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Repair in arterial tissue 2 years after a severe single dilatation injury: the regenerative capacity of the rabbit aortic wall

The importance of endothelium and of the state of subendothelial connective tissue to reconstitution of the intimal barrier

John Chemnitz and Bent Collatz Christensen

Institute of Medical Biology, Department of Anatomy and Cytology, Odense University, Campusvej 55, DK-5230 Odense M, Denmark

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Summary. The thoracic aortae from 11 rabbits that survived a single severe dilatation injury for 2 years were studied by vital staining with Evans blue, immunohistochemistry and transmission electron microscopy. Our results have shown almost total restitution of the thoracic aorta. Six of the 11 rabbits submitted to an injury had no blue-stained areas, indicating total reendothelialization. Five rabbits had a few blue areas often on the ventral side of the aorta. The reendothelialization from the first to the seventh pair of intercostal arteries ranged from 82% to 100%. There was intimal thickening inside the original internal elastic lamina in both white and blue areas. All blue areas had a surface composed of smooth muscle cells. Reendothelialized areas consisted of mature endothelium, reticular basal membrane, layered smooth muscle cells and an extracellular matrix consisting of pre-elastin, elastin, collagen and proteoglycans. An effective barrier had apparently been formed against penetration of macromolecules, judged from the absence of fibrinogen/fibrin and unmasked fibronectin. Intimal thickenings without endothelial cover were covered with smooth muscle cells without intercellular junctions. Our results indicate that an extracellular matrix of fibrin and fibronectin plays a role in forming an intimal thickening, and it is suggested that proteoglycans may modulate the biological role of the extracellular matrix in the healing process.

Key words: Experimental arteriosclerosis – Fibrin – Fibronectin – Intimal barrier – Proteoglycans

Introduction

Intimal thickening based on proliferation of and connective tissue formation by smooth muscle cells (SMCs) is a common and non-specific response of arterial tissue to a single dilatation injury (Ross and Glomset 1977; Collatz Christensen et al. 1979a, b). Collatz Christensen and Chemnitz (1983) have suggested that endothelial cells (ECs) alone do not form an effective barrier in the absence of differentiated subendothelial connective tissue, as a result of a pilot study on 2-year-old dilatation injury. This is significant since according to the myogenic theory of atherosclerosis (Ross and Glomset 1973) the role of ECs is to act as a barrier to lipoproteins and factors present in the blood which stimulate proliferation of SMCs.

The object of our long-term study was to examine the reendothelialization of the thoracic aorta, the differentiation of reendothelialized connective tissue and to evaluate the importance of ECs and/or the state of connective tissue in the barrier function, for 2 years after injury. Our observations showed an almost total reendothelialization and restitution of the thoracic aortic wall. Immunohistochemical tracing of the distribution of fibrinogen/fibrin in intimal thickenings covered by either ECs or SMCs supported the assumption that the state of the luminal connective tissue has an important role in the intimal barrier function.

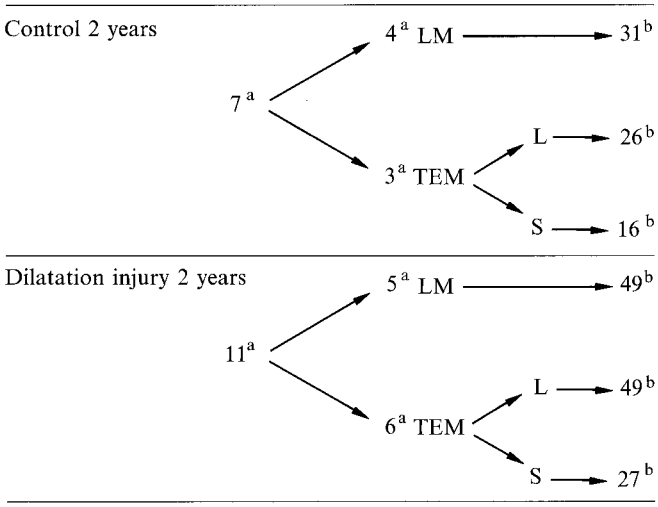
In the healing arterial wall, the state of neointimal connective tissue seems to be a major determinant. In a comparative study (Rasmussen et al. 1989) we concluded that the femoral artery quickly formed an effective barrier against penetration of fibrinogen with a well-organized intimal thickening. Thrombosis and occlusion were not seen. In contrast, the healing response of the carotid artery was retarded, the neointimal connective tissue seemed poorly organized, no effective barrier was established against fibrinogen/fibrin, and thrombosis, occlusion and destruction of the arterial wall resulted.

Materials and methods

Eighteen male albino rabbits of the Danish country strain, 2–3 months of age and each weighing 2.5 kg, were used (Table 1). Under general anaesthesia 11 rabbits were submitted to a single dilatation injury. An embolectomy catheter (12-080-5F) was advanced to the aortic arch through a left femoral artery. The balloon

Offprint requests to: J. Chemnitz

Table 1. Number of animals and specimens



LM, Light microscopy; TEM, Transmission electron microscopy; L, Low-temperature embedding; S, Standard embedding

[a] number of animals
[b] number of specimens

of the catheter was inflated with 3 ml air, and the catheter violently withdrawn till it was arrested by the resistance offered by the diaphragm. Following deflation of the balloon and withdrawal of the catheter, the femoral artery was ligated and the skin incision closed (Helin et al. 1971; Collatz Christensen and Garbarsch 1973). Seven rabbits served as controls. Two years after the lesion all rabbits were vital-stained with Evans blue, perfusion-fixed with 4% formaldehyde [light microscopical (LM) and immunohistochemical investigations] and with 2.5% glutaraldehyde [transmission electron microscopical investigations (TEM)] for 30 min at 4° C. The fixatives were dissolved in phosphate-buffered saline (PBS), pH 7.2; fixative vehicle: 300 mosmol.

Evans blue (T-1824, Merck, Darmstadt, FRG), 0.5% (w/v) dissolved in physiological saline, was injected slowly into an ear vein 1 h before anaesthesia and perfusion fixation in a dosage of 3 ml/kg body weight. The aorta was removed atraumatically; it was cleaned of peri-adventitial tissue, cut open on the ventral side, pinned on a plate of dental wax, examined for macroscopical changes, and finally photographed. Planimetry was carried out by measuring the extent of unstained and stained areas from Evans blue in percentage of the total area between the first and seventh intercostal artery (IA).

Specimens for LM and TEM were generally excised from the thoracic aorta close to the IAs and between IAs. The number of animals and specimens excised from unoperated and operated animals is shown in Table 1.

The specimens excised for immunohistochemical examinations were immersion-fixed in 4% formaldehyde in PBS for 4–6 h at 4° C, rinsed overnight in PBS at 4° C, dehydrated in ethanol and xylene, and embedded in Paraplast at 56° C. All sections (5 μm) were placed on ethanol-cleaned glass slides. Goat anti-rabbit fibronectin (Cappel, Malvern, PA, USA) in dilutions of 1:200 or 1:400 (50 or 25 μg/l) and sheep anti-rabbit fibrinogen (Capple) in dilutions of 1:200 and 1:400 (100 and 50 μg/l) were used for incubation of trypsinized and untrypsinized sections (Chemnitz and Collatz Christensen 1983a). The incubations were carried out at 37° C for 30–60 min, followed by incubation with peroxidase-conjugated rabbit anti-goat IgG (Dakopatts, Copenhagen, Denmark) and stained with diaminobenzidine. The sections were dehydrated and mounted with Eukitt (O Kindler GmbH, Freiburg, FRG). Control sections were incubated with the IgG fraction from non-immunized goats.

The specimens excised for TEM were immersion-fixed in 2.5% glutaraldehyde for 24 h at 4° C. The specimens were processed for standard TEM embedding and low-temperature embedding. For standard embedding the specimens were post-fixed in 1% osmium tetroxide, pH 7.2 for 2 h at 4° C, pre-stained with 1% (w/v) uranyl acetate in re-destilled water for 1 h. The specimens were then dehydrated in ethanol and embedded in Araldite. Ultrathin sections were contrasted with lead citrate. For low-temperature embedding the specimens were dehydrated to 90% in a graded series of ethanol in water at −20° C, embedded in Lowicryl resin K4M at −35° C and polymerized overnight by indirect ultraviolet irradiation at −35° C (Chemnitz and Collatz Christensen 1983b).

Results

After vital staining with Evans blue the 7 control animals had macroscopically and microscopically normal aortae; the intimal surface was unstained (white) after exposure to Evans blue (Fig. 1a). Only 1 of the 11 rabbits submitted to a dilatation injury had severe macroscopical arteriosclerotic changes in the thoracic aorta, consisting of a thin wall, bean shaped bulges and transverse folds.

In the experimental group the intimal surface of the thoracic aorta from 6 rabbits was unstained (Fig. 1b), whereas 5 showed blue intimal areas often on the ventral side of the thoracic aorta (Fig. 1c). The percentage of unstained intimal surface from the first to the seventh pair of IAs was calculated. A mean of 92% (range 82–100%) of unstained surface was found.

On light microscopy the control group showed a normal intima; ECs were close to the internal elastic lamina (IEL) or focally separated from it by a narrow space. In the experimental group the thickness of the neointimal hyperplasia was increased in reendothelialized areas and was thickest in a zone bordering SMC-covered neointima. In these non-reendothelialized areas the inti-

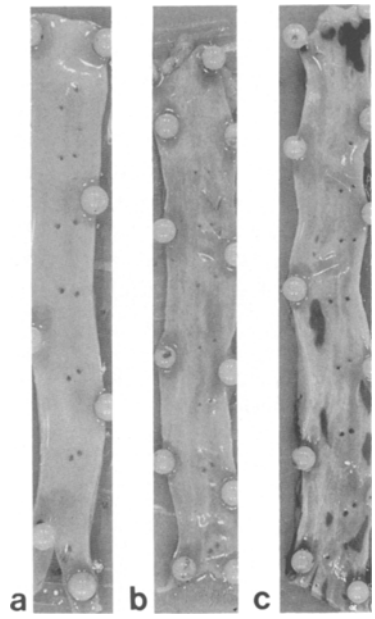


Fig. 1a–c. Rabbit thoracic aorta, opened longitudinally to show the staining with Evans blue. Control animal (**a**) and 2 years after injury (**b, c**). Unstained intimal surface indicates total reendothelialization (**b**) and small stained intimal areas indicate incomplete reendothelialization (**c**)

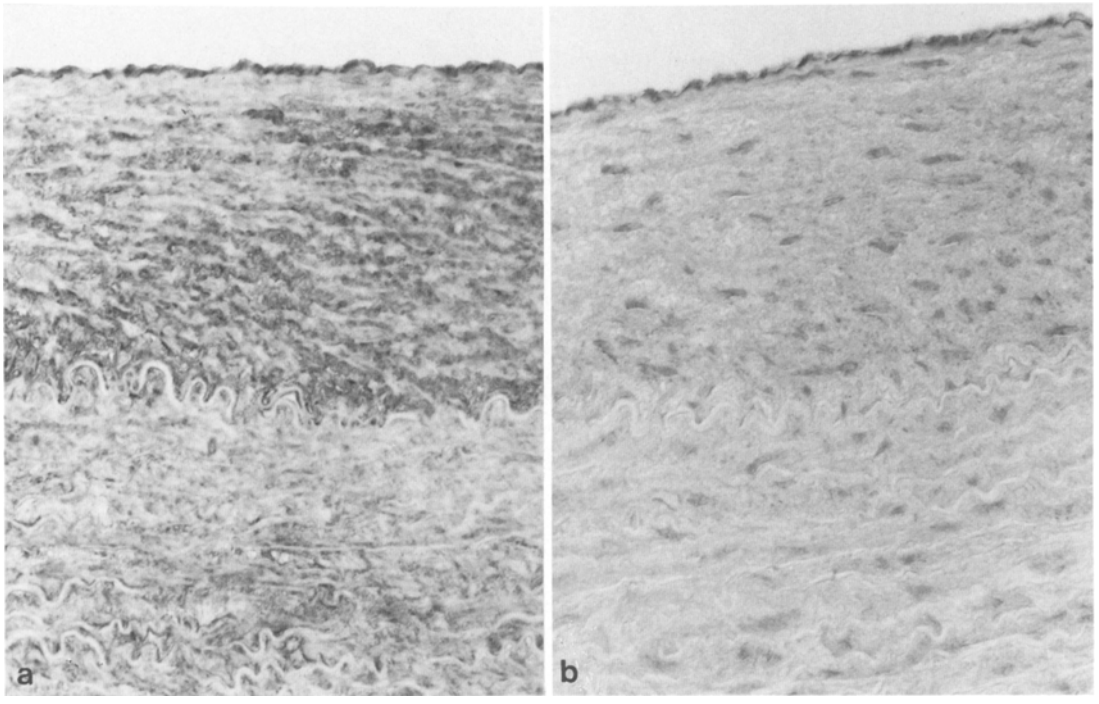


Fig. 2a, b. Reendothelialized intimal thickenings. Formaldehyde fixation. Immunoperoxidase staining for fibronectin. ×400. After proteolytic treatment a strong positive staining reaction is seen in the intimal thickening and in the media (**a**). Without proteolytic treatment the reaction is minimal (**b**)

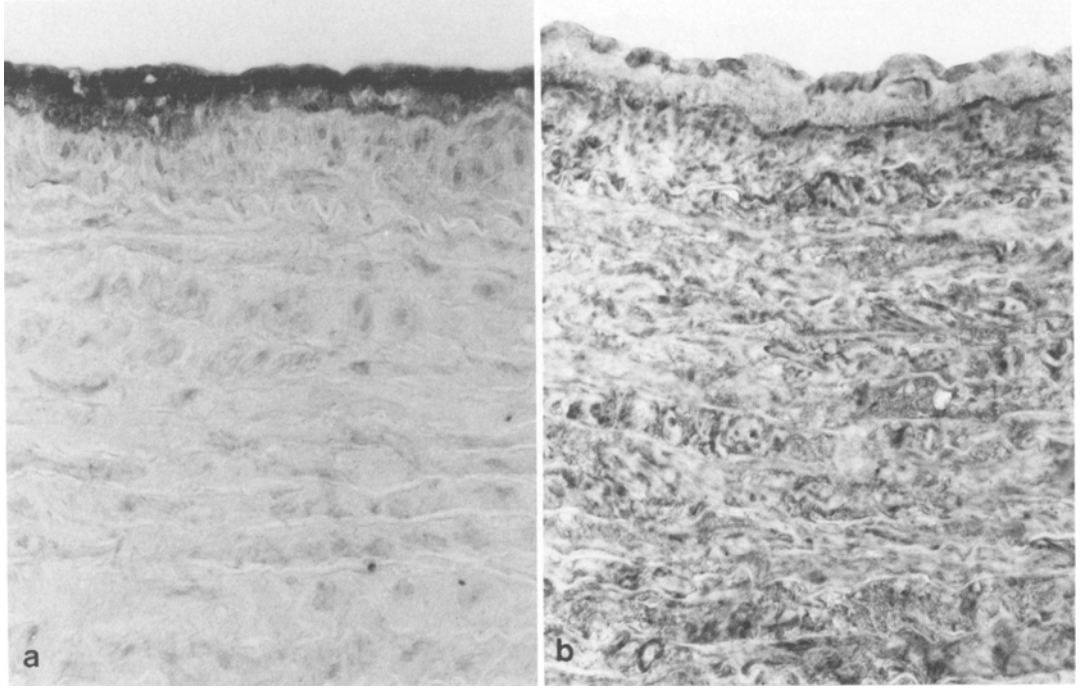


Fig. 3a, b. Non-reendothelialized intimal thickenings. Formaldehyde fixation. Immunoperoxidase staining for fibronectin. ×400. Without proteolytic treatment fibronectin is seen in the luminal zone of the intimal thickening (**a**). Proteolytic treatment increased the staining reaction in the well-organized zone of the intimal thickening and in the media (**b**)

mal thickening generally was thinner and looser in texture (compare Figs. 2, 3, 4 and 5).

Immunohistochemically, proteolytic digestion increased the staining intensity for fibronectin in the normal intima and media compared to staining of tissue without treatment. The reaction product was located in the subendothelial connective tissue, and in the extracellular matrix (ECM) between the elastic laminae around the SMCs. No staining was observed in sections incubated with IgG fractions from non-immunized goats or

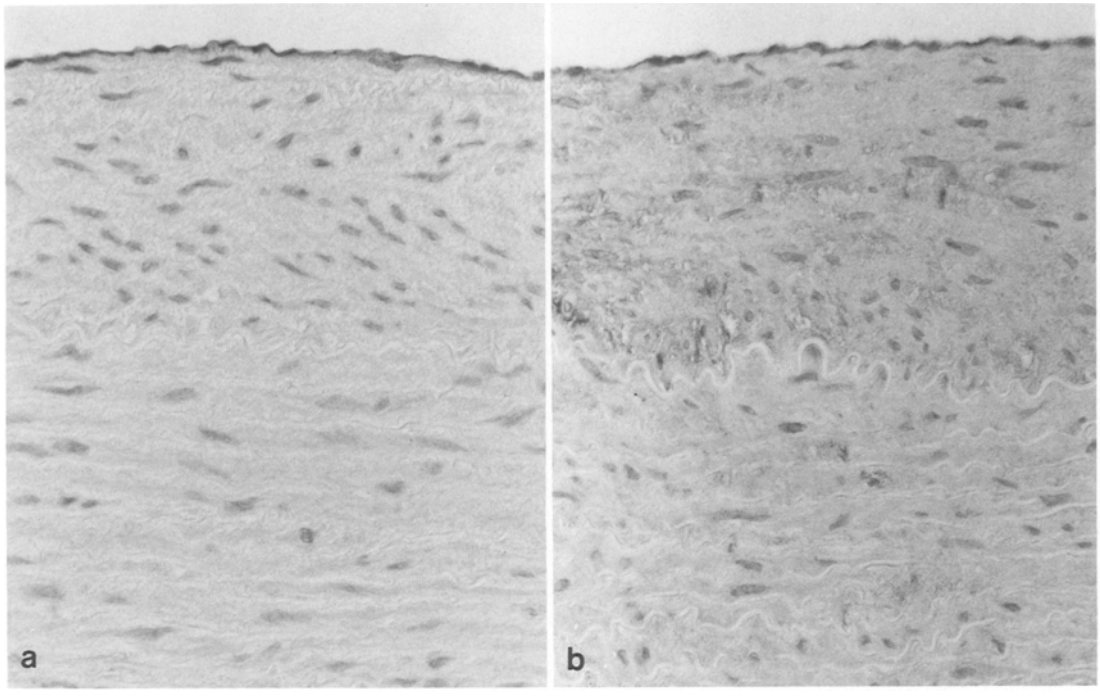


Fig. 4a, b. Reendothelialized intimal thickenings. Formaldehyde fixation. Immunoperoxidase staining for fibrinogen/fibrin. ×400. Without proteolytic treatment no staining is observed in the intimal thickening and in media (**a**). With proteolytic treatment a weak staining in the intimal thickening is seen. No staining is seen in the media (**b**)

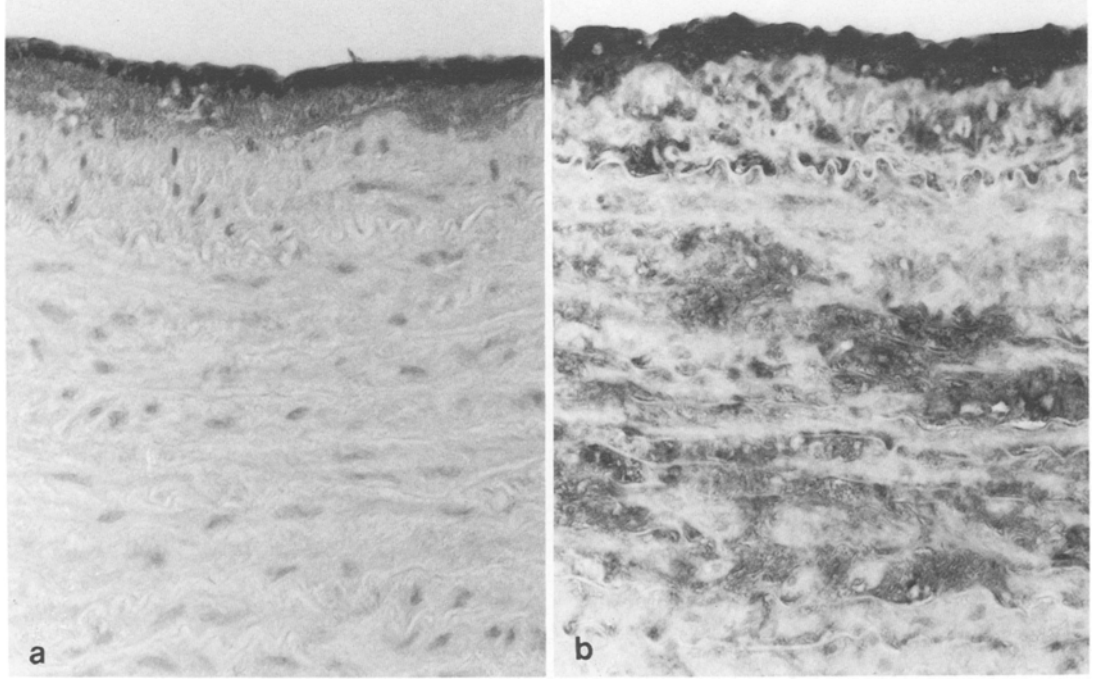


Fig. 5a, b. Non-reendothelialized intimal thickenings. Formaldehyde fixation. Immunoperoxidase staining for fibrinogen/fibrin. ×400. Without proteolytic treatment a conspicuous observation is a heavy staining of the luminal zone of the intimal thickening (**a**) With proteolytic treatment fibrinogen/fibrin is found in the basal zone of the intimal thickening and in the media (**b**)

when the primary antibody was replaced by PBS. No staining reaction for fibrinogen/fibrin was observed in normal intima and media except in sporadically occurring subendothelial areas, where a positive reaction was present.

In the experimental animals the reendothelialized intimal thickenings found 2 years after injury showed increased staining intensity for fibronectin after proteolytic digestion when compared with untreated sections (Fig. 2a, b). The reaction product was observed in SMCs

and in the ECM. Intracellular staining was seen as small grains; in the ECM the staining as rod-like bands parallel to elastic grains and membranes. In media beneath reendothelialized areas proteolytic treatment increased the positive staining reaction for fibronectin which was observed in ECM between the elastic laminae.

Non-reendothelialized intimal thickenings (covered by SMCs) were divided in two distinct zones microscopically; a luminal zone, loose and poorly organized, and a basal zone close to IEL, which showed a more compact arrangement of the connective tissue. The difference in the state of the two zones was reflected in immunohistochemical observations. Without proteolytic treatment a heavy staining for fibronectin was found in the luminal zone in contrast to a weaker staining in the basal and more organized zone (Fig. 3a). After proteolytic treatment faint staining for fibronectin was seen in the luminal zone, while in the basal zone an intense staining was observed (Fig. 3b). In media beneath non-reendothelialized areas the staining reaction for fibronectin was similar to that observed in reendothelialized areas.

In reendothelialized intimal thickenings without proteolytic treatment no staining reaction was observed for fibrinogen/fibrin except at the surface of the endothelium (Fig. 4a). After proteolytic treatment the staining reaction was generally weak. No reaction for fibrinogen/fibrin was observed in media (Fig. 4b).

In non-reendothelialized intimal thickenings without proteolytic treatment the poorly organized luminal zone

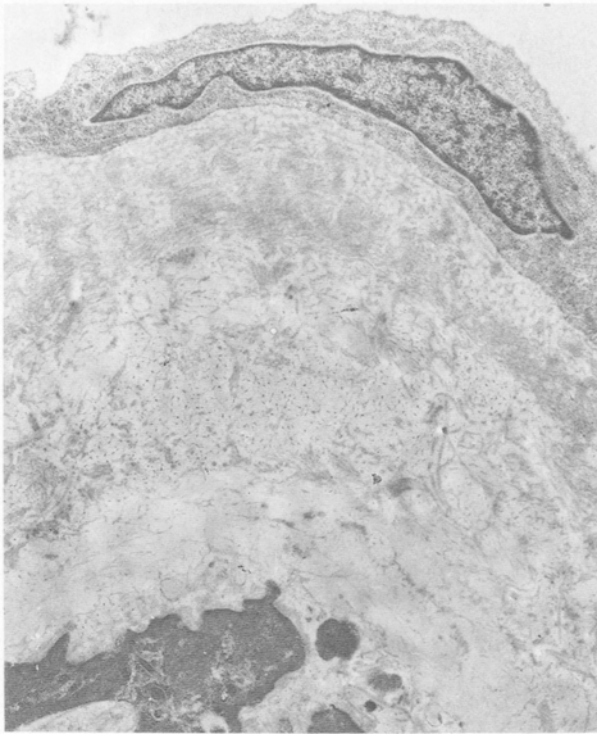


Fig. 6. Intima of a normal thoracic aorta. Low-temperature embedding. In the thickened subendothelial zone between the endothelial cells and the internal elastic lamina proteoglycans occupied a great proportion of the extracellular matrix. × 7000

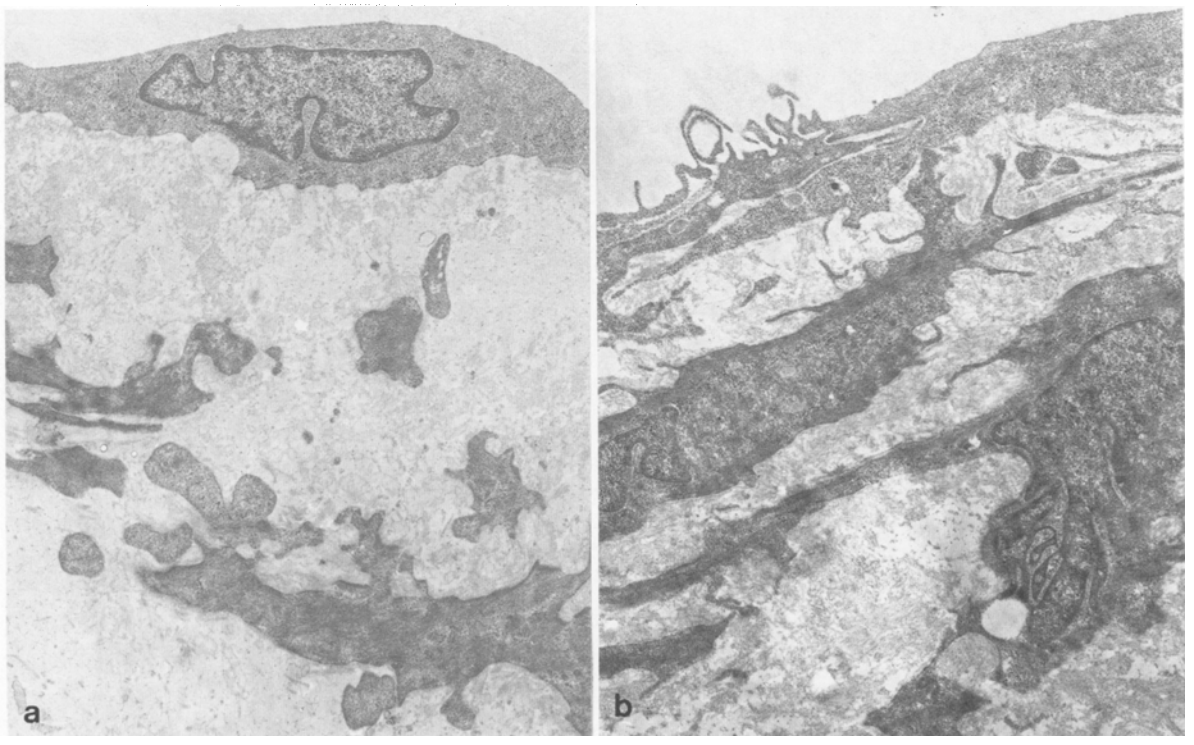


Fig. 7a, b. Intimal thickenings 2 years after a dilatation injury. Low-temperature embedding. × 7000. Reendothelialized intimal thickening consists of a multilayered myo-elastic tissue. Microfibrils, elastic grains, collagen, and proteoglycans are seen in the extracellular matrix (ECM) (**a**). Non-reendothelialized intimal thickening covered by smooth muscle cells shows a loose texture of ECM which consists of microfibrillar substance with a low concentration of proteoglycans and elastin (**b**)

showed an intense diffuse staining reaction for fibrinogen/fibrin. Usually no staining was observed in the basal zone (Fig. 5a). After proteolytic treatment the basal zone showed staining for fibrinogen/fibrin. In media, areas devoid of SMCs, judged from the absence of nuclear staining, were visible as lacunae with positive staining reaction (Fig. 5b).

TEM of control thoracic aorta showed an endothelium tightly apposed to the IEL. The narrow zone between the IEL and the abluminal surface of the ECs contained microfibrillar substance without the presence of TEM-visible large proteoglycans. Areas with endothelium separated from the IEL were also observed. By TEM these areas consisted of microfibrils, collagen, elastin, proteoglycans and frequently SMCs (Fig. 6). Media contained branched SMCs of the contractile phenotype between the elastic laminae. The ECM around SMCs was formed by microfibrils, pre-elastin, elastin, collagen and lacunae with proteoglycans.

Two years after the dilatation injury the thickened reendothelialized neointima consisted of multilayered myoelastic tissue. By TEM, the ECM between the SMCs contained microfibrils, elastic grains and large amounts of proteoglycans forming a lattice joining together the components of ECM (Fig. 7a). All the non-reendothelialized intimal areas, vital stained with Evans blue, were covered by SMCs. A loose texture of the ECM components was observed. In the superficial layers almost no proteoglycans were found, and all components of the ECM were randomly mixed (Fig. 7b). Bundles of collagen microfibrils and microfibrillar substance were conspicuous and the few elastic components appeared as coarse smooth grains. In the deeper layers a more tightly organized ECM with proteoglycans could be observed.

In media beneath both re- and non-reendothelialized areas the composition and spatial arrangement of the ECM between SMCs were almost similar to that observed in the media of the normal thoracic aortae.

Discussion

The extent of healing of the arterial tissue 2 years after a severe single dilatation injury was remarkable. Reendothelialization, as described in previous studies (Collatz Christensen et al. 1977, 1979a, b) was rapid during the first weeks then slowed and was not completed after 240 days. The present study has evidenced that a total reendothelialization is possible after 2 years. The structure of ECs and differentiation of connective tissue in reendothelialized intimal thickenings seemed identical 2 months (Collatz Christensen et al. 1977, 1979a, b) and 2 years after the dilatation injury. The significant source of the proliferation and migration of ECs is the surviving endothelium in and around the intercostal orifices (Collatz Christensen et al. 1977, 1979a, b). A location of stained, blue areas on the ventral aortic wall 2 years after injury is in accordance with the assumption that the ventral side opposite the IAs represents the areas last reendothelialized from the intercostal orifices.

Other workers (Poole et al. 1958; Minick et al. 1977; Reidy et al. 1982) have shown that rabbit ECs did not completely repopulate a denuded aorta. The discrepancy between their data and ours may be explained by our longer observation time, variation in severity of the injury and/or by variation in the sources for new endothelial outgrowth from intercostal orifices and small areas of endothelium which have survived the injury. Reidy et al. (1983) showed that the rat and rabbit carotid artery (with no side branches) did not completely reendothelialize after denudation; by 12 weeks a large zone remained devoid of endothelium.

Fibronectin is a genuine ECM component of the arterial wall (Stenman and Vaheri 1978; Natali et al. 1981; Chemnitz and Collatz Christensen 1983a; Jensen et al. 1983; Voss and Rauterberg 1986; Shekhonin et al. 1987; Rasmussen et al. 1989). The dilatation injury from the embolectomy catheter produces a lesion of both intima and media, implying de-endothelialization, and stretching of the media. Positive immunoreaction for fibronectin may depend on penetration into the arterial wall of plasma fibronectin, or it may come from secretion of matrix fibronectin from SMCs in the media and in the intimal thickening, engaged in an intense inflammatory and reparatory process. Fibronectin is present in human aortic intima and atherosclerotic lesions (Smith and Ashall 1986; Shekhonin et al. 1987). Our own studies and those of others have demonstrated increases in glycosaminoglycans in rabbit reendothelialized intimas when compared with non-reendothelialized intima (Minick et al. 1977; Collatz Christensen et al. 1979b; Richardson et al. 1980; Chemnitz and Collatz Christensen 1983b; Wight et al. 1983). We have previously presented evidence for masking of immunoglobulin access to fibronectin binding sites by proteoglycans in formaldehyde-fixed tissue and demonstrated the unmasking effect of enzymes (Chemnitz and Collatz Christensen 1983a). Other workers (Weiss and Reddi 1980, 1981; Murray and Culp 1981; Harrison et al. 1984) have also reported masking of fibronectin.

It has been suggested that the interactions between fibronectin and proteoglycans may have a biological role during tissue differentiation (Weiss and Reddi 1980, 1981; Harrison et al. 1984). Lewandowska et al. (1987) have demonstrated that the binding of dermatan sulphate proteoglycans to the fibronectin molecules had a marked influence on the adhesive responses of fibroblasts to select extracellular matrices. The unmasking of fibronectin by proteolytic enzymes in reendothelialized intimal thickenings has led to the assumption that fibronectin is or has become a matrix fibronectin embedded in the ECM, especially in proteoglycans. Similar results have been found in intimal thickenings 21 days after the lesion (Chemnitz and Collatz Christensen 1983a). The weak or faint staining observed after 2 years without proteolytic treatment for both fibronectin and fibrinogen/fibrin or their degradation products support the assumption that no obvious penetration of plasma fibronectin and fibrinogen into the reendothelialized intimal thickening has occurred. The unmasking of fibrinogen/fibrin and their degradation products in the reen-

dothelialized intimal thickenings may represent previously penetrated plasma fibrinogen now trapped, embedded in the organized ECM.

The difference in the organization of the intimal thickenings in non-reendothelialized areas compared with areas covered with ECs reflects the poor barrier function of the superficial layers with little proteoglycan, in contrast to the deeper layers with a higher amount of proteoglycans and a better organization of the ECM. The intense staining for fibrinogen/fibrin without proteolytic treatment (unmasked form) in the luminal layer of non-reendothelialized intimal thickenings and the observed masking in the deeper layers support this assumption.

After dilatation injury the deendothelialized subendothelium is thrombogenic. The interaction of blood components with the injured aorta is associated with reendothelialization and neointimal formation. We have previously demonstrated that plasma fibrinogen is available to SMCs following injury and furthermore have suggested that fibrin and fibronectin may be factors in SMC migration and proliferation (Chemnitz and Collatz Christensen 1984). In vitro experiments on the effects of fibrinogen and fibrin on the migration of SMCs suggest that this plasma protein has a role in the pathogenesis of intimal thickening, atherosclerosis and organization of thrombus (Ishida and Tanaka 1982; Naito et al. 1990). Work by Fischer and Christensen (1988) supports the role of fibrinogen/fibrin in the neointimal formation in human small-sized arteries. In normal and hypertensive patients they analysed the intimal deposition of albumin, fibrinogen, IgG, IgM, and beta-lipoprotein by immunoperoxidase staining. Their data indicate that fibrinogen was the most reliable marker of intimal thickening in small-sized human arteries. It has also been emphasized that fibrin may be involved in the progression of the early gelatinous lesion into the typical fibrous plaque (Smith 1982).

The relevance of our experimental model with regard to human arteriosclerosis has been confirmed by Lorenzen (1963) describing similarities in human and experimental arteriosclerosis depending on unspecific injury and repair processes in arterial connective tissue. It is generally accepted that a key event in atherogenesis is proliferation of SMCs (Ross and Glomset 1976). The factor or factors that initiate and sustain this proliferation have not yet been fully explored. The infiltration of plasma fibrinogen into the intima and subsequent transduction of fibrinogen to fibrin may initiate intimal thickening.

Interactions between proteoglycans and fibrous proteins may be of importance for ECM organization, which affects the permeability of blood components across the arterial wall. Our data on the increasing masking of fibronectin and/or fibrinogen/fibrin by proteoglycans may reflect a modulation of the biological activity of the ECM network in the healing process.

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