Myofibroblasts in the Lamina Propria of Human Seminiferous Tubules are Dynamic Structures of Heterogeneous Phenotype*

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Summary. Myofibroblasts of the lamina propria of human seminiferous tubules were studied in testes having normal or slightly reduced spermatogenesis by means of electron microscopy, confocal laser microscopy and immunocytochemistry. Myofibroblasts are large, flat individual cells braced in a network of microfibrils and collagen fibrils in the tubular wall. They are arranged in discontinuous cell layers with interposed layers of an extracellular matrix.

Myofibroblasts of the lamina propria exhibit a unique cell shape with the peripheral cytoplasm split up in two or more layers. After FITC-phalloidin staining and by means of confocal laser microscopy, actin filaments of variable orientation are visible in their cytoplasm. The thickness and preferential direction of actin filaments differ in the outer and innermost cell layers. The myofibroblasts express both antigens of smooth muscle cells (α -smooth muscle actin, pan-actin, desmin, GB 42, smooth muscle myosin), and of connective tissue cells (vimentin, fibroblast surface protein). The variable expression of these antigens evidenced the existence of different phenotypes of myofibroblasts.

Immunoreactivity for *basic fibroblast growth factor* and *transforming growth factor β* as well as for components of the extracellular matrix indicate that these agents may be important for the phenotypic differentiation of the lamina propria cells. The detection of *CNPase* and *galactocerebroside*-immunoreactivity in a number of lamina propria cells and some cells of the intertubular tissue gives rise to the hypothesis that components of the testicular tissue share some structural similarities with glia cells of the nervous system.

Finally, immunoreactivities for the *neuronal and endothelial nitric oxide synthase*, *soluble guanylyl cyclase*, *cyclic GMP*, *calmodulin*, *calcium-dependent protein kinase II* and *glutamate* indicate that the

contractility of myofibroblasts in the lamina propria of human seminiferous tubules may be in part modulated by the NO/cGMP-system.

Seminiferous tubules of the human testis are surrounded by 3–5 inner layers of myofibroblasts and additional 1–2 outer layers of fibroblasts (BUSTOS-OBREGÓN and HOLSTEIN, 1973; DE KRETZER et al., 1975; DAVIDOFF et al., 1990). These cells are separated by thin layers of extracellular glycosaminoglycans, proteoglycans and collagen fibres (DAVIDOFF et al., 1990; KREIS and VALE, 1993; UNGEFROREN et al., 1995). In contrast with results in experimental animals, the exact structure and function of myofibroblasts is still not well known. This is due in part to the complicated texture of the lamina propria of seminiferous tubules in the human testis. In the rat testis the peritubular tissue consists of only one layer of myoid cells, and therefore has been well investigated (CLERMONT, 1958; LACY and ROTBLAT, 1960; LEESON and LEESON, 1963; ROSS, 1967; TOYAMA, 1977; VIRTANEN et al., 1986; MAEKAWA et al., 1991, 1995). The myofibroblasts of the rat seminiferous tubules possess bundles of filaments (stress fibres) and express the cytoskeletal proteins desmin, α -smooth muscle actin and smooth muscle myosin (SKALLI et al., 1986; BENZONANA et al., 1988; SAPPINO et al., 1990, for review).

It is assumed that myofibroblasts originate from fibroblasts and that the myofibroblasts of different vertebrate tissues represent a heterogeneous cell population in terms of their content of cytoskeletal proteins (GABBIANI et al., 1981; SKALLI et al., 1980;

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SAPPINO et al., 1990). Four cell types are characterized relative to myofibroblasts in connective tissue during wound healing (BENZONANA et al., 1988; SKALLI et al., 1989). KOHNEN et al. (1995) described the gradual differentiation of different forms of myofibroblasts from fibroblasts that are originally vimentin immunoreactive, during the normal development of the human placenta.

The innermost myofibroblasts of the lamina propria of human seminiferous tubules are attached to the basal lamina of the germinal epithelium. Earlier studies showed that myofibroblasts of the human lamina propria express vimentin, desmin, actin and to a lesser degree, smooth muscle myosin (LONGTINE et al., 1985; VIRTANEN et al., 1986; DAVIDOFF et al., 1990). A more detailed characterization concerning the actin-immunoreactivity was performed recently by KOHNEN et al. (1995) who showed that cells in the lamina propria of human testis exhibit α -smooth muscle actin-, GB-42- (see HSI and YEH, 1988), desmin- and vimentin-immunoreactivity.

Under pathological conditions the basal lamina and the connective tissue layers between the myofibroblasts may be thickened (SÖDERSTRÖM, 1986; CHRISTL et al., 1988; HOLSTEIN et al., 1988; CHRISTL, 1990), indicating that the myofibroblasts are not only contractile cells but also exhibit secretory activity. Most myofibroblasts of the thickened lamina propria of the human testis have lost their ability to express desmin (DAVIDOFF et al., 1990), suggesting that they may change their phenotype (SKALLI et al., 1989). Changes in the phenotype of myofibroblasts were observed previously in connective tissue during experimental wound healing (see TOMASEK, 1988; SAPPINO et al., 1990) and the normal development of the placental stroma (KOHNEN et al., 1995).

Some cytokines and components of the extracellular matrix of connective tissue may exert important influences on the differentiation of myofibroblasts (see SAPPINO et al., 1990, for review). There is little information in the literature concerning the location of such components in the lamina propria of the human testis (HADLEY et al., 1985; DAVIDOFF et al., 1990; UNGEFROREN et al., 1995). It is believed that the structural and biochemical heterogeneity of myofibroblasts reflects different functional features of these cells (see SAPPINO et al., 1990). In the lamina propria of the seminiferous tubules the contractile myofibroblasts may be involved in the transport of spermatozoa towards the rete testis and the epididymis (ROOSEN-RUNGE, 1951). There is no evidence concerning the innervation or regulation of the contractile activity of myofibroblasts in the lamina propria of human seminiferous tubules. Recently, it was

presumed that the NO/cGMP system, revealed in the Leydig cells of the human testis, may be involved in the motility of the seminiferous tubules (DAVIDOFF et al., 1995).

The aim of the present investigation was: 1) to give a detailed and precise description of the structural features and the phenotype diversity of the connective tissue cells and some components of the extracellular matrix of the lamina propria of the human testis by means of light and electron microscopy as well as immunocytochemistry; 2) to establish to what extent components of the NO/cGMP pathway (nitric oxide synthase I, soluble guanylyl cyclase, cyclic GMP, glutamate, calmodulin, calcium-calmodulin dependent protein kinase II) are located within structures of the lamina propria and contribute to the functional activity of myofibroblasts; and 3) to establish the location of some cytokines (basic fibroblast growth factor, transforming growth factor- β) and extracellular matrix components (laminin, collagen IV, fibronectin, heparan sulphate- and chondroitin sulphate-proteoglycan) that may be important for the differentiation of heterogeneous phenotypes of myofibroblasts.

MATERIALS AND METHODS

Testes were obtained from 18 men, aged 65–78 years, who were undergoing orchidectomy as a primary treatment for prostatic cancer before the onset of hormonal therapy. The spermatogenic activity in these testes was evaluated by means of semithin sections using a score count (DE KRETZER and HOLSTEIN, 1976), which was in the range of SC 8–10 representing slightly reduced or full spermatogenesis.

The testes were processed as follows: Small pieces were fixed in 5.5% glutaraldehyde in 0.05 M phosphate buffer (pH 7.2, 820 mOsm) for 2 h. After dehydration in ascending alcoholic series, the material was embedded in Epon 812. Semithin sections for light microscopic examination were free floating, stained with toluidine blue/pyronine G (ITO and WINCHESTER, 1963) or with azur II-methylene blue/basic fuchsin (LACZKÓ and LÉVAI, 1975) and covered with Caedax (Darmstadt, Germany). Ultrathin sections were contrasted with uranyl acetate and lead citrate and investigated in a Phillips EM 300.

The arrangement of myofibroblasts in the lamina propria of seminiferous tubules and dimensions of their size were analyzed on electron micrographs of cross and longitudinally sectioned seminiferous tubules. Four series of 38, 44, 45 and 58 tangentially oriented serial thin sections provided the basis for the drawing of Figure 1.

Fig. 1. A large flat myofibroblast is braced by many bundles of microfibrils emanating from the cell surface. The periphery of the myofibroblast body often appears (Figs. 11-5) splitting up in two or more lamellae. Semischematic drawings on the basis of electron micrographs.

Fig. 2. Tangential section of the central part of a myofibroblast. Intracytoplasmic stress fibres and microfilaments bracing the cell in the extracellular collagen fibrils texture are visible. *Arrows* indicate the direction of microfilaments bracing the myofibroblast in the surrounding connective tissue. $\times 5,000$

Other portions of the testes were fixed in 3% paraformaldehyde with 0.1 M phosphate buffer or in the Bouin's solution. This material in part was embedded in paraffin for immunocytochemistry.

Part of the material was used for preparing frozen sections or was immersed in 0.1% collagenase (Wako Co, Japan) for the isolation of single seminiferous tubules (MAEKAWA et al., 1994). For the demonstration of actin filament bundles (stress fibres), frozen sections of 25 μm thickness or collagenase-pretreated isolated tubules were incubated with 7×10^{-5} M FITC-labeled phalloidin (Sigma, USA) for 1 h at room temperature. Additionally, propidium iodide (Sigma) was used for staining the nuclei of myofibroblasts. The specimens were washed with phosphate-buffered saline (PBS), mounted with Perma Fluor (Immunon, USA) and investigated with a confocal laser microscope (Bio-Rad, MRC 600). Planes at a 0.6 μm thickness were pictured. The lamina propria of tubule fragments of the testes of three patients was selected

and dissected by the confocal laser microscope in 16 up to 24 images. In part, 5–7 images were combined to result in one computer image. In frozen sections the peritubular lamina propria of the testes of 5 patients was dissected by the microscope in 16 up to 23 images.

Three additional testes were perfused via the testicular artery with 2% paraformaldehyde in PBS (960 mOsm); after overnight immersion in 20% sucrose in 0.1 M phosphate buffer and freezing with liquid nitrogen, 20 μm thick sections were mounted onto poly-L-lysine precoated slides. The sections were then incubated with 2 $\mu\text{m}/\text{ml}$ FITC- or TRITC-phalloidin (Sigma) in PBS for 45 min. After rinsing with PBS in a part of the sections, the nuclei were stained for 20 min at 37°C with 0.2 μg DAPI in 1 ml PBS. After rinsing, the sections were covered with Mowiol and observed in a fluorescence microscope using appropriate filter combinations.

For the immunocytochemical investigations 10 μm

Fig. 3. Tangential section of peripheral parts of myofibroblasts. The following layers of the lamina propria are identifiable: 1) basal part of a spermatogonium; 2) basal lamina; 3) collagen fibrils of the first extracellular connective tissue layer; 4) myofibroblast; 5) second extracellular layer with a lower content of collagen fibrils; 6) myofibroblast, etc. $\times 3,500$

frozen or 6 μm paraffin sections were used. The sections were incubated with the following primary antisera directed against: *smooth muscle myosin* (monoclonal, Sigma; optimal dilution 1:200 for paraffin sections treated with microwaves, 1:500 for frozen sections), α -*smooth-muscle-actin* (monoclonal, Sigma, USA, 1:400), GB 42 (monoclonal, recognizing an isoform of actin, KOHNEN et al., 1995; staining performed in cooperation with Dr. KOHNEN, Aachen, Germany, 1:10), *desmin* (monoclonal D 33, Dakopatts, Denmark, 1:300), *actin* (polyclonal; Sigma, USA; 1:100), *actin* (monoclonal; Amersham, England; 1:1000), *vimentin* (monoclonal, Dakopatts, Denmark, 1:10), *fibroblast surface protein* (monoclonal, Sigma, USA, 1:500), *basic fibroblast growth factor* (polyclonal Sigma, USA, 1:2000), *transforming-growth factor- β* (monoclonal, Serotec,

France, 1:700), *fibroblast surface protein* (monoclonal, Sigma, USA, 1:500), *nitric oxide-synthase I* (kindly provided by Dr. MAYER, Graz, Austria, 1:1000), *endothelial nitric oxide synthase* (polyclonal, Biomol, USA, 1:250), *glutamate* (polyclonal, Sigma-USA, 1:1000), *cyclic guanosine-monophosphate* (polyclonal, Biogenesis, USA, 1:500), *soluble guanylyl cyclase* (kindly provided by Dr. KOESLING, Berlin, Germany, 1:200), *calmodulin* (polyclonal, Polysciences Inc., USA, 1:200), *calcium-calmodulin-dependent protein kinase II* (monoclonal, Boehringer, Germany, 1:1000), *CN-Pase* (2',3'-cyclic nucleotide-3'-phosphodiesterase; Boehringer, Germany, 5 $\mu\text{g}/\text{ml}$), *laminin* (monoclonal, Sigma, USA, 1:2000), *collagen type IV* (monoclonal, Dakopatts, Denmark, 1:100), *fibronectin* (monoclonal, BioGenex, USA, 1:40), *heparan sulphate* (monoclonal, Boehringer, Germany, 4 $\mu\text{g}/\text{ml}$),

Fig. 4. Cross section of the lamina propria. 1) Basal part of the germinal epithelium, 2) basal lamina, 3) first extracellular layer of collagen fibrils, 4) myofibroblast, 5) second extracellular layer with a lower content of collagen fibrils, 6) myofibroblast, 7) extracellular connective tissue layer, 8) myofibroblast, 9) extracellular connective tissue layer, 10), 11), 12) fibroblasts. $\times 11,500$

Fig. 5. Sections of the peripheral cytoplasm of myofibroblasts. Only punctuated contacts exist between neighbouring cells (arrows). Contractile filaments (F) cross the cytoplasm. The cell membrane exhibits small spiny processes or ridges where the microfilaments of the connective tissue are fixed (double arrow). $\times 40,000$

chondroitin sulphate (monoclonal, Sigma, USA, 1:200), *galactocerebroside* (monoclonal, Boehringer, Germany, 5 $\mu\text{g}/\text{ml}$), and S 100 protein (polyclonal, Sigma, 1:100).

For the visualization of the antigens under study, an amplification procedure (PAP-ABC) previously described by DAVIDOFF and SCHULZE (1990) with or without additional amplification with nickel (ZÁBORSKI and LÉRÁNT, 1985) was applied. In the second step of the immunostaining, biotinylated anti-mouse- or anti-rabbit IgG (Dakopatts, Denmark, 1:250) were used and in the third and fourth steps, rabbit-PAP (1:200) or mouse-PAP (1:100) and a preformed Elite avidin-biotin-peroxidase complex (Vector, USA, 1:250) were

employed.

As controls, sections were used in which: 1) the primary, secondary or tertiary antibodies were replaced by PBS; 2) only the peroxidase activity was visualized; 3) for negative controls some of the antibodies had been preabsorbed for 24 h at 4°C with 5–50 μg of the corresponding antigens/ml antiserum at its working dilution; 4) instead of the primary antisera, sections were incubated with normal mouse- or rabbit serum (Sigma) or purified mouse- or rabbit IgGs (Sigma) at concentrations of 0.01% to 0.1%; or 5) sections were incubated with antiserum against *vasoactive intestinal polypeptide* and *somatostatin*.

Fig. 6. Tangential section of a bundle of microfibrils (*arrowheads*) of the connective tissue attached to the cell membrane of a myofibroblast. $\times 40,000$

Fig. 7. Obliquely sectioned myofibroblast showing many microfibrils of the connective (*Mf*) tissue attached to the cell membrane. $\times 40,000$

RESULTS

Structure and phenotype of the myofibroblasts

Myofibroblasts of the lamina propria of seminiferous tubules in the human testis are flat, multangular cells of 40–60 μm in diameter (Fig. 1). Their diameter was only measurable in low magnified electron micrographs of cross sectioned seminiferous tubules. Because of their size, their thickness of 1–4 μm and spatial orientation to the curved lamina propria, tangential sections comprised only parts of a myofibroblast, e.g. the nucleus and perinuclear cytoplasm (Fig. 2) or a ring-like aspect of the peripheral cytoplasm (Fig. 3). In electron micrographs, the flat and often bean-shaped nucleus was about 10 μm in diameter and surrounded by many small mitochondria, a small Golgi complex, some cisterns of rough endo-

plasmic reticulum and abundant free ribosomes. Many strands of smooth muscle filaments (actin filaments, myosin filaments, dense patches) crossed the cytoplasm, some of them encircling the nucleus. These filaments were affixed at the attachment plaques of the cell membrane which in part protruded in the form of short spiny processes or ridges (Fig. 5). Most of the attachment plaques of the myofibroblasts face small areas of their discontinuous basal lamina, where microfibrils of the extracellular tissue are fixed (Figs. 6, 7). These microfibrils, at some distance from the myofibroblast, are intermingled with collagen fibrils and therefore apparently brace the myofibroblasts between the connective tissue layers.

Many of the cytoplasmic ridges follow the direction of contractile intracytoplasmic filaments and external adjacent microfibrils (Fig. 2). The number of ridges was lower in the region of the nucleus and

Fig. 8. Semithin section of two neighbouring seminiferous tubules stained by the Laczkó-Lévai-method. The first extracellular tissue layer of the lamina propria is intensely red stained (*arrows*); the outer layers only show patchily stained components (*double arrow*). $\times 800$

Fig. 9. Phalloidin-FITC stained stress fibres in the outer (**a**) and the inner (**b**) myofibroblasts of the lamina propria. The thickness and arrangement of fibres are different. Figure **a** represents the 5th and **b** the 15th of 21 planes of the lamina propria dissected by the confocal laser microscope. The nuclei of myofibroblasts are stained red by propidium iodide. $\times 600$

increased in the cell periphery. Parts of the periphery appeared asterisk-like. Between the areas of spiny cytoplasmic processes, the plasma membrane exhibited a number of typical caveolae.

The myofibroblasts are individuals and do not form continuous cell layers. Only punctuated cellular contacts between partly overlapping neighbouring cell processes were visible (Fig. 5). The cytological aspect of the myofibroblasts is highly complicated by the fact that the periphery of the large flat cell body was often split up into two or more layers (Fig. 1). In the lamina propria of seminiferous tubules with normal spermatogenesis, uni- or bi-layered myofibroblasts were seen. Under pathological conditions of reduced spermatogenesis and thickened lamina propria, multi-layered myofibroblasts appeared.

Optimal visualization of the actin bundles of the myofibroblasts was obtained with the FITC-phalloidin/propidium iodide staining method with subsequent investigation in a confocal laser-microscope. The best results showed whole-mounted seminiferous tubules prepared free from the intertubular tissue by collagenase digestion.

In the confocal laser-microscope, planes of 0.6 μm exhibit bright fluorescent bundles of actin filaments (Fig. 9). They are arranged parallel and cross a myofibroblast in a preferential direction. Only in a few neighbouring cells do the actin filaments continue in the same direction, but not all cells of the same layer of the lamina propria show the same orientation of filaments. In the images of all planes, rectangular crossing actin filaments appeared, representing parts of identical myofibroblasts with bilayered peripheral cell bodies. The actin filaments in different cytoplasmic layers of one myofibroblast followed different directions.

Following the series of dissected planes, it becomes evident that the actin filaments in the outer myofibroblast layers were longer, thicker and stained stronger than in the innermost myofibroblasts (Fig. 9a, b). In the layers next to the germinal epithelium, the actin filaments were rather short and thin and showed no preferential direction.

For a more detailed cytological analysis and to establish some functional features of the myofibroblasts, a number of cellular antigens were investigated. A strong immunoreactivity for the intermediate filament protein *vimentin* was observed within the myofibroblasts and fibroblasts of all layers of the lamina propria, the outermost fibroblast layer showing the strongest staining intensity (Fig. 10). A strong vimentin immunoreactivity possessed—in addition—the Sertoli cells, the majority of the connective tissue cells of the intertubular tissue and the tunica al-

buginea. Vimentin staining was observed also in the endothelial cells, the adventitia of all blood vessels and only the tunica media of larger arteries. Immunoreactivity for *α -smooth muscle actin* and *GB-42 antigen* was detected in the innermost three layers of myofibroblasts of the lamina propria (Fig. 11), the smooth muscle cells of the blood vessel walls and the myofibroblast bundles of the tunica albuginea of the testis. The GB 42-immunoreactivity (the antigen is a 43 kD protein, sharing great similarity with γ -smooth muscle actin; KOHNEN et al., 1995), showed a lower staining intensity within the myofibroblasts of the lamina propria, moderate staining in the myofibroblasts of the tunica albuginea and very strong intensity of staining in the smooth muscle cells of the blood vessels. *Pan actin*-immunoreactivity, containing the β - and γ -cytoplasmic isoforms of actin, was seen within the innermost layers of myofibroblasts, the fibroblasts of the outer layers of the lamina propria, cells of the tunica albuginea, the Sertoli cells of the seminiferous tubules, some cells of the intertubular tissue and—with a stronger intensity of the staining—in the tunica media of the larger blood vessels. Immunoreactivity for *smooth muscle myosin* was found in the three or four layers of myofibroblasts situated beneath the germinal epithelium as well as in the myofibroblasts of the tunica albuginea, and with stronger staining intensity in the smooth muscle cells of arteries and arterioles of different sizes (Fig. 12). The best results for the visualization of this antigen were obtained after pretreatment of paraffin sections with citrate buffer in a microwave oven. The same location as for the smooth muscle myosin was observed for the intermediate filament protein *desmin* with the difference that lower numbers of myofibroblasts were immunoreactive (Fig. 13). *Fibroblast surface protein* immunoreactivity was found predominantly in the cells of the outer layers of the lamina propria (Fig. 18) as well as in a number of connective tissue cells of the intertubular tissue. Staining for the *basic fibroblast growth factor* was seen in a number of cells of the lamina propria and in a larger number of surrounding connective tissue cells, within most Sertoli cells, the germ cells and cells of the intima and media of larger arterial vessels. Immunoreactivity of *transforming growth factor- β* (TGF- β) was detectable in single cells of the innermost lamina propria as well as in fibroblasts, macrophages and monocyte-like cells of the intertubular tissue and in the tunica albuginea. Distinct staining was seen also for a number of Sertoli cells. Immunoreactivity for *CNPase* was observed in myofibroblasts and with stronger staining intensity within the fibroblasts of the outer layer of the lamina propria and the

Figs. 10-24. Segments of the lamina propria of human seminiferous tubules after immunocytochemical staining for different antigens. Magnification of all figures: $\times 630$

Fig. 10. Vimentin-immunoreactivity in two adjacent tubules. Distinct staining shows numerous peritubular cells and several Sertoli cells. Note the stronger staining intensity of the outer peritubular cells and especially the fibroblasts (*arrow*) at the periphery of the tubules.

Fig. 11. α -Smooth muscle actin-immunoreactivity in myofibroblasts of the lamina propria and smooth muscle cells of a small blood vessel.

Fig. 12. Smooth muscle myosin-immunoreactivity in the innermost 3-4 layers of the lamina propria and in smooth muscle cells of a blood vessel.

Fig. 13. Desmin-immunoreactivity in myofibroblasts of the innermost layers of the lamina propria.

Fig. 14. NOS I-immunoreactivity within cells located predominantly at the periphery of the lamina propria.

Fig. 15. NOS III immunoreactivity located within myofibroblasts and fibroblasts of all layers.

Fig. 16. cGMP-immunoreactivity within peritubular cells of all layers.

Fig. 17. S 100-immunoreactivity in a thin nerve fibre bundle running at the periphery of a seminiferous tubule (*arrow*).

Fig. 18. Fibroblast surface protein-immunoreactivity within some fibroblasts of the outer layers.

Fig. 19. CNPase-immunoreactivity located within a number of peritubular cells, preferentially of the outer layers.

Fig. 20. Galactocerebroside-immunoreactivity is seen within a number of cells situated in different layers.

Fig. 21. Chondroitin sulphate-immunoreactivity in the extracellular matrix of all layers.

Fig. 22. Laminin-immunoreactivity is distributed most strongly in the basal lamina and with a lower staining intensity in some segments of the outer layers.

Fig. 23. Collagen type IV-immunoreactivity in two adjacent seminiferous tubules. The reaction precipitate is located in the basal lamina and is discontinuous in the extracellular matrix of most layers.

Fig. 24. Fibronectin-immunoreactivity is predominantly located in the extracellular matrix of the outer layers, but in some tubules a strong staining intensity is seen in the layer beneath the basal lamina.

connective tissue cells of the interstitium (Fig. 19). The endothelial cells of some vessels also exhibited CNPase immunoreactivity. *Galactocerebroside*-immunoreactivity was located within a large number of cells of all layers of the lamina propria as well as in numerous cells of the intertubular tissue (Fig. 20).

Immunoreactivity for the representatives of the NO/cGMP system as well as substances regulating the constitutive NO-synthases was observed as follows: low to moderate *neuronal nitric oxide synthase*-immunoreactivity (NOS-I) was detected in single cells of the inner but predominantly of the outer layers of the lamina propria (Fig. 14), whereas lamina propria cells in all layers were sGC- and cGMP-immunoreactive (Fig. 16). Moderate to low NOS-I staining intensity was observed in a number of myofibroblasts of the tunica albuginea and single cells in the interstitium of the testis. In contrast to NOS-I, a larger number of myofibroblasts of the tunica albuginea possessed moderate to strong sGC- and cGMP immunoreactivities. The endothelial cells of some blood vessels and smooth muscle cells of their walls also contained sGC and cGMP-immunoreactivities. Moderate NOS-I immunoreactivity was seen in the Sertoli cells of the seminiferous tubules. Immunoreactivity for the *endothelial nitric oxide synthase* (NOS-III) was seen in a larger number of myofibroblasts in the innermost three layers of the lamina propria (Fig. 15) and in the myofibroblasts of the tunica albuginea, in the endothelial cells of large blood vessels as well as in smooth muscle cells of large and small vessels. The staining intensity of the endothelial cells of the small vessels (arteries, arterioles and capillaries) was lower. A number of cells in the connective septula testis and in the interstitium also possessed moderate NOS-III immunoreactivity. Moderate *calmodulin* immunoreactivity showed a number of lamina propria cells distributed in all layers. In addition, endothelial cells and cells of the tunica media and adventitia of blood vessels and connective tissue cells of the interstitium possessed strong staining intensity. Numerous germ cells, especially some spermatids, were also positive. Immunoreactivity for the *calcium/calmodulin-dependent protein kinase II* appeared predominantly in the cells of the outer layers of the lamina propria and in connective tissue cells of the interstitium. In these cells immunoreactivity for *glutamate* was also detected. Electron microscopically, no direct innervation of myofibroblasts was found. However, light microscopically (immunoreactivity for *S100 protein*) in the lamina propria of single seminiferous tubules, small nerve bundle fragments and single nerves could be observed (Fig. 17).

Heterogeneity of myofibroblasts

Taken together the results obtained provide evidence for the existence of a distinct heterogeneity of myofibroblasts in the human testis:

1. There were clear differences in the morphology and distribution pattern of actin filaments and the other contractile proteins between the innermost and outermost cellular layers of the lamina propria.
2. Only a small number of cells of the lamina propria exhibited GB 42 immunoreactivity, in contrast to the larger number of cells which were immunoreactive for smooth muscle myosin, α -smooth muscle actin and vimentin, allowing the presumption of the existence of a special GB 42 immunoreactive population of myofibroblasts.
3. The outer cell layers of the lamina propria possessed a strong fibroblast surface protein immunoreactivity.
4. Small numbers of cells in the outer layers of the lamina propria exhibited the neural type of nitric oxide synthase (NOS-I)-, calcium/calmodulin dependent protein kinase II- and glutamate immunoreactivity. In contrast, a larger number of innermost layer myofibroblasts predominantly expressed the endothelial form of nitric oxide synthase (NOS-III).

Extracellular matrix of the lamina propria of seminiferous tubules

The germinal epithelium of seminiferous tubules was underlined by a bilamellar basal lamina of 250 nm thickness, readily visible in electron micrographs. The first layer of the extracellular matrix adjacent to the basal lamina showed densely packed collagen fibrils mostly oriented parallel to the long axis of the seminiferous tubule. They were embedded in an amorphous fine granular ground substance. In the following layers of the intercellular matrix, the number of collagen fibrils was remarkably reduced (Fig. 4). Small strands of collagen fibrils were intermingled with bundles of microfibrils and formed a loosely organized network. The microfibrils were attached to the basal lamina fragments. They faced the above described small spiny processes or ridges of the membrane of myofibroblasts (Figs. 1, 6), bracing them in the fibre network. The amount of microfibrils was highest in the outer extracellular layers (Fig. 7). Typical aspects of elastin fibres as described in the tunica dartos (HOLSTEIN et al., 1974) were missing.

Under the light microscope, the peritubular extracellular matrix showed a quite different aspect in comparison to the intertubular extracellular matrix. After staining the semithin sections with toluidine-

blue/pyronin or with azur II-methylene blue/basic fuchsin, the first layer of the lamina propria attached to the germinal epithelium showed a strong staining intensity (Fig. 8). The remaining layers exhibited only low, patchy staining. Immunohistochemically the following antigens were identified: *Laminin* was located in the lamella of the basal lamina situated beneath the germinal epithelium and with lower staining intensity in the matrix of some peripheral layers (Fig. 22). *Collagen type IV* was seen in both lamellae of the basal lamina and with a lower staining intensity in all peritubular layers. The strongest staining intensity of collagen type IV was located in the connective tissue layer surrounding blood vessels of the intertubular space (Fig. 23). *Heparan sulphate* and *chondroitin sulphate* showed a strong staining intensity mainly in the inner layers of the lamina propria, but reaction precipitate was seen also in the remaining layers (Fig. 21). A lower staining intensity was observed in the tunica media and adventitia of blood vessels. Chondroitin sulphate immunoreactivity was also visible on the surface of some Leydig cells. Immunoreactivity for the extracellular adhesive glycoprotein *fibronectin* was predominantly found (Fig. 24) in the outer layers of the lamina propria.

DISCUSSION

Myofibroblasts of the lamina propria of human seminiferous tubules are large, flat and slightly bent individuals braced in a network of microfibrils and collagen fibrils. They represent discontinuous cell layers with interposed layers of an extracellular matrix. Many myofibroblasts show a splitting up of their cytoplasm in two or more layers where actin filaments of divergent directions are enclosed.

This unique cell shape of a myofibroblast surrounding a human seminiferous tubule differs markedly from other myofibroblasts in the human genital tract, e.g. in the coat of the ductuli efferentes or of the ductus epididymidis (HOLSTEIN, 1969), in the tunica dartos (HOLSTEIN et al., 1974) and from testicular peritubular myoid cells of other species excluding some higher vertebrates and primates (CLERMONT, 1958; LACY and ROTBLAT, 1960; LEESON and LESSON, 1963; ROSS, 1967; CHRISTL, 1990; MAEKAWA et al., 1991; PALOMBI et al., 1992). The myoid nature of the cells of the lamina propria of the guinea pig, rat and human seminiferous tubules was earlier proved (GRÖSCHEL-STEWART and UNSICKER, 1977; VIRTANEN et al., 1986; SKALLI et al., 1986; BENZONANA et al., 1988). The electron microscopic study by ROSS and LONG (1966) revealed the "contractile-type" of human

peritubular connective tissue. BÖCK et al. (1972) concluded, on the basis of electron microscopic results, that the myoid cells of the lamina propria of human seminiferous tubules are myofibroblasts. Recently this was confirmed for the cells of the innermost layers of the lamina propria of the human testis by immunocytochemistry. It was shown that these cells co-express smooth muscle cell- and connective tissue cell-antigens (DAVIDOFF et al., 1990). KOHNEN et al. (1995) reported that, in human myofibroblasts, in addition to vimentin and desmin, α -smooth muscle actin and GB-42 immunoreactivity could be established. However, the results of the present study showed that in the human testis, GB-42 possessed lower staining intensity and occurred in a lower number of cells than α -smooth muscle actin-, smooth muscle myosin- and desmin-immunoreactivities. This fact raised the possibility of the existence of two types of myofibroblasts (KOHNEN et al., 1995). As for the distribution of the actin isoforms, the myofibroblasts of the lamina propria differ from the smooth muscle cells of the vessel walls, in contrast with the results obtained for the human placenta (GABBIANI et al., 1981). The myofibroblasts of the lamina propria showed lower GB-42-, pan-actin- and higher vimentin-immunoreactivities, providing evidence for substantial differences in the phenotype between smooth muscle cells and myofibroblasts.

The results of the present investigation show that the myofibroblasts of the lamina propria of the human seminiferous tubules exhibit micro- and intermediate filament proteins characteristic for both fibroblasts and smooth muscle cells such as vimentin, smooth muscle myosin, desmin, α -smooth muscle actin, cytoplasmic actin, GB-42 antigen. The outer layers of myofibroblasts contain thick and parallel oriented actin filaments (stress fibres), whereas in the cytoplasm of the innermost myofibroblasts, fine-structured actin filaments could be observed. These differences probably reflect the presence of various isoforms of actin filaments and allow one to define the first three cell layers situated beneath the basal lamina of the human seminiferous tubules as true myofibroblasts. Our earlier studies showed that, in addition to cells that co-express vimentin- and desmin-immunoreactivities (see VIRTANEN et al., 1986), there were cells of the lamina propria positive only for desmin or only for vimentin (DAVIDOFF et al., 1990). In addition, in cases with a thickening of the lamina propria, the myofibroblasts show only vimentin-like immunoreactivity, indicating that their phenotype changed towards true fibroblasts. Thus, in accordance with previous findings (see BENZONANA et al., 1988; TOMASED, 1988; SAPPINO et al., 1990; DARBY et

al., 1990), it can be presumed that the myofibroblasts of the lamina propria of the human testis represent a heterogeneous cell population. Their heterogeneity most probably reflects differentiation stages of these cells and the corresponding diversity of their functional features (SKALLI et al., 1989; KOHNEN et al., 1995). Interestingly enough, the myoid cells of the tunica albuginea of the human testis possess the same immunocytochemical characteristic, thus providing evidence for their myofibroblastic nature.

The outer one or two layers of the lamina propria cells could be defined as pure fibroblasts because they do not exhibit antigens characteristic for smooth muscle cells. They possess stronger vimentin- and fibroblast surface protein-immunoreactivity. The functional significance of these cells is not known. They may represent a supporting sheath important for the stability of the seminiferous tubules. On the other hand, these fibroblasts constitute a border layer that demarcates a narrow space between the smooth surface of the seminiferous tubules and the interstitial tissue sheet facing the tubules. This space may be important for the motility of the seminiferous tubules.

The myofibroblasts of the lamina propria of seminiferous tubules of the rat and dog presumably are contractile structures responsible for the peristaltic activity of the tubules transporting spermatozoa towards the rete testis and epididymis (ROOSEN-RUNGE, 1951; BÖCK et al., 1972; HARGROVE et al., 1977). Movements of the human seminiferous tubule have never been analyzed *in vivo*. Consequently, physiological data on the human tubular movements are not known, in contrast to the spontaneously rhythmic and local contractions of 0.2 Hz that has been recorded in the rat seminiferous tubule (NAGANO et al., 1994). Presumably, the myofibroblasts of the human testis resemble functionally contractile cells of other organs and myoepithelial cells of exocrine glands (see SKALLI et al., 1986; BENZONANA et al., 1988). The expected contractile function of the myofibroblasts of the lamina propria of the human testis may also influence the permeability and transport throughout the lamina propria. In comparison with several laboratory animals, the multilayered lamina propria of human seminiferous tubules probably allows a more precise regulation of the transport processes between the intertubular space and the seminiferous tubules.

An unresolved problem concerns the regulatory mechanisms of the contractile activity of myofibroblasts in the lamina propria. Our electron microscopic observations showed that the myofibroblasts are not directly innervated by peripheral nerves. Thus, their paracrine influence via neuroendocrine substances

released by nerves running at the periphery of the lamina propria or by Leydig cells must be taken into consideration. There is evidence that endothelin I initiates the contraction of the peritubular myofibroblasts (FILIPPINI et al., 1993). Presumably the neurohypophyseal hormones vasopressin and oxytocin that are produced in the testis (PICKERING et al., 1989) lead to contraction of myofibroblasts. HOWL et al. (1995) described the expression of vasopressin receptors in myofibroblasts of the rat lamina propria. There is also evidence that after TGF- β application the shape of testicular peritubular cells in culture changed and the degree of their contractility was markedly enhanced (AILENBERG et al., 1990). As the present study shows, in addition to Sertoli cells, some of the peritubular cells also express TGF- β . This fact supports the idea that TGF- β may act, among other factors, as a messenger between adjacent peritubular cells, affecting their contractile state and contributing to the rhythmic contractions of the seminiferous tubules. Concerning the relaxation of myofibroblasts, the present study suggests that their contractile tone may be partially influenced by the NO/cGMP system. The fact that myofibroblasts of the lamina propria of seminiferous tubules and the tunica albuginea of the testis exhibit NOS-III, NOS-I, sGC- and cGMP-like immunoreactivities as well as immunoreactivities for agents that are known to regulate the constitutive NOS-activity such as calmodulin, calcium/calmodulin dependent protein kinase II and glutamate, shows that cells of the lamina propria are able to produce NO. This newly discovered gaseous messenger may be active in intracrine, autocrine and paracrine fashions and may influence the contractility of myofibroblasts. Additionally, NO produced by the interstitial Leydig cells may also influence the contractile tone of the lamina propria myofibroblasts (DAVIDOFF et al., 1995).

In addition, NO was established in other organs to mediate the action of vasopressin (OTA et al., 1993; YASIN et al., 1993). New findings have shown that endothelin-1 and NO both participate in regulating the basal tone of cerebral arteries in dogs (HIROSE et al., 1995). Moreover, as established for the cerebral vessels of the cat, endothelin-1 in higher concentrations mediates its action through the ET_B receptor subtype that leads to the subsequent induction of the NOS and production of NO (KOBARI et al., 1994). It is noteworthy that the peristaltic waves of the lamina propria and the tunica albuginea of the testis are driven by complex regulatory mechanisms involving a number of autocrine and paracrine factors.

The present investigation shows that cells of the lamina propria of human seminiferous tubules

express the cytokines *fibroblast growth factor* and *transforming growth factor- β* and *extracellular matrix* components such as *laminin*, *collagen type IV*, *fibronectin*, *chondroitin sulphate* and *heparan sulphate* (in addition see HADLEY et al., 1985; DAVIDOFF et al., 1990). Recently, it was confirmed that cells of the lamina propria express also the small proteoglycans *biglycan* and *decorin* (UNGEFROREN et al., 1995). The relationship between these agents and their exact functional significance in the lamina propria is still not known. However, in addition to their morphogenetic role and contribution to the structural integrity of connective tissue architecture (UNGEFROREN et al., 1995) their presence in the lamina propria may be of importance for the differentiation of multiple myofibroblast phenotypes (SAPPINO et al., 1990). Altered expressions of these substances may be responsible for the thickening of the lamina propria and myofibroblast dedifferentiation of the human seminiferous tubules. Concerning the existence of elastic fibres in the lamina propria, distinct differences exist between light- and electron microscopic results. Similar differences were earlier described by WIEDMER-BRIDEL et al. (1978), who showed that elastic fibres could be demonstrated in the lamina propria of human testis only after araldite embedding of the material. However, in the lamina propria of the human testis, the amount of elastin seems to be very low and does not correspond to the amount of "pseudo-elastica"-positive material seen under the light microscope (BÖCK, 1978).

One interesting result is represented by the established immunoreactivity for CNPase and galactocerebroside in the cells of the lamina propria and in some cells of the intertubular tissue. These antigens were already used as markers for oligodendrocytes and Schwann cells of the nervous system (REGAN, 1988). In general, the CNPase- and galactocerebroside-immunoreactivity of the lamina propria and intertubular cells may manifest some structural and biochemical similarities with glia cells of the nervous system. Under the presumption that Leydig cells in part may be derivatives of the neural crest (SCHULZE et al., 1987; DAVIDOFF et al., 1993), it could be supposed that some concomitant connective tissue cells of the testis also originate from the neural crest.

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