Immunocytochemistry of Extracellular Matrix in the Lamina Propria of the Rat Testis: Electron Microscopic Localization

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ABSTRACT

The distribution of laminin, type IV collagen, heparan sulfate proteoglycan, and fibronectin was investigated in the rat testicular lamina propria by electron microscopic immunocytochemistry. Distinct patterns were observed for each antigen within the extracellular matrix (ECM) layers of the lamina propria. Laminin, type IV collagen, and heparan sulfate proteoglycan all localized to the seminiferous tubule basement membrane. Type IV collagen and heparan sulfate proteoglycan, but not laminin, localized to the seminiferous tubule side of the peritubular myoid cells. All four of the antigens were localized between the peritubular and lymphatic endothelial cells. Failure to localize fibronectin in the ECM layer between the Sertoli and peritubular myoid cells tends to support the concept that adult Sertoli cells do not produce this protein in vivo. Intracellular immunostaining was insufficient to allow unambiguous identification of the cellular source of any of the ECM molecules.

INTRODUCTION

It has become increasingly clear over the last three decades that the extracellular matrix (ECM) is more than an inert scaffolding around cells. ECM profoundly influences the development and expression of differentiated cell structures and functions (for reviews, see Kleinman et al., 1981; Gospodarowicz, 1984; Hay, 1984; Reid and Jefferson, 1984). In the adult rat testis, the seminiferous tubules are separated from the interstitium by a series of noncellular and cellular layers collectively called the lamina propria. Although a number of investigators (Leeson and Leeson, 1963; Dym and Fawcett, 1970) have described the ultrastructural appearance of this boundary tissue, little was known until recently about the nature of the ECM, which occupies the noncellular layers of the testicular lamina propria.

Paranko et al. (1983) and Pelliniemi et al. (1984) have described the developmental appearance of fibronectin, laminin, and type IV collagen during testicular cord formation in the fetal rat. Tung et al. (1984) localized fibronectin at the light microscopic level to the boundary tissue of adult rat seminiferous tubules. Hadley et al. (1985b) have demonstrated laminin, types I and IV collagens, and heparan sulfate proteoglycan in the lamina propria of the human seminiferous tubules, and fibronectin and type I collagen in the interstitial tissue. Heparan sulfate was also shown to specifically stain the Charcot-Bottcher crystalloids within the human seminiferous epithelium (Hadley et al., 1985b).

In this study, we demonstrate the ultrastructural location of laminin, type IV collagen, heparan sulfate proteoglycan, and fibronectin within the lamina propria of the rat testis. This is the first electron microscopic immunocytochemical localization of these ECM components in the adult testis.

MATERIALS AND METHODS

Antisera

Laminin, type IV collagen, and fibronectin antisera used in this study were a gift of Dr. Hynda K. Kleinman (NIDR, NIH, Bethesda, MD). Antibodies to heparan sulfate proteoglycan were kindly provided by Dr. J. R. Hassell (NIDR, NIH). The antisera were raised in rabbits against laminin, type IV collagen, and heparan sulfate prepared from Engelberth-Holm-Swarm (EHS) mouse tumor (Kleinman et al.,
1982) and fibronectin prepared from mouse serum (Rennard et al., 1981). After purification by affinity chromatography, antibody specificity was determined by enzyme-linked immunoassay (ELISA) and antisera were shown to have no cross-reactivity with other extracellular matrix components (Hassell, 1980; Laurie et al., 1982).

Preparation of Tissue for Immunostaining

Electron microscopic localization was performed using the technique of Laurie et al. (1982) with minor modification. The testes of 70-day-old rats (Sprague-Dawley, Charles River Breeding Laboratories, Inc., Wilmington, MA) were fixed by vascular perfusion with 4% paraformaldehyde and 0.2% glutaraldehyde in 0.08 M sodium phosphate buffer (pH 6.0) plus 0.05% saponin. Fixed testes were removed and sliced longitudinally into quarters, then postfixed for 2 h by immersion in the same fixative at 4°C. The tissue was subsequently sliced into 2 × 2 × 8-mm pieces and washed in phosphate-buffered saline (PBS), plus 0.05% saponin (PBSS) for 2 h (5 changes) at 4°C. Next, the tissue was coated in 7% agar at 50°C and allowed to cool to 4°C. The excess agar was trimmed and the tissue cut to 60-μm sections with a Tissue-Chopper (Smith & Farquhar-Sorvall, Newtown, CT). Selected sections were collected and immersed in 0.5% sodium borohydride in PBSS at 4°C for 1 h. The sections were washed in PBSS (two 5-min washes), incubated in PBSS + 30% normal goat serum (NGS) for 20 min, and then washed in PBSS + 1% NGS (PBSSG; two 5-min washes). Twenty sections were placed into 10-ml glass vials for each experimental treatment or control group. Primary antisera and controls (controls included primary antisera pre-absorbed with specific antigen or normal rabbit serum substituted for primary antisera), each in PBSSG, were added in appropriate dilution (dilutions ranged between 1/50 to 1/400) for the various antisera) at a final volume of 800 μl. The vials were then incubated on a rotating wheel overnight at 4°C. The next day, the slices were washed (three 30-min washes) in PBSSG. Eight hundred microliters of a 1/50 dilution of goat anti-rabbit antibody (GAR; Cappel, Malvern, PA) in PBSSG was then added to each vial and incubated for 30 min at room temperature. The sections were washed (three 20-min washes) in PBSSG; then incubated with a 1/50 dilution of peroxidase anti-peroxidase complex (PAP; Cappel) in PBSSG for 10 min at room temperature. The sections were washed (two 5-min washes) in PBSSG and then (two 5-min washes) in PBSS. The sections were postfixed in 2.5% glutaraldehyde in PBSS for 15 min at room temperature. After postfixing, the sections were washed (two 5-min washes) in PBS (no saponin) and then (two 5-min washes) in 0.05 M tris(hydroxymethyl)aminomethane (Tris) HCl buffer, pH 7.6 (TRIS). The sections were incubated for 10 min in 0.05% 3,3-diaminobenzidine tetrahydrochloride (DAB) plus 0.02% H2O2 in TRIS, then washed 15 min with TRIS. Next, the sections were postfixed in 1% OsO4(aq) at room temperature, washed in water (two 10-min washes), dehydrated 5 min each in 50, 70, and 95% ethanol, and then 3 times, for 30 min each time, in 100% ethanol. The sections were then placed in straight propylene oxide (two 5-min immersions) and infiltrated with propylene oxide: Epon, 1:1 (2–4 h), and then Epon alone (8 h). Finally, the sections were sandwiched in a drop of fresh Epon between a piece of Teflon® and a pre-cast Epon block and cured at 60°C. Unstained ultramicrotome sections (<90 nm) were prepared for electron microscopy. Additional controls included omitting either the primary or secondary antibodies, PAP complex, or the DAB one at a time from the reaction sequence.

RESULTS

The ECM of the seminiferous tubule lamina propria, as well as the basement membrane of testicular capillaries, showed a strong reaction when immunostained with antibodies to laminin, type IV collagen, heparan sulfate proteoglycan, or fibronectin. Rarely was reaction product detected within the cells associated with these basement membranes (Fig. 1). Although each of these antisera localized within the lamina propria, the staining patterns of individual components differed.

The rat testicular lamina propria has two ECM layers (Fig. 2a). The first is sandwiched between the seminiferous epithelium and peritubular myoid cells; the second lies between the peritubular myoid and lymphatic endothelial cells. These two ECM layers have been described previously as the inner and outer noncellular layers, respectively (Leeson and Leeson, 1963). The first layer, or inner noncellular layer, has three components: a basement membrane directly subjacent to the seminiferous epithelium, a network of collagen fibrils underlying the basement membrane, and an amorphous matrix material between the
collagen fibrils and the peritubular myoid cells. The second layer, or outer noncellular layer, usually consists of a single band of amorphous matrix material.

Normal rabbit serum and preabsorbed primary antisera controls, as well as the omission controls of either primary or secondary antisera, or PAP, or DAB, all showed no background staining of peroxidase reaction product in the testis (Fig. 2b).

Laminin localized to two distinct bands within the testicular lamina propria (Figs. 1 and 2c). One band corresponded to the seminiferous epithelial basement membrane, within the inner noncellular layer. The second band of laminin staining was within the outer noncellular layer, directly subjacent to the peritubular myoid cells. In addition, there was spotty laminin staining in the inner noncellular layer beneath the seminiferous epithelial basement membrane. However, this spotty staining may have been artifactual, representing diffusion of peroxidase reaction product, since this staining was absent in samples incubated for only 5 min in DAB/H₂O₂.

Type IV collagen localized within both the inner and outer noncellular layers (Fig. 2d). The antisera intensely stained the seminiferous epithelial basement membrane, and less intensely stained the amorphous matrix adjacent to either side of the peritubular myoid cell layer (Fig. 2d). The staining between the myoid and lymphatic endothelial cell layers appeared most often as a single, wide lamina (approximately 200 nm in width), but occasionally as two, separate, narrow laminae (approximately 100 nm each in width; Fig. 2d).

Heparan sulfate proteoglycan localized to both the inner and outer noncellular layers of the lamina propria in much the same way as did type IV collagen (Fig. 2e). However, heparan sulfate proteoglycan in the outer noncellular layer stained as a single broad lamina, and not as two separate lamina, as was observed occasionally for type IV collagen.

Fibronectin did not localize to any structure in the inner noncellular layer (Fig. 2f), but it stained intensely within the outer noncellular layer as a single lamina
FIG. 2. A series of high-power electron micrographs indicating the ultrastructural immunostaining patterns (arrows) of antibodies directed against extracellular matrix molecules in the testicular lamina propria. (A) Conventional electron microscopic section counter-stained with lead citrate and uranyl acetate. (B) A normal rabbit serum control in a testis prepared for immunostaining. Immunolocalization is shown for laminin (C), type IV collagen (D), heparan sulfate proteoglycan (E), and fibronectin (F). No intracellular staining was observed. (L) is lipid. X30,000.

approximately 200 nm in width. Fibronectin also localized diffusely to the interstitial region of the testis (not shown).

Striated collagen fibrils (presumably type I collagen) were distributed in the lamina propria as a network subjacent to the seminiferous epithelial basement membrane and as scattered single fibrils in the outer noncellular layer. This pattern has been described in the adult rat by Leeson and Leeson (1963).

A summary of the electron microscopic immunolocalization of laminin, type IV collagen, heparan sulfate proteoglycan, and fibronectin within the lamina propria of the seminiferous tubules of the adult rat is shown in Figure 3. Although a small amount of sporadic staining by each of the antisera was observed within cells of the testis, the inconsistent nature of this staining prevented an unambiguous interpretation.

DISCUSSION

In the testis, the lamina propria of the seminiferous tubule is strategically located between the systemic circulation and the basal plasma membranes of the Sertoli cells and spermatogonia (Dym and Fawcett, 1970). The ECM of the lamina propria may function as an important part of the blood-testis barrier by forming a nonspecific barrier to cells and macromolecules, just as the glomerular basement membrane functions as part of the filtration system keeping blood proteins from urinary excretion (Farquhar,

FIG 3. A photographic montage of the testicular lamina propria summarizing the distribution of extracellular matrix molecules, as determined by electron microscopic immunocytochemistry. X100,000.
In addition, cell-cell interactions between Sertoli, peritubular myoid, and Leydig cells are thought to be essential for spermatogenesis (reviewed by Fritz, 1984). Each of these interactions must be communicated through the ECM of the testicular lamina propria. Thus, it is important to have a knowledge of the molecular composition of the testicular ECM.

Early in this decade, several laboratories including our own began to investigate the role of the ECM on Sertoli cell structure and function in culture (Mather et al., 1984; Suarez-Quian et al., 1984; Tung and Fritz, 1984). The ECM has since been shown to be involved in the phenotypic expression of polarized Sertoli cell structure (Suarez-Quian et al., 1984; Tung and Fritz, 1984; Hadley et al., 1985a), secretory function (Hadley et al., 1985a, 1987), transepithelial transport (Djakiew et al., 1986), as well as organogenesis and germ cell differentiation (Hadley et al., 1985a).

Skinner et al. (1985) and Borland et al. (1986) have reported that Sertoli cells in culture secrete type IV collagen, laminin, and heparan sulfate proteoglycan. Tung et al. (1984) and Skinner et al. (1985) have shown that fibronectin, type I collagen, and type IV collagen were secreted by peritubular myoid cells in culture. Proteoglycans containing chondroitin and heparin glycosaminoglycan chains have been identified as secretory products of both Sertoli and myoid cells in culture (Elkington and Fritz, 1980; Skinner and Fritz, 1985).

There are certain discrepancies concerning ECM secretion by cultured testicular cells. Tung et al. (1984) and Skinner et al. (1985) have reported that myoid, but not Sertoli cells, produce fibronectin, whereas Borland et al. (1986) have reported that Sertoli cells produce fibronectin. Skinner et al. (1985) have reported that Sertoli cells cultured on plastic do not secrete type I collagen. We have demonstrated that type I collagen is secreted by Sertoli cells when cultured on a reconstituted basement membrane matrix, but confirmed that it is not secreted when the cells are cultured on plastic (Hadley et al., 1985a; Byers et al., 1986). Therefore, cultured cells may secrete ECM components differently from their in vivo counterparts depending on the in vitro conditions. To elucidate unequivocally the source of the ECM components of the lamina propria, it is necessary to localize them within the protein-synthesizing organelles of testicular cells.

Failure to observe significant intracellular staining of ECM antigens in this study indicates that the rates of turnover and synthesis of these molecules in the adult rat testis are low, and undetectable within the sensitivity of our immunocytochemical technique. Peroxidase reaction product was occasionally observed within vesicular structures of testicular cells. However, no staining was observed within Golgi or rough endoplasmic reticulum (Fig. 2). Perhaps at an earlier stage of testicular development, the rate of ECM synthesis is greater and could possibly be detected by this immunocytochemical technique.

Using isolated seminiferous tubule extracellular matrix sleeves, prepared by squeezing and then detergent extracting isolated tubules, Enders et al. (1986) have demonstrated that fibronectin stains in a hexagonal pattern and laminin stains diffusely. Based on the diameter of the hexagonal staining pattern, they have predicted that fibronectin localizes to the edges of the peritubular myoid or lymphatic endothelial cells. Their interpretation is consistent with our observation that fibronectin localizes exclusively to the outer noncellular layer of the lamina propria (Fig. 2f).

The lack of any fibronectin staining within the inner noncellular layer indicates that Sertoli cells and spermatogonia probably do not secrete this molecule in vivo. It might be argued that fibronectin within the inner noncellular layer may be masked by other ECM components such that the fibronectin-specific antibodies are unable to bind. However, this is unlikely since sodium borohydride was used to reduce disulfide bonds and thus expose antigenic sites. In addition, the fibronectin antibody is polyclonal and is specific for numerous determinants on the molecule. It is unlikely that all of the antigenic sights on a molecule as large as fibronectin could be masked. However, it is important to emphasize that a negative immunocytochemical finding alone is not sufficient to rule out the possibility that Sertoli cells may in fact secrete fibronectin under certain circumstances.

Since peritubular myoid cells produce fibronectin in culture (Tung et al., 1984; Skinner et al., 1985), it is likely that fibronectin is selectively secreted in a polarized manner to the outer noncellular layer by these cells in vivo. The immunocytochemical staining of the outer noncellular layer may also represent serum fibronectin deposited by the interstitial fluid since the fibronectin antibody used in this study does
not distinguish between serum and cellular fibronectins.

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REFERENCES


