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Matrix production of smooth muscle cells from rat aorta *in vitro*

B. Stiemer[1], G. Springmeier[1], L. El-Jarad[1] and C. Schröter-Kermani[2]
[1]Institute of Anatomy and [2]Institute of Toxicology and Prenatal Pharmacology, Free University of Berlin, Berlin, Germany

Summary. Immunofluorescence microscopic methods served to demonstrate the production of the following matrix components in cultures of vascular smooth muscle cells from rat aorta: fibronectin; nidogen; heparan sulphate-proteoglycan (HS-PG); laminin; and collagen types I, III, IV, V, and VI. A time-dependence of synthesis and secretion could be shown for a number of components of the extracellular matrix (ECM), such as laminin. The results revealed the following estimated quantitative differences of the collagen types: type I > type III > types V and VI. A filamentous/fibrillar matrix and also occasionally a typical basal lamina could be demonstrated electron microscopically around the smooth muscle cells.

Key words: Smooth muscle, Rat, Aorta

Introduction

Despite intensive investigations neither the reasons nor the mechanisms leading to the development of hypertension and arteriosclerosis have been fully elucidated so far. Both diseases are accompanied by proliferation of smooth vascular cells and «sclerosis» of the arterial tissue. The changes in the connective tissue are mainly attributed to changes in matrix production of smooth vascular cells (Lee et al., 1983; Borel et al., 1987; Kindy et al., 1988). Numerous biochemical investigations on quality and quantity of the matrix components of aortae have so far been performed using autopsy material from man (McCullagh and Balian, 1975; McCullagh et al., 1980; Bondjers et al., 1986; Borel et al.,, 1987; Kwan et al., 1987) and from animals (Jurukova, 1980; Ledet and Vuust, 1980; Halila and Peltonen, 1984; Deyl et al., 1987; Opsahl et al., 1987). However, impurities due to intima and adventitia cannot be excluded. The study of the synthesis of matrix components in the smooth muscle cells of the media is only possible in pure cultures of such cells.

Cell cultures of smooth muscle cells from the media have already been investigated biochemically and immunomorphologically with regard to various matrix components, such as fibronectin (Chemnitz and Christensen, 1983), collagen types I and III (Barnes et al., 1976; Mayne et al., 1978; Kindy et al., 1988), dermatan sulphate-proteoglycan (Sandell et al., 1988) and basal lamina-like material (Heickendorf and Ledet, 1983).

Systematic investigations in dependence on the time and the method of isolation and cultivation of the cells with as many matrix components as possible are still outstanding. Therefore, the aim of this study was to investigate smooth vascular muscle cells from rat media *in vitro* regard to their capability to produce various matrix components.

Materials and methods

Cell culture

The cells were obtained 1) from explant cultures and 2) through enzymatic isolation. For the explant cultures the aortae were freed from adventitia and endothelium, cut into 2 mm x 2 mm pieces which were placed on the bottom of a culture flask. Subsequently, the flask was carefully filled with medium so that the pieces did not float away. After a 3-week culture period the emigrating cells had reached confluency and could be transferred. Enzymatic cell isolation was performed in a mixture of collagenase (2.5 mg/ml) and elastase (0.7 mg/ml) modified according to Chamley-Campbell et al. (1979). After incubation with only elastase (0.7 mg/ml) for 30 min the 2 mm x 2 mm aorta pieces were incubated in the collagenase/elastase mixture before they were centrifuged at 600g for 10 min and seeded into primary culture dishes. The cells were incubated in MEM Dulbecco medium with the following substances being added: 10% FCS; 2 mM glutamine, 100 IU penicillin;

Offprint requests to: Dr. B. Stiemer, Institute of Anatomy, Free University of Berlin, Königin-Luise-Str. 15, D-1000 Berlin 33, Germany

and 100 µg streptomycin. Seeding was performed at a cell density of 1 x 10^6/ml on glass and Thermanox cover slips. The cells were prepared in the 2nd and 4th passage (see below).

Immunofluorescence staining

Anti-α-actin

For these experiments vascular smooth muscle cells (SMC) and embryonal mouse skin fibroblasts were seeded onto glass cover slips and used 7 to 14 days after seeding. The culture medium was removed by rinsing the cells three times with PBS (phosphate buffer). Subsequently, the cells were fixed with methanol (-20°C) for 3 min. The methanol was removed by rinsing the cells again three times with PBS. The cells were then incubated for 90 min at room temperature with a monoclonal antibody raised in mouse against smooth muscle-specific α-isoactin (Fa. Bio Makor, Rehovot, Israel) in a dilution of 1:100 in PBS (primary antibody). After rinsing three times with PBS the cells were incubated for another 60 min with a FITC-conjugated anti-mouse-Ig-antibody (Fa. Dakopatts, Hamburg, FRG) in a 1:40 dilution in PBS (secondary antibody). Subsequently, the cells were thoroughly rinsed with PBS and thereafter embedded in glycerin-α-phenylene-diamine (Platt and Michael, 1983) to inhibit the fading of fluorescence. The cells were evaluated and photographed using a Zeiss-Photomicroscope III with an epi-fluorescence device at a magnification of 250. Control experiments were performed using either PBS or a pre-immune serum instead of the primary antibody.

Antibody production against matrix components

Laminin, nidogen and heparan sulphate proteoglycan BM-1 were isolated and purified from EHS sarcoma (Timpl and Rohde, 1979; Hassel et al., 1980; Paulsson et al., 1987), the type I and type III collagens from the skin of newborn rats according to Trelstad et al. (1976). Type IV and V collagens were isolated from human placenta (Sage and Bornstein, 1979; Sage et al., 1979). Collagen type VI also originating from pepsin-solubilized human placenta was purchased from Heyl (Berlin, FRG). Fibronectin was isolated from rat serum (Engvall and Ruoslahti, 1977).

Antibodies against these components were raised in rabbits following standard protocols (Kittelberger-Ewert et al., 1988) and purified by immunoadsorption (March et al., 1974). The monospecificity of the antibodies was assayed by ELISA (Engvall and Pearlmann, 1971) and by immunoblotting (Towbin et al., 1979).

Immunofluorescence of matrix components

Cells obtained from explants and enzymatic isolation were stained for the determination of the matrix components.

Before staining, the cover slips were rinsed with PBS. When demonstrating extracellular material, fixation could be omitted. Demonstration of intracellular antigens requires fixation with pure methanol. The procedure of the immunofluorescence method is analogous to that of α-actin staining. However, the first antibody was used in different dilutions: fibronectin 1:5; HS-PG 1:6; nidogen 1:3; laminin 1:2; collagen types I and III 1:2.5; collagen type IV 1:4; collagen types V and VI 1:5.

Since the first antibody was obtained from rabbits, the second antibody was an FITC-conjugated antirabbit antibody that was used in a dilution of 1:30 (Dakopatts, Hamburg, FRG).

Transmission electron microscopy (TEM)

The monolayer cells on the Thermanox cover slips were fixed for 1 to 3 hrs in tannic acid solution (1% tannic acid, 2% glutaraldehyde in 0.1M phosphate buffer) and washed twice for 15 min each in 0.1M phosphate buffer. For postfixation we used cacodylate-buffered 2% OsO_4 at pH 7.2. Subsequently, the cells were washed in 0.1M cacodylate buffer, pH 7.2., dehydrated in acetone (30, 50, 70, 100%) and embedded in Mikropal (Ferak, Berlin FRG). Thin sections were prepared using a Reichert OmU2. Contrasting with uranyl acetate and lead citrate (Reynolds, 1963) was followed by electron microscopic inspection (Zeiss EM 10 and 109).

Scanning electron microscopy (SEM)

The cells were centrifuged (600g/min for 10 min) directly after enzymatic isolation, decanted and the pellet was fixed in 2.5% glutaraldehyde for 1 h. Subsequently, the cells were washed in 75 mM cacodylate buffer. Dehydration was performed in the ascending alcohol series up to absolute alcohol. The cells were placed on a filter, followed by critical point drying with CO_2. The preparations were sputtered with gold palladium. This was followed by inspection on an ETEC Autoscan SEM.

Results

The typical elongated, round and spindle-like appearance of the enzymatically isolated cells, as revealed by scanning electron microscopic inspection, indicates smooth vascular muscle cells (Fig. 1a). Also in culture the cells largely maintained their long spindle-like shape (Fig. 1b).

Immunofluorescence microscopic α-actin staining was performed prior to each determination of the matrix components. The staining was specific of smooth muscle cells (Gown et al., 1985; Skalli et al., 1986). Typical were the heavily-stained parallel actin bundles (Fig. 1c). Compared with this situation, actin staining of fibroblasts was only very weak and unspecific. Hence, this technique served to largely exclude any impurities

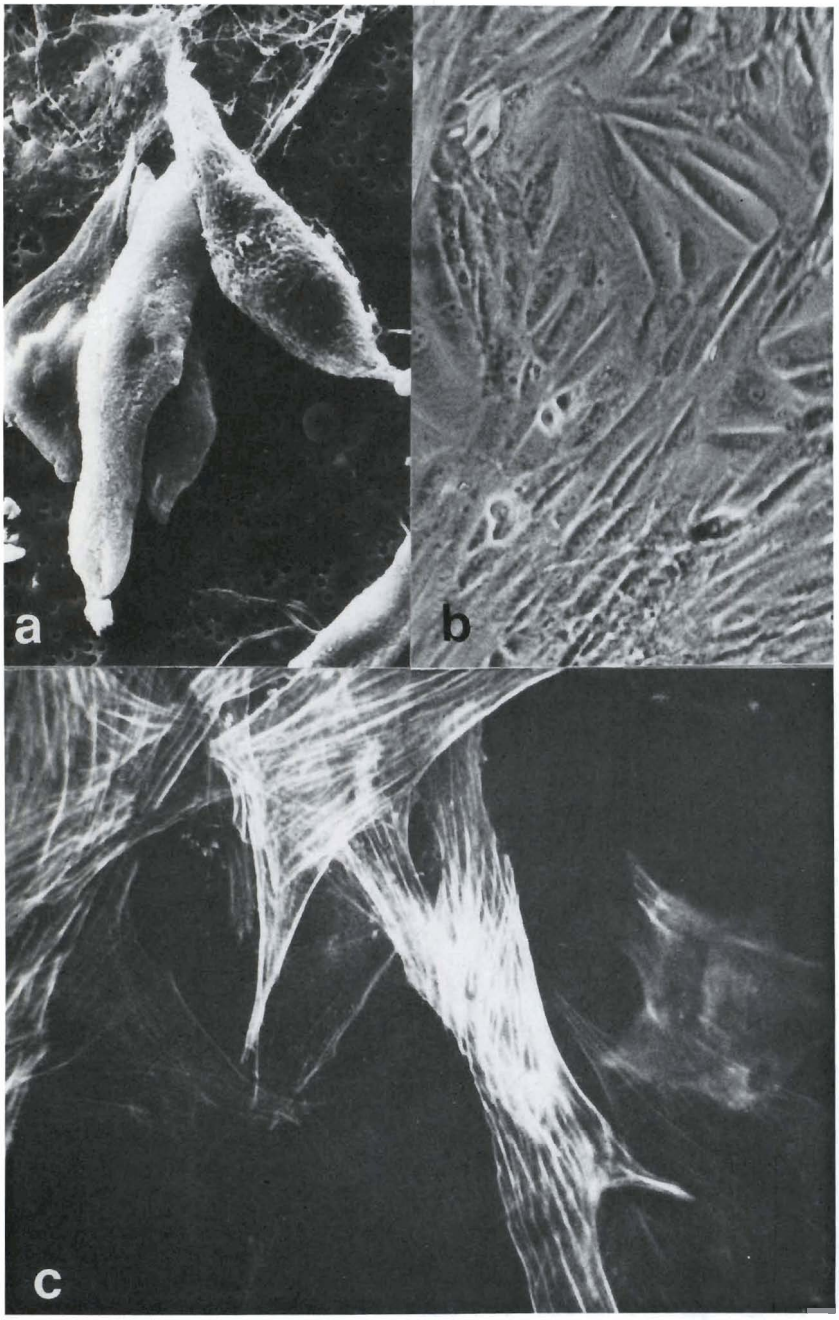

Fig. 1. a. Spindle-shaped vascular muscle cells immediately after enzymatic cell isolation as seen by scanning electron microscopy. x 1,500. **b.** Culture of smooth vascular muscle cells as seen by phase contrast microscopy after 6 days in culture. x 250. **c.** Binding of antibodies against α–actin of smooth vascular muscle cells. Parallel-arranged actin filament bundles are seen. x 250

by fibroblasts.

Matrix staining did not reveal any differences between cells from explant cultures and cells from enzymatic isolation. Thus, the following description applies to both culture techniques.

Collagen type IV was predominantly characterized by fibre bundles, occasionally also by very delicate fibres that were arranged either parallel to the cells or in a ring-shaped manner around the latter (Fig. 2a). Fibronectin appeared in the form of a fibrous network from day 4 onwards. The partly delicate fibres were mainly located in the region of the cells and occasionally formed rather long fibre bundles between the cells (Fig. 2b). Heparan sulphate-proteoglycan (HS-PG) mainly appeared in the form of delicate, cross-wise-arranged fibres, occasionally also as fibre bundles. Striking was the distribution which was almost exclusively seen at the edge of the cells or around them. In contrast to other

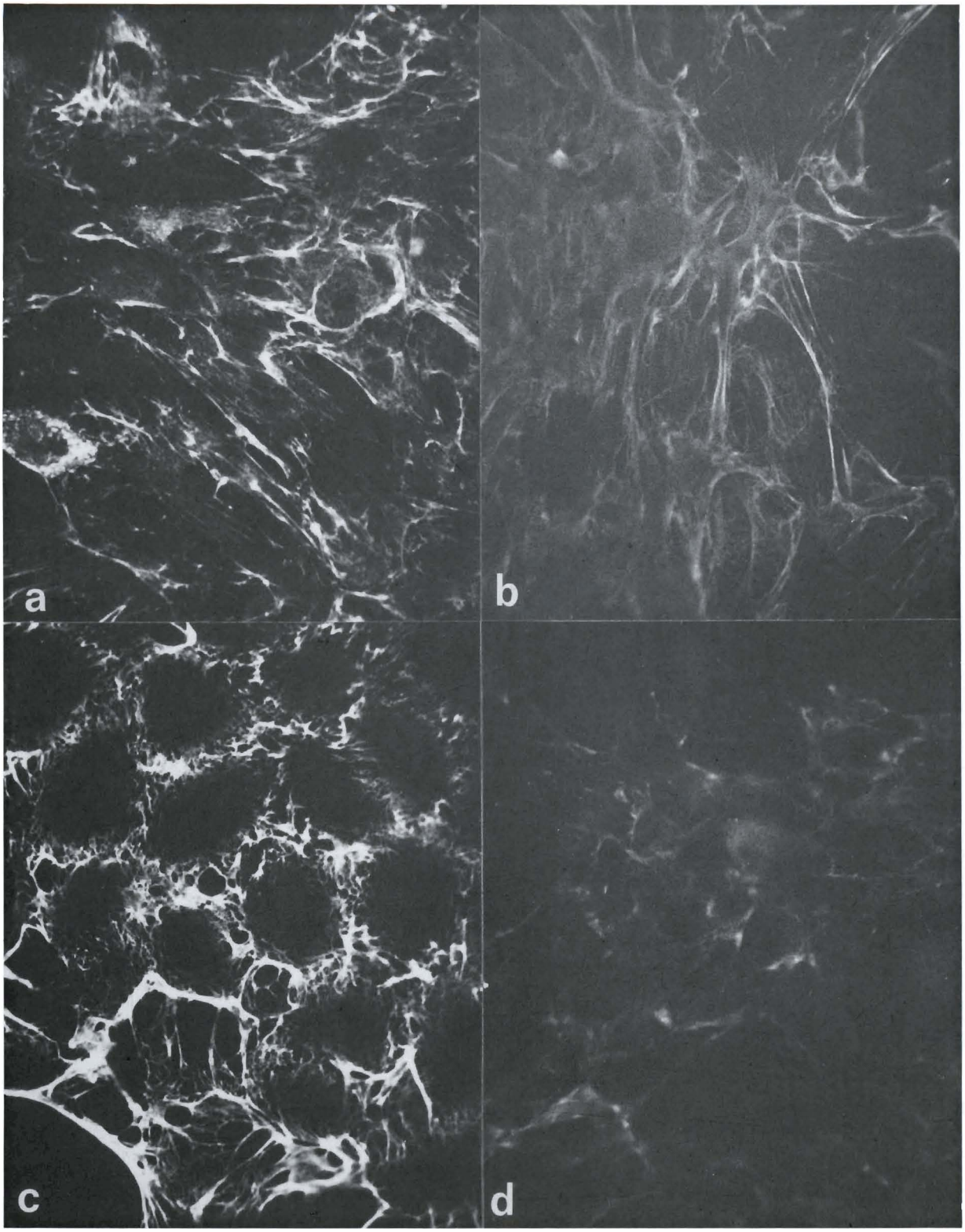

Fig. 2. a. Anti-fibronectin, fibrous network, occasionally rather long fibre bundles. x 250. **b.** Anti-collagen type IV, predominantly short fibre bundles arranged parallel to the cells or in the periphery of the cells. x 250. **c.** Anti-heparan sulphate-proteoglycan fibres and fibre bundles that are located either between the cells or at the edge of the cells, regularly distributed. x 250. **d.** Anti-nidogen, very thin, delicate fibrils often showing a ring-shaped structure. x 250.

stainings, the arrangement of HS-PG seemed to be very regular (Fig. 2c).

Compared with HS-PG, nidogen was only weakly stainable. Mostly short fibre pieces or plaques were seen that were arranged in a ring-shaped manner (Fig. 2d), less regular, however, than in the case of HS-PG. Collagen type IV, HSPG and nidogen were always demonstrable from the 4th day onwards after seeding.

The formation of extracellular structures of laminin proceeded depending on the time the cells were allowed to stay in the culture dish. After a culture period of 7 days, a relatively weak intracellular staining was observed. After a 3-week culture period, however, abundant punctiform and fibrous material was perceptible extracellularly (Figs. 3a,b).

Investigations of collagen type I (Figs. 4a-c) revealed a clear-cut time-dependence of occurrence and shape of extracellular structures. After a 1-day culture period mainly intracellular granular material became recognizable with methanol fixation, but firstly fibres were also seen extracellularly. After a 3-day culture period fine fibrous structures were perceptible in the region of the cells and often, in the form of coarser aggregates, between the cells. But it was only after a 3-week culture period that a dense network of partly thick fibres was seen extracellularly (without methanol

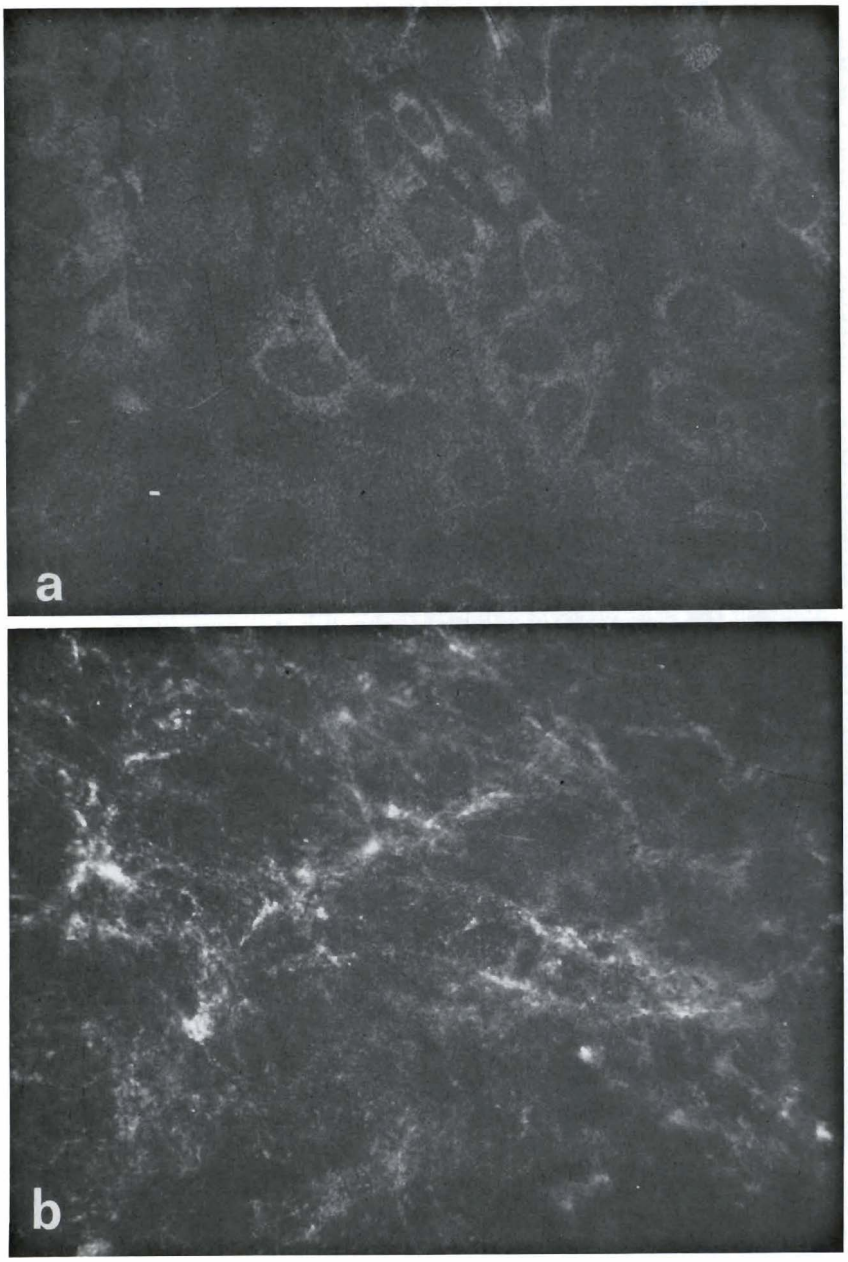

Fig. 3. a. Anti-laminin. Weak intracellular stainability after 7 days. **b.** After 3 weeks point-like and fibrous material seen extracellularly. x 250

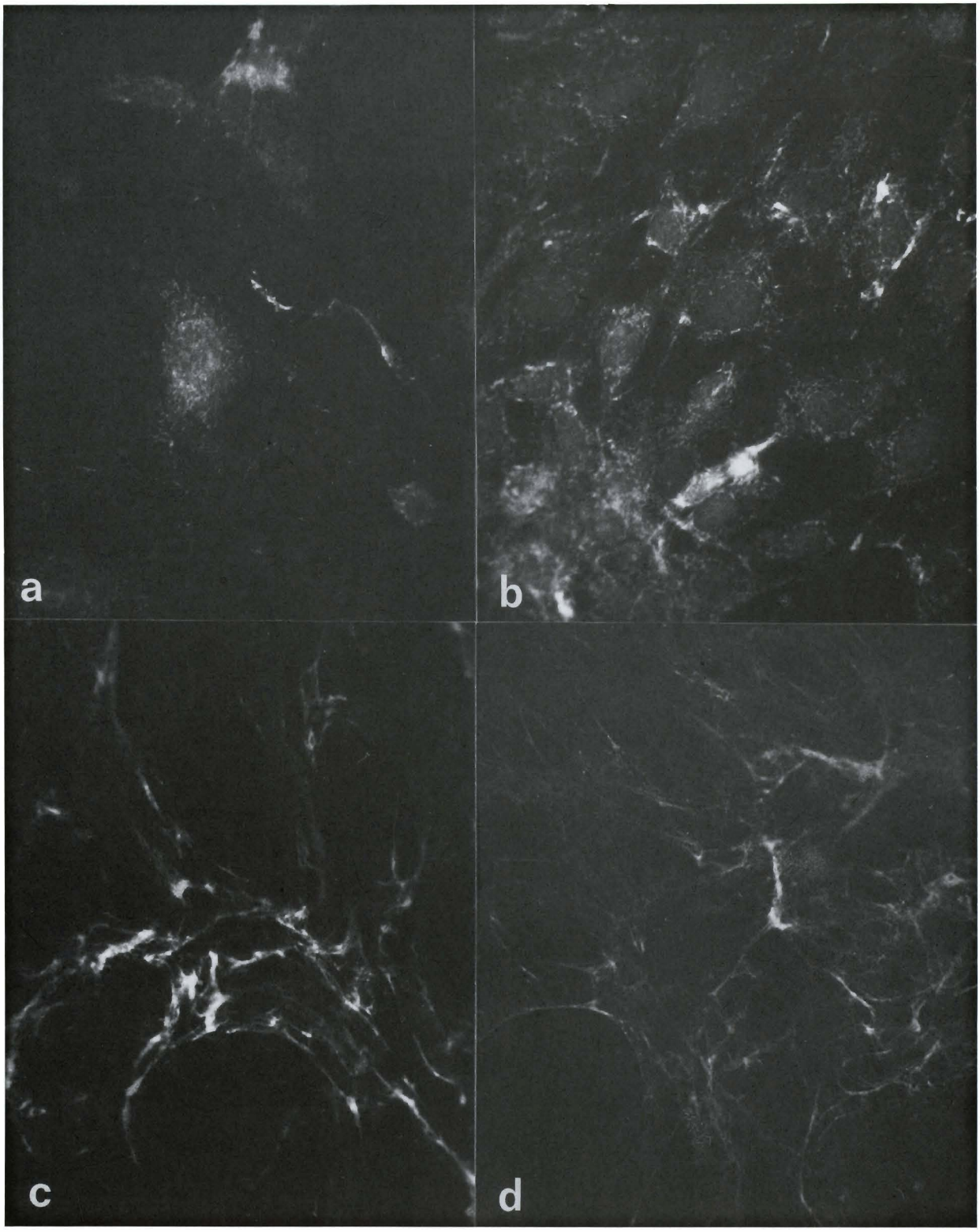

Fig. 4. a-c. Anti-collagen type I, x 250. **d.** Anti-collagen type III, x 250. **a.** After 1 day - intracellular material, first fibrils extracellularly. **b.** After 3 days- increase in intra- and extracellular material (A and B - methanol fixation). **c.** As late as the 3rd week - dense fibrous network extracellularly (without methanol fixation). **d.** Collagen type III forming a dense network, but only fibrils, rarely as dense as in this picture.

fixation). The cell areas were mostly free of fibres which formed an irregular network of varying density at the edges of the cells or in the spaces between them.

Collagen type III showed fibrils that also formed a dense network. They, too, preferred the periphery of the cells or the spaces between them (Fig. 4d). Generally, the staining of collagen types III was weaker than that of type I. Compared with collagen types I and III the expression of collagen types V and VI was clearly weaker. After a culture period of more than 3 weeks only little extracellular material was seen. Both collagen types exhibited a network; the bundling of filaments being somewhat more pronounced in the case of collagen type VI (Figs. 5a, b). Striking was the varying extent of staining. Some cell groups showed a dense network, others were not stained at all.

Transmission electron microscopy (TEM)

The transmission electron microscopic pictures

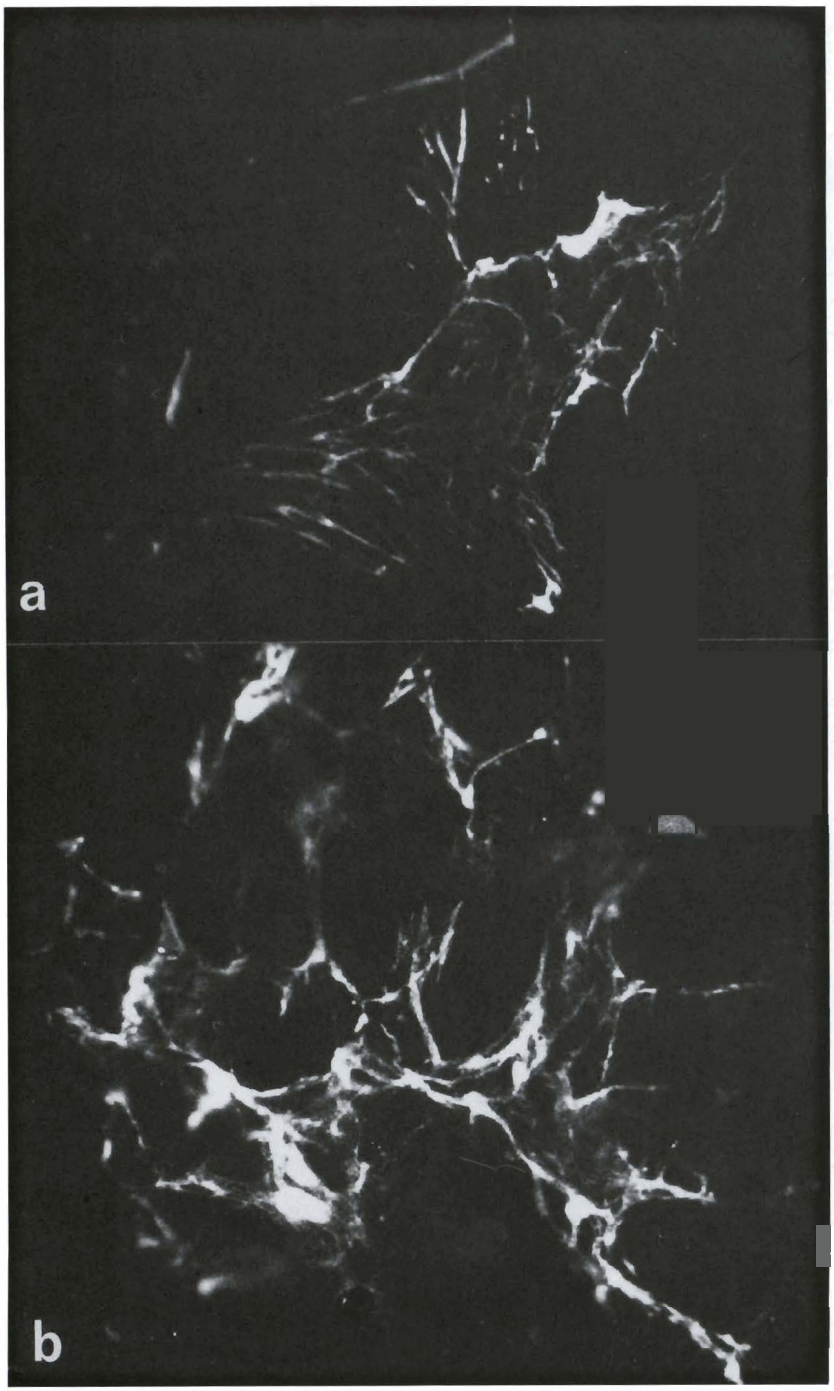

Fig. 5. a. Anti-collagen type V, very delicate fibrils, stainability mostly very weak. x 250. **b.** Anti-collagen type VI, bundling of fibres partly somewhat stronger than with type V. x 250. Figs. 5a,b are an exception. Numerous cell groups show a weaker antibody binding.

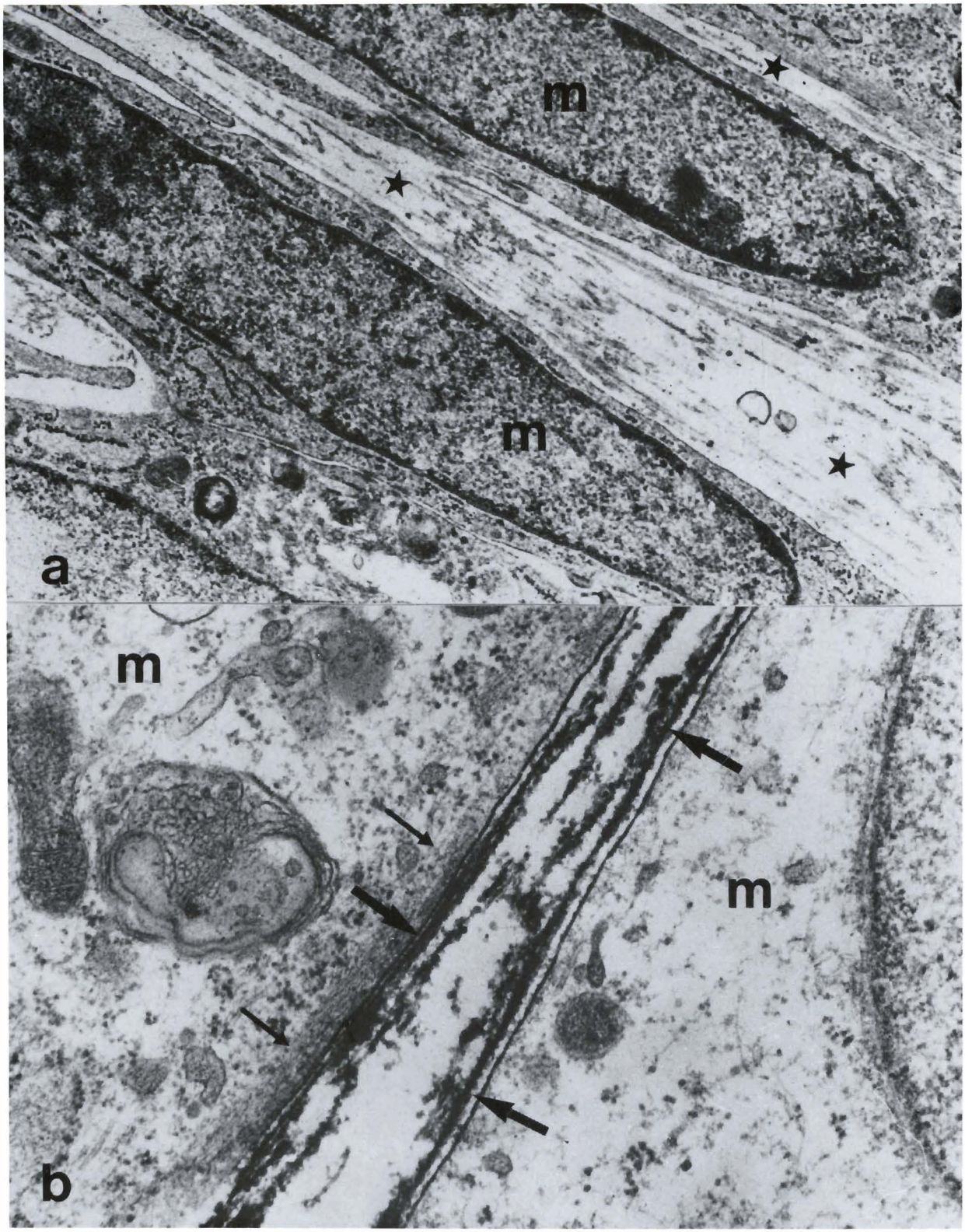

Fig. 6. Transmission electron microscopy. **a.** After 1 week, multi-layered arrangement of smooth muscle cells (m) with extracellular material (*). x 8,000. **b.** After 3 weeks, increase in extracellular material; basal lamina perceptible in certain areas (arrow). Filament bundles in smooth muscle cells (small arrow). x 55,000

showed a multilayered arrangement of the smooth muscle cells as early as after a 1-week culture period. Extracellular material was clearly recognizable in the intercellular spaces (Fig. 6a). This material was arranged in the form of single or bundled filaments (smaller than 15 nm) and cross-striated fibrils (larger than 15 nm). They were arranged parallel to the surface of the cells. In addition, fine (smaller than 10 nm) single and irregularly arranged filaments and small granules were seen that were occasionally aggregated in the form of irregularly-bordered plaques of varying size. Such plaques were preferentially located in the vicinity of or at the cell membranes. Close to these cell membranes segments of basal lamina-like material varying in length were seen (Fig. 6b). This material consisted of a Lamina densa (±50 nm) that proceed parallel to the cell membrane at a distance of about 30 nm. This bright zone (Lamina rara) was bridged at regular intervals by fine filaments. Such basal lamina segments occasionally revealed a length of several µm.

Discussion

Our findings have shown that vascular smooth muscle cells at the confluent state in culture form smooth muscular-specific α-actin also after four passages. These results are in agreement with investigations by Owens et al. (1986). Matrix production is independent of the way the cells were obtained (explants or enzymatic isolation). Like α-actin, it increases considerably once confluence has been reached. It is specially collagen type I that can be demonstrated as early as 1 day after seeding intracellularly and, to a lesser extent, extracellularly in the culture of smooth vascular cells from aorta. Hence, production of this type of collagen sets in shortly after seeding of the cells. And yet it takes 3 weeks before a dense extracellular network is formed. This shows the discrepancy between the onset of synthesis and formation of fibrils. The *in vitro* conditions might be responsible for this situation. *In vitro*, a deficiency of aggregation-promoting substances, such as proteoglycans (Lilja and Barrach, 1983), may occur, the degree of hydroxylation may be low or there may be a lack of vitamin C in the medium.

In contrast to collagen type I, laminin can only be demonstrated with certainty after 3 weeks in culture. Two weeks after seeding the presence of laminin is still questionable. Collagen type I and laminin are characterized by the time of the pronounced extracellular organisation, i.e., 3 weeks after seeding.

This result does not say anything about a synthesis and following secretion into the medium. Such soluble substances need not necessarily be retained in the pericellular matrix, but can also reach the medium directly. The presence of matrix components in the medium of our cultures could recently be demonstrated (Unpublished data).

A comparison with electron microscopic pictures confirms the key function that laminin has for the formation of the basal lamina (Merker, 1987). It is only after the formation of a sufficient amount of laminin, i.e., after 3 weeks, that electron microscopic inspection reveals a basal lamina that is still not continuous, although the other components (nidogen, heparan sulphate-proteoglycan, collagen type IV) are present extracellularly as early as the 7th day.

Immunofluorescence microscopic investigations do not allow any statements as to quantitative differences. However, repeated experiments have shown that obviously more collagen type I than III is aggregated extracellularly and, on the other hand, more collagen type III than types V and VI. These assumptions are in agreement with investigations performed by Mayne et al. (1978) who were able to demonstrate biochemically more collagen type I than type III and by Rauterberg et al. (1977) who found that type III occurs predominantly in the medium of cell cultures and type I between the cellular layers and in the medium.

The limited extracellular occurrence may be due to the fact that less than 1% of collagen type V and VI were found in the aortae (Schuppan and Hahn, 1987). Figure 6 shows exceptions to this situation. It shows cell groups with distinct and strong fibres.

Of the hitherto investigated collagen types only collagen types I and III have been discussed in connection with hypertension and arteriosclerosis. In spontaneously hypertense rats that developed hypertension at the 4th week after birth due to a genetic defect, an increased amount of collagen type III could be demonstrated in the prehypertense phase, while type I predominated in the hypertonic phase (Deyl et al., 1987). Similar findings were obtained for arteriosclerosis. In the early atherosclerotic plaque collagen type III strongly increases, as compared with type I, while the late plaque shows more collagen type I (Gay and Miller, 1978).

Collagen type I appears to play a role in the migration process (Ooshima, 1981) and possibly influences, given a changed fibrous structure in the extracellular matrix, the migration of smooth muscle cells into the intima during the development of arteriosclerosis.

Of special interest for further investigations should be changes in the basal lamina composition, since these normally regulate permeability, i.e., those substances are selected that reach the media where they may influence the behaviour of the smooth muscle cells (Borel et al., 1987).

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