ECM between the elastic lamellae. The positive staining reaction of areas of necrosis was only slightly increased, whereas the giant cells lying close to the necroses remained unstained. No staining was observed in sections incubated in control serum (Fig. 9).

Discussion

Re-endothelialization of neo-intimal hyperplasia, following an embolectomy catheter lesion, has previously been studied by TEM correlated with Evans

Figs. 7a, b. Re-endothelialized and non-re-endothelialized neo-intima. Formaldehyde fixation. No proteolytic treatment. ×400. **a** EC-covered neo-intima. Minimal reaction for fibronectin. **b** ASMC-covered neo-intima, strongly positive reaction for fibronectin

Fig. 8. EC-covered neo-intima. Formaldehyde fixation. Pepsin treatment. Strongly positive reaction for fibronectin in neo-intima. Comparison between Fig. 7a and 8 illustrates the unmasking effect of proteolytic treatment concerning the staining reaction for fibronectin in EC-covered neo-intima. ×400

Fig. 9. EC-covered neo-intima. Formaldehyde fixation. Pepsin treatment. Incubation with control serum. No staining reaction. ×400 *Asterisk* indicates original internal elastic membrane

blue vital staining (Collatz Christensen et al. 1979a and b). EC-covered neo-intima (21 days after lesion) showed a remodelled and more mature ECM than ASMC-covered neo-intima. A notable observation was the reduced amount of AGAGs (proteoglycans) in ASMC-covered neo-intima compared to EC-covered neo-intima (Chemnitz and Collatz Christensen 1981). Similar results have been shown by Richardson et al. (1980) in a morphometric study using ruthenium red stained sections.

The remarkable differences between morphology and histochemical composition of neo-intima covered with EC and ASMC were reaffirmed in the present work. Staining with Alcian blue (critical electrolyte concentration method) indicated a high content of AGAGs in EC-covered neo-intima. Immunohistochemical detection of fibronectin revealed a high content of fibronectin in all neo-intimal connective tissue. However, in EC-covered neo-intima fixed in formaldehyde, fibronectin immunoreactivity was weak, unless the tissue was pre-treated with proteolytic enzymes.

Recently Hølund et al. (1981), have shown that proteolytic digestion with pepsin of formaldehyde-fixed human gastro-intestinal tract prior to immunoperoxidase staining for fibronectin was identical to results obtained on unfixed frozen section.

The unmasking of antigenic determinants by trypsin or pepsin is still a subject of discussion. Formaldehyde fixatives immobilize the antigens by the formation of cross-links, resulting in some modification of the antigenic determinants of proteins (Brandtzaeg 1981), and/or forming a steric hindrance for the antibody to penetrate, especially when the actual antigen is mixed with a high concentration of other proteins (Brandtzaeg 1981).

The unmasking effect of enzymes observed in our investigation may depend on a proteolytic cleavage between fibronectin and other proteins. The hypothesis that proteoglycans mask the antigenic sites of fibronectin by steric hindrance, is supported by results presented by Weiss and Reddi (1981). They found that hyaluronidase treatment of cartilage rendered endogeneous fibronectin morphologically visible by immunofluorescence. Murray and Clup (1981) have presented evidence for masking of synthesized fibronectin and cold insoluble globulin in fibroblast cell-substratum adhesion sites in vitro, which made these sites unavailable for antibody binding. It was suggested that cross-links between fibronectin and multivalent heparan proteoglycans prevented antibodies from gaining access to the fibronectin.

The staining reaction in neo-intima covered with ASMCs is almost independent of enzymatic treatment. The reason may be free binding sites for anti-fibronectin in a poorly organized ECM with a decreased content of proteoglycans (Richardson et al. 1980). A possible explanation which has to be taken into consideration is that plasma fibronectin could have penetrated into the neo-intima due to a defect barrier function of ASMCs. A more speculative theory is that penetrated albumin interfers with the fibronectin-proteoglycan interactions, and so minimizes steric hindrance. Vital staining with Evans blue has shown an increased permeability of Evans blue-albumin complex in ASMC-covered neo-intima in contrast to EC-covered neo-intima (Collatz Christensen et al. 1977).

In ethanol-fixed tissue and in frozen sections of unfixed material the intensity of staining reaction was almost equal in EC-covered and ASMC-covered neo-intima. It may depend on the non-additive fixation which to some extent could minimize steric hindrance of immunoglobulin access to antigenic binding sites. Extraction of AGAGs before immunoreaction is not likely as the staining for AGAGs by Alcian blue at varied pH still is present in sections of ethanol-fixed tissue.

Evidence for masking of immunoglobulins access to fibronectin binding sites by proteoglycans in both ethanol- and formaldehyde-fixed tissue has been obtained from experiments using pre-treatment of the sections with hyaluronidase or chrondroitinase ABC (Chemnitz and Collatz Christensen, in preparation).

TEM examinations (Chemnitz and Collatz Christensen 1981) have shown a minimal content of AGAGs in ASMC-covered neo-intima and the failure to form a remodelled mature ECM could possibly implicate a defect in knitting together by proteoglycans the other connective tissue components. It has been proposed that proteoglycan-induced self-assembly of insoluble collagen-fibronectin-proteoglycan complexes could, together with fibril formation, be of basic importance for the formation of ECM (Oldberg and Ruoslahti 1982).

In conclusion we have demonstrated fibronection in normal and healing arterial tissue and found that immunoperoxidase staining depends on fixation and tissue preparation. The present and previous investigations allow us to suggest that the coincidental occurrence in the healing aortic wall of EC, increasing barrier function, high contents of proteoglycans and of fibronectin, are prerequisites for the reconstruction of ECM with intact elastogenesis. In ASMC-covered neo-intima the coincidental absence of EC and proteoglycans expresses a failing regulation of ASMC secretory mechanism and/or ECM coupling of matrix components.

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