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# Injury and repair of smaller muscular and elastic arteries

## Immunohistochemical demonstration of fibronectin and fibrinogen/fibrin and their degradation products in rabbit femoral and common carotid arteries following a dilatation injury

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**Summary.** Indirect immunoperoxidase staining for fibrinogen/fibrin and fibronectin was performed on normal and healing arterial tissue of muscular and smaller elastic arteries. Fibronectin was observed in the wall of the normal arteries, whereas fibrinogen/fibrin could not be demonstrated. Fibronectin was observed in the intima as well as the media deposited in a similar fashion in the femoral and carotid artery during repair. Apart from the early occurrence of fibrin/fibrinogen in the media of both arteries the distribution of fibrinogen/fibrin and degradation products differed. In the femoral artery a progressively weakening positive reaction for fibrinogen/fibrin and degradation products towards the lumen was observed in the intima and the media 7 and 14 days after the lesion. By 28 days the reaction in the media was negative. No thrombus formation was observed. In contrast, all the specimens examined from the common carotid arteries were obliterated by luminal thrombi 28 days after the lesion. The thrombus as well as the damaged intimal thickening and the compressed media were loaded with fibrinogen/fibrin and degradation products. The deposition of fibronectin, fibrinogen, and degradation products in the carotid artery was similar to that previously reported in experimental aortic arteriosclerosis in rabbits as well as in giant cell arteritis.

**Key words:** Common carotid artery – Experimental arteriosclerosis – Femoral artery – Fibrin – Fibronectin

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### Introduction

Injury of blood vessels is followed by repair processes in which proliferation of smooth muscle cells (SMCs) and accumulation of extracellular matrix (ECM) components are central events (Lorenzen 1963; Garbarsch 1975; Ross and Glomset 1977). This has been shown repetitively in experimental models. Immediately after the lesion fibrinogen/fibrin is observed in the vessel wall (Chemnitz and Collatz Christensen 1984) followed by accumulation of fibronectin extracellularly as shown in experimental models on rabbit aorta (Chemnitz and Collatz Christensen 1983; Jensen et al. 1983). These substances are also observed in injured human muscular vessels, for example the temporal arteries in giant cell arteritis (Chemnitz et al. 1987).

Fibronectin is a high molecular weight multifunctional adhesive glycoprotein found on cell surfaces, in ECM, and in body fluids (Ruoslahti 1981). Matrix fibronectin has a wide distribution. It is associated with basement membranes and the connective tissue matrix around individual cells including those in the normal aorta (Stenman and Vaheri 1978; Natali et al. 1981; Chemnitz and Collatz Christensen 1983; Jensen et al. 1983). Fibronectin is believed to take part in the formation of the ECM of an atherosclerotic plaque (Stenman et al. 1980).

Fibrinogen has not been demonstrated immunohistochemically in the normal human or rabbit aortic wall (Haust 1971; Chemnitz and Collatz Christensen 1984). However, immunoelectrophoresis has shown the presence of fibrinogen in the normal intima, while an intact internal elastic

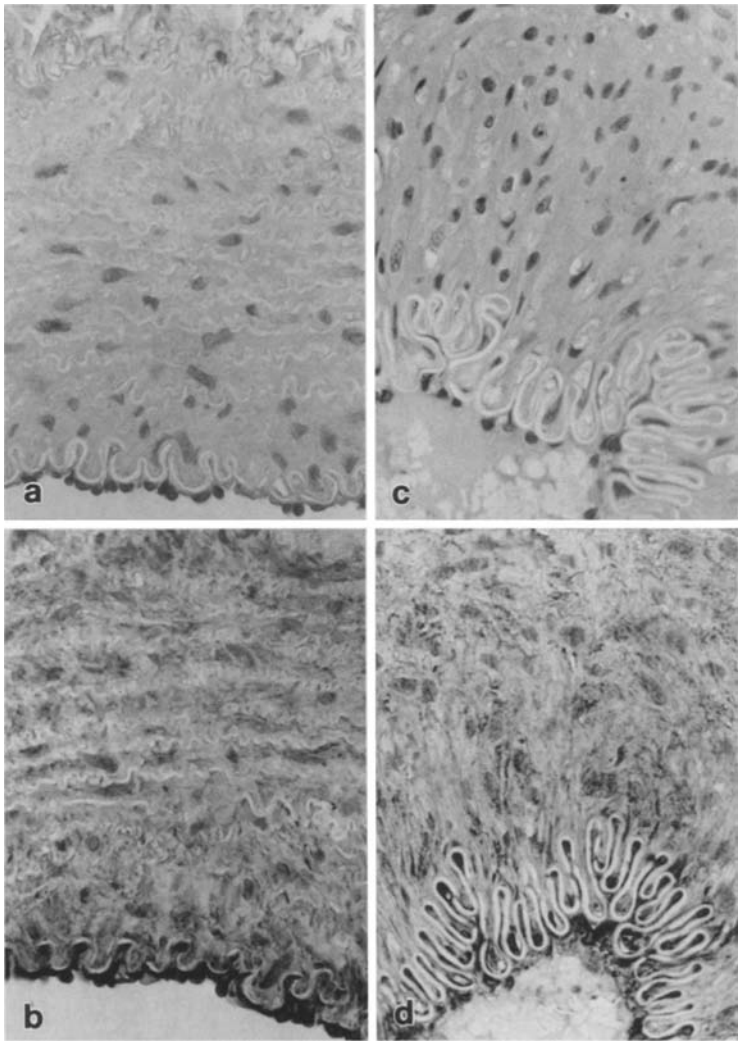


**Fig. 1.** Normal femoral (**a, b**) and common carotid (**c, d**) arteries. Immunoperoxidase staining for fibronogen/fibrin (**a, c**) and fibronectin (**b, d**). Fibronectin is seen in the subendothelial zone and between SMCs in media. No staining reaction for fibrinogen/fibrin is seen in intima and media. ×400

lamella (IEL) reduces the infiltration of plasma fibrinogen into the media (Smith and Staples 1980). The IEL therefore seems to provide the major barrier to outward transport of plasma macromolecules across the aortic wall (Smith 1986).

The different healing patterns of rabbit femoral and carotid arteries following dilatation injuries have recently been described (Rasmussen et al. 1987). The aim of the present study was to examine the distribution of fibronectin, fibrinogen/fibrin, and their degradation products in a muscular and smaller elastic artery following a severe dilatation injury. Furthermore, the observations were compared to the findings in giant cell arteritis (Chemnitz et al. 1987) and to previous studies on injury and repair in the rabbit aorta (Chemnitz and Collatz Christensen 1983).

## Materials and methods

Twenty-nine rabbits of the Danish country strain 2–3 months of age each weighing 2.5 kg were studied after a dilatation injury performed as previously described (Rasmussen et al. 1987). The arteries on the left, the common carotid and femoral artery, were submitted to a single dilatation injury, whereas the corresponding right sided arteries served as controls. From the carotid arteries on the left 4, 3, 6, and 7 segments were investigated 2, 7, 14, and 28 days after lesion together with 5 control segments from the right arteries. From the left femoral arteries 5, 4, 6, and 5 segments were studied besides 9 control segments from the right arteries.

Goat immunoglobulins to rabbit fibronectin and sheep immunoglobulins to rabbit fibrinogen were purchased from Microtiter, Dynatech (Cappel Laboratories, Inc.). The antibodies were used in dilutions 1:100 and 1:200. The primary antibodies were dissolved in phosphate buffered saline (PBS) containing 3% (w/v) bovine serum albumin (BSA) and 0.1% (v/v) Tween 20 (SIGMA). The control reagents were the immunoglobulin fraction of serum from non-immunized goats (dilution 1:50)

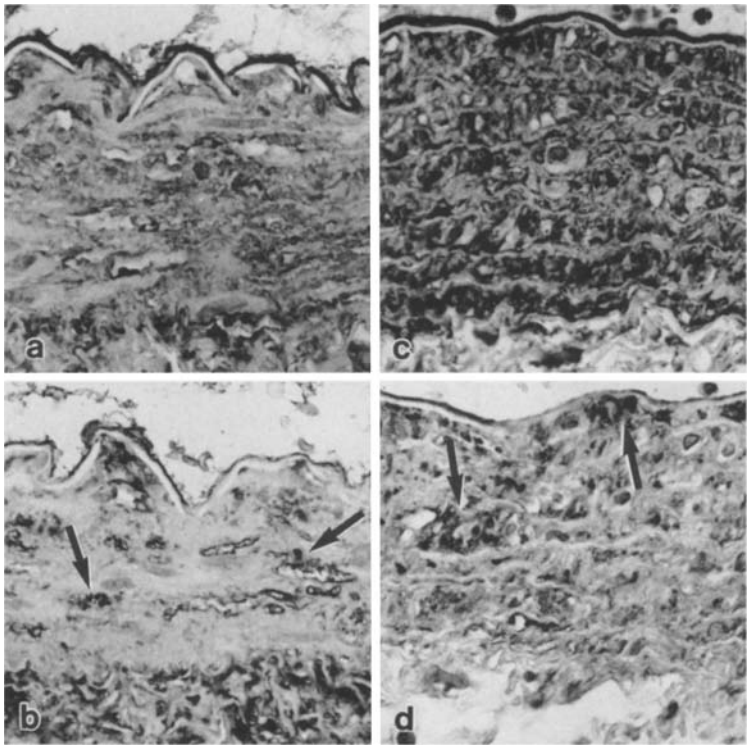


**Fig. 2.** Injured femoral (**a, b**) and injured common carotid (**c, d**) arteries 2 days after lesion. Immunoperoxidase staining for fibronectin (**a, c**) and fibrinogen/fibrin (**b, d**). A denuded endothelial surface stained for fibronectin and fibrinogen/fibrin is seen in both arteries. In both arteries a positive reaction for fibrinogen/fibrin is seen in medial areas with disintegrated SMCs and ECM (*arrows*) (**b, d**). × 400

or PBS. The secondary antibodies were peroxidase conjugated rabbit immunoglobulins to goat (DAKOPATT's, code P 160) or sheep immunoglobulins (DAKOPATT's, code P 163) diluted 1:50 in PBS containing 3% (w/v) BSA and 0.1% (v/v) Tween 20.

Specimens for immunohistochemistry were removed from dilated and undilated femoral and common carotid arteries and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4 for 6–12 h, followed by dehydration and embedding in Paraplast. Five μm thick transverse sections were cut for immunohistochemistry. Deparaffinized and rehydrated sections were incubated for 10 min at 37° C in the following proteolytic solutions: 1) 0.05% (w/v) trypsin (DIFCO 1:250) in 0.1% (w/v) $CaCl_2$ adjusted to pH 7.4 with 0.1 M NaOH and 2) 0.4% (w/v) pepsin (SIGMA, type II) in 0.01 M HCl. The indirect immunoperoxidase technique was used. Tween 20 0.1% (v/v) was added to all antibody solutions and rinsing buffers in order to reduce nonspecific staining. The steps in the staining procedure have been described previously (Chemnitz et al. 1987).

For semithin sections specimens were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4 for 24 h, postfixed in 1% $OsO_4$, dehydrated and embedded in Araldite. Semithin sections (0.5 μm) were cut on a Reichert ultramicrotome (Chemnitz et al. 1987).

## Results

In both the femoral and common carotid arteries fibronectin was observed in the intimal zone between the endothelial cells and the IEL in normal vessels. In media fibronectin was observed in the ECM around the SMCs. In contrast, fibrinogen/fibrin could not be demonstrated in the two arteries (Fig. 1a, b, c, d).

Two days after injury fibronectin and fibrinogen/fibrin were present on the denuded luminal surfaces of both arteries. Areas in the media of both arteries with injured and disintegrated SMCs showed a strong positive reaction for fibrinogen/fibrin. In apparently undamaged areas fibronectin was observed intracellularly and around the SMCs, whereas fibrinogen/fibrin could not be demonstrated (Fig. 2a, b, c, d).

Seven days after injury only the femoral arteries showed intimal thickening. Fibronectin was present in all the layers of the intimal change. Fibrinogen/fibrin showed a luminal and focal localization with varied depth and intensity and/or extension. Fibronectin was observed in the media of both arteries, whereas a minimal to no staining reaction for fibrinogen/fibrin was observed in intact media, however, damaged areas visible as disintegrated architecture of SMC-pattern were positive for fibrinogen/fibrin.

Fourteen days after injury both arteries showed intimal thickenings with strong staining reaction for fibronectin. Fibrinogen/fibrin was observed in the intimal thickening of both arteries as a luminal staining. A focal staining reaction for fibrinogen/fibrin was more often found in media of the com-

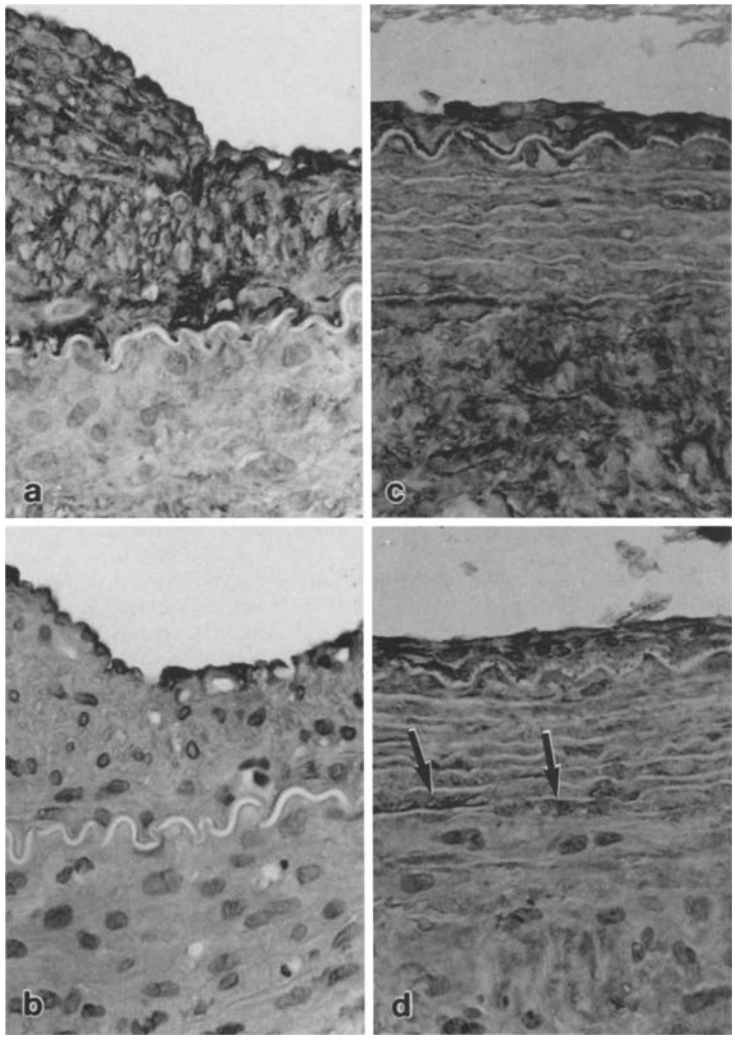


**Fig. 3.** Femoral (**a, b**) and common carotid (**c, d**) arteries with intimal thickening 14 days after lesion. Immunoperoxidase staining for fibronectin (**a, c**) and fibrinogen, fibrin (**b, d**). A strong positive reaction for fibronectin is present in the intimal thickening of both arteries. In the femoral artery fibrinogen/fibrin (**b**) is only seen in the luminal part of the intimal thickening. Fibrinogen/fibrin (*arrows*) is seen in media of the common carotid artery (**d**). × 400

mon carotid artery than in the media of the femoral artery (Fig. 3a, b, c, d).

Twenty-eight days after injury a pronounced intimal thickening could be demonstrated in the femoral artery. A strong reaction for fibronectin was found both in the intimal thickening and in an intact media, free of necrosis. A minimal to negative staining reaction for fibrinogen/fibrin was observed in the media, whereas in the intimal thickening a luminal zone of positive reaction was visible (Fig. 4a, b).

All the examined specimens from common carotid arteries were obliterated by luminal thrombi. In the zone of contact between the thrombus and arterial wall no intimal thickening was visible, the IEL was absent or fragmented, and the media apparently compressed (Fig. 5). In the borderzone between thrombus, remnants of intima and compressed media a process of organization was in play with ingrowth of SMCs and giant cells (Fig. 5). A strong reaction for fibronectin was conspicuous in the thrombus as well as in the media. Fibrinogen/fibrin presented a positive reaction in the thrombus, and all segments showed a moderate to strong staining reaction for fibrinogen/fibrin (Fig. 4c, d).

A conspicuous difference in the reactive pattern of the common carotid and femoral artery apparently is thrombotic occlusion and persistence of fibrinogen/fibrin in all layers of the carotid artery four weeks after injury.

## Discussion

The purpose of the present study was to trace the distribution of fibronectin and fibrinogen/fibrin in the rabbit carotid and femoral arteries, in which a different healing pattern following a single dilata-

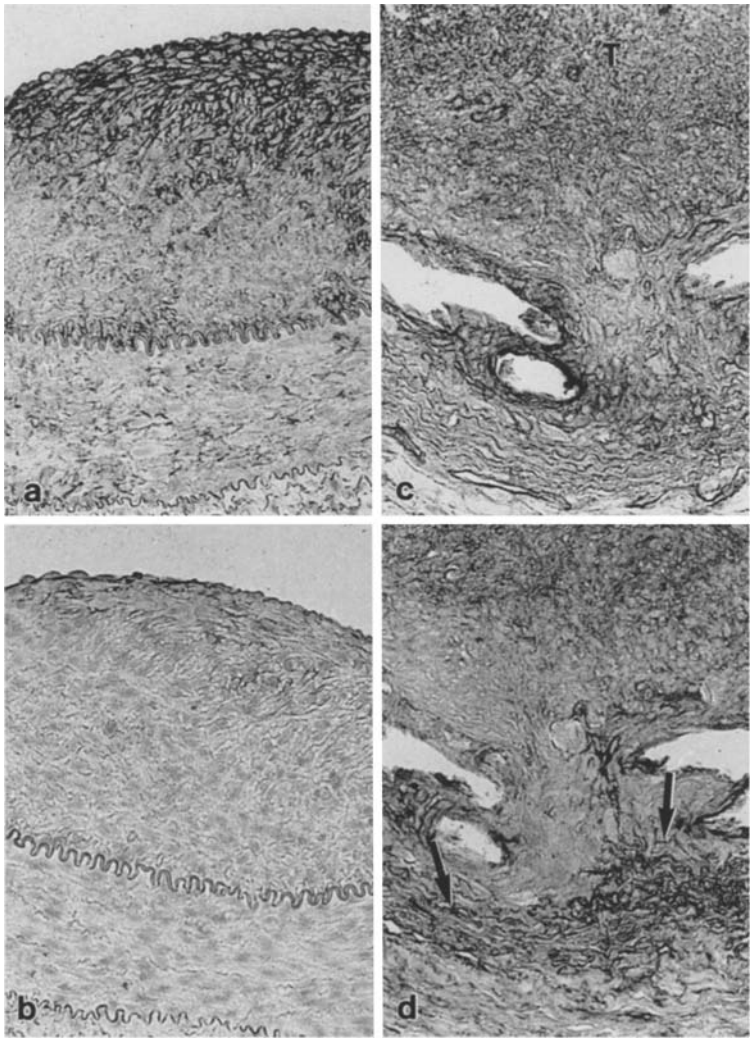


**Fig. 4.** A femoral artery (**a, b**) with intimal thickening 28 days after lesion. A strong positive reaction for fibronectin (**a**) is seen in the intimal thickening, strongest in the luminal part. In media fibronectin is seen as fine dark lines around the SMCs. Fibrinogen/fibrin and degradation products are only present in the luminal part of intima (**b**). A common carotid artery obliterated by an organized thrombus (T) containing fibronectin (**c**) and fibrinogen/fibrin. Fibrinogen/fibrin (*arrows*) is seen in media (**d**). ×160

tion injury has been demonstrated previously (Rasmussen et al. 1987). The dilatation injury of the carotid and femoral arteries used in the present investigation will allow plasma constituents such as fibrinogen and fibronectin to leak into the media, and loss of the non-thrombogenic surface of endothelial cells will allow platelets to adhere and release growth factors of which platelet derived growth factor is known to stimulate the proliferation of arterial SMSs in vitro (Ross et al. 1986).

In the present study the elastic carotid artery and muscular femoral arteries had in common an inflammatory and proliferative response with intimal thickening. In the carotid artery, however, the intimal thickening occurred later than in the femoral artery. After 28 days the femoral artery had healed with a pronounced intimal thickening, whereas all the examined carotid artery segments were occluded by thrombosis in continuity with the destroyed intimal thickening exhibiting inflammatory reaction.

Fibronectin, being a genuine ECM component in the arterial wall, has been demonstrated in the intima and media of the normal arterial wall (Stenman and Vaheri 1978; Natali et al. 1981; Chemnitz and Collatz Christensen 1983; Jensen et al. 1983). Distinction between plasma fibronectin and matrix fibronectin is not possible by the immunohistochemical method used in the present work. Accordingly a positive immunoreaction may depend on penetration into the arterial wall of plasma fibronectin from the lumen as well as on secretion of matrix fibronectin from cells in the media and intimal thickening.

Fibrinogen/fibrin is by light microscopic methods not traceable in the normal rabbit thoracic aorta (Chemnitz and Collatz Christensen 1984) or in the normal human aortic wall (Haust 1971).

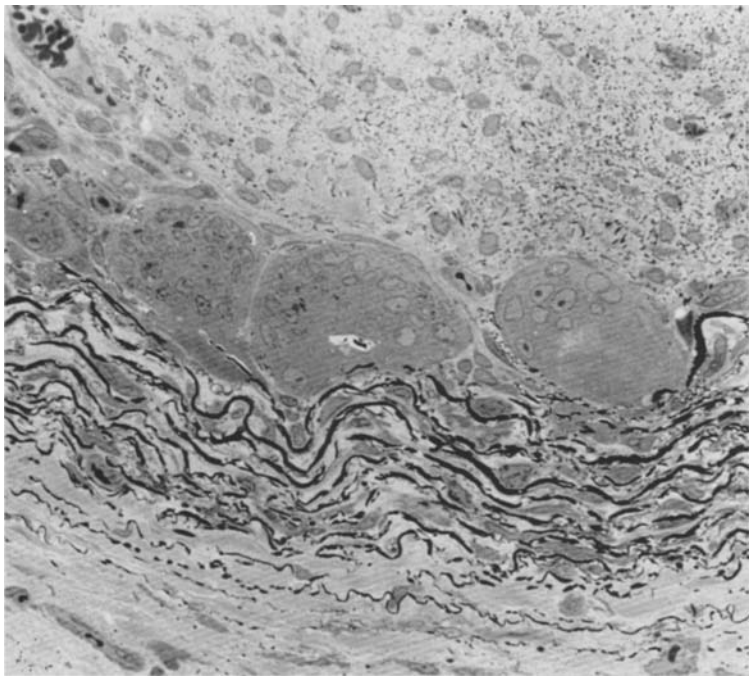

**Fig. 5.** A semithin section (0.5 μm) of the borderzone between thrombus and media. A fragmented IEL and several large giant cells are seen. ×400

This is also true of the undilated arteries in our study. However immunoelectron-microscopy on the normal rabbit coronary artery has shown that fibrinogen can be found in the caveolae and vesicles of the endothelial cells and in very small amounts in the subendothelial space (Kurozumi et al. 1983). When intact, the IEL seems to provide the major barrier to outward transport of plasma macromolecules across the arterial wall as they become trapped between the IEL and endothelium (Smith 1986).

The femoral artery, representative of a muscular type, responds to a single dilatation injury with a thick intimal thickening rich in SMC, covered with EC, and in the present investigation no occlusive thrombosis is seen after four weeks. The persistent positive reaction for fibronectin, most conspicuous in the intimal thickening, may be ascribed to proliferative processes in play, as well as to penetration of plasma fibronectin, as long as the intimal barrier remains incomplete.

Decreasing amounts of fibrinogen/fibrin in the intimal thickening, and its disappearance from the media, are in accordance with establishment of an intimal barrier from reendothelialization and ripening of neointimal connective tissue. Effects from an increased fibrinolytic activity may also be suggested. Both mechanisms conform with the observation of decreasing amounts of fibrinogen/fibrin and with a gradient in staining intensity from luminal layers towards the depth of the intimal thickening.

The carotid artery, representative of an elastic type, responds in quite a different way. The present study has shown occlusion of the artery after four weeks and the underlying intimal thickening is transformed into an inflammatory border zone against a thinned out media. The overall high concentrations in media of fibronectin and fibrinogen/fibrin may be attributed to inflammation and/or to a missing intimal barrier function. Compared to the femoral artery, the intimal thickening is retarded in growth and apparently ineffective in preventing expansion of thrombotic masses, which occlude the lumen. Failure of fibrinolytic activity is possible.

Our previous studies on repair processes of the aortic wall in rabbits (Chemnitz and Collatz-Christensen 1983, 1984) as well as the findings in giant cell arteritis in human (Chemnitz et al. 1987) showed a pattern of distribution of fibronectin, fibrinogen/fibrin, and degradation products similar to that of the rabbit carotid artery and the early stage of repair process of the femoral artery. Therefore, the morphological and immunohistochemical changes in the present study probably reflect a general pattern of arterial repair.

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