Cellular Origin of Fibronectin in Interspecies Hybrid Kidneys

HANNU SARIOLA, PENTTI KUUSELA, and PETER EKBLOM*
Department of Pathology and Bacteriology and Immunology, University of Helsinki, 00290 Helsinki 29, Finland; and *Friedrich-Miescher-Laboratorium der Max-Planck-Gesellschaft, D-7400 Tübingen, Federal Republic of Germany

ABSTRACT The cellular origin of fibronectin in the kidney was studied in three experimental models. Immunohistochemical techniques that use cross-reacting or species-specific antibodies against mouse or chicken fibronectin were employed. In the first model studied, initially avascular mouse kidneys cultured on avian chorioallantoic membranes differentiate into epithelial kidney tubules and become vascularized by chorioallantoic vessels. Subsequently, hybrid glomeruli composed of mouse podocytes and avian endothelial-mesangial cells form. In immunohistochemical studies, cross-reacting antibodies to fibronectin stained vascular walls, tubular basement membranes, interstitium, and glomeruli of mouse kidney grafts. The species-specific antibodies reacting only with mouse fibronectin stained interstitial areas and tubular basement membranes, but showed no reaction with hybrid glomeruli and avian vascular walls. In contrast, species-specific antibodies against chicken fibronectin stained both the interstitial areas and the vascular walls as well as the endothelial-mesangial areas of the hybrid glomeruli, but did not stain the mouse-derived epithelial structures of the kidneys. In the second model, embryonic kidneys cultured under avascular conditions in vitro develop glomerular tufts, which are devoid of endothelial cells. These explants showed fluorescence staining for fibronectin only in tubular basement membranes and in interstitium. The avascular, purely epithelial glomerular bodies remained unstained. Finally, in outgrowths of separated embryonic glomeruli, the cross-reacting fibronectin antibodies revealed two populations of cells: one devoid of fibronectin and another expressing fibronectin in strong fibrillar and granular patterns. These results favor the idea that the main endogenous cellular sources for fibronectin in the embryonic kidney are the interstitial and vascular cells. All experiments presented here suggest that fibronectin is not synthesized by glomerular epithelial cells in vivo.

Fibronectin is a high molecular weight glycoprotein composed of two disulphide-bonded 210,000–250,000-mol-wt polypeptides, and it displays interactions with several kinds of macromolecules, cells, and bacteria (1–6). It is found in interstitial connective tissue matrix (1), but also in association with basement membranes (2). It is still uncertain whether, in all cases, it is an integral part of the basement membrane, and it has not been directly shown that cells attached to the basement membrane produce fibronectin in vivo (7).

It has been suggested that fibronectin could be an integral component of embryonic but not adult basement membranes (for reviews, see references 5 and 7). We have previously studied the histogenesis of embryonic basement membranes by using the developing kidney as a model. Such studies have shown that fibronectin gradually disappears as embryonic mesenchymal cells are converted into epithelial kidney tubules (8–11) and suggest that kidney epithelial cells once differentiated do not produce fibronectin. However, during subsequent stages and later in adult life, it is again found in close association with epithelial basement membranes. Fibronectin can also be found in glomeruli and in vascular walls, where it is located in the endothelial basement membranes, around smooth muscle cells, and in loose connective tissue of adventitia (2). Immuno-electron microscopy has shown that in glomeruli fibronectin is present in the mesangial area and in the lamina rara of the glomerular basement membrane, but there is little if any fibronectin present in the lamina densa (12, 13). Similar ultrastructural localization techniques suggest that the tubular basement membrane does not contain fibronectin (14). Although these morphological studies have shown how fibronectin is distributed in the kidney, it is still not known with certainty whether the cells attached to base-
ment membranes produce fibronectin. Experimental model systems that mimic normal histogenesis are therefore of value. We have recently shown that it is possible to construct hybrid glomeruli that consist of cells from different species. When undifferentiated, not yet vascularized embryonic mouse kidneys are cultured on avian choioallantoic membranes (CAMS), they differentiate into epithelial kidney tubules, but the vasculature forms from cells of the avian choioallantoic vessels. Thus, hybrid interspecies glomeruli form that consist of mouse glomerular epithelial cells and avian endothelial-mesangial cells (15, 16). Such grafts gave us an opportunity to study the origin of the glomerular basement membrane. Use of species-specific antibodies against type IV collagen revealed that both epithelial cells and endothelial cells deposit basement membrane components (17). In the present study, the same model is used to analyze the cellular source of fibronectin in the embryonic kidney. In addition, the expression of fibronectin is investigated in avascular glomeruli that consist of podocytes only, and in primary cultures of isolated embryonic glomeruli.

MATERIALS AND METHODS

Choioallantoic Grafts: Undifferentiated and avascular 11-d mouse embryonic kidneys were transplanted and cultured on the CAMs of 7-d quail and chick embryos. The technical details have been previously described (15, 16). Briefly, a small window was cut on the surface of the egg and the shell membrane was carefully scraped off. By using a Pasteur pipette, the kidney explant was then introduced onto the CAM close to an area that marked the division of the large blood vessels. The egg window was then sealed with a piece of Scotch tape, and the eggs were incubated in a humidified atmosphere for 7 d, after which the grafts were harvested. For immunohistochemical studies, the grafts were frozen in liquid nitrogen or fixed in cold alcohol. They were then embedded in paraffin and serially sectioned at 5 μm. After deparaffinization in xylene the sections were washed three times in phosphate-buffered saline (PBS) for 10 min. For Feulgen staining the grafts were fixed in Carnoy’s solution.

Transfilter Experiments: In vitro transfilter cultures were performed as described earlier (18, 19). Separated 11-d mouse metanephric mesenchymes were placed on the upper side of Nucleopore filters (pore size, 1.0 μm; Nucleopore Corp., Pleasanton, CA), and pieces of dorsal spinal cord were glued to the lower side of the filter. The explants were cultured for 7 d in a Trowell-type organ culture in Eagle’s medium supplemented with 10% horse serum. The glomeruli were then separated mechanically after a 15-min incubation with a further 30 min. After three washes in PBS the samples were mounted in an epi-illuminator for fluorescence.

Controls: For testing the antibodies in immunohistochemistry, pieces of 18-d mouse embryonic and adult kidneys, 7-d quail and chick CAM, 14-d embryonic and adult quail and chick kidneys, as well as livers, were frozen in liquid nitrogen. 7-μm sections of each tissue were cut and stored at -20°C.

Immunohistochemistry and Lectin Stainings: The histological sections or cell colonies were incubated with the predeterminated dilution of the primary antibodies for 30 min, washed three times in PBS for 10 min, and then incubated with fluorescein isothiocyanate-conjugated sheep anti-rabbit IgG for a further 30 min. After three washes in PBS the samples were mounted in buffered glycerol and monitored in a Zeiss Universal microscope equipped with an epi-illuminator for fluorescence.

To detect the glomerular anionic coat in histological sections (10) and cell cultures (20), tetramethylrhodamine isothiocyanate-conjugated wheat germ agglutinin (TRITC-WGA) was used. After incubation with TRITC-WGA for 30 min, the specimens were washed three times for 10 min in PBS and processed as above. The immunofluorescence staining of single specimens was carried out before the lectin staining.

For immunoperoxidase staining, the biotin-avidin staining kit (Vector Laboratories, Inc., Burlingame, CA) was used. Briefly, the sections were first incubated in 0.3% H2O2 in methanol for 30 min. After a 15 min wash in PBS, they were incubated with the primary antisera for 30 min followed by a second 30-min incubation with diluted biotinylated antibody. A biotinylated horseradish peroxidase-avidin complex was then added and the sections incubated for a further 60 min. After each incubation, the reagent was flushed off the slide with PBS and slides were washed in PBS for 10 min. The staining was developed by 3,3′-diaminobenzidinetetrahydrochloride, washed in PBS for 10 min, incubated in tap water for 5 min, and mounted. When the primary antisera was omitted or when preimmune normal serum was used in its place, no staining was seen in the tissues.

Antibodies: Rabbits were immunized with chicken or mouse plasma fibronectins purified by a modification of affinity chromatography on gelatin Sepharose (21, 22). For absorptions the antisera were diluted 1:100 in PBS containing 1% (wt/vol) bovine serum albumin and incubated with lyophilized mouse or chicken extracts for 3 h at room temperature. After incubation tissue extracts were pelleted by centrifugation and the preabsorbed antisera were absorbed further. Antiserum against chicken fibronectin was incubated with pooled mouse serum and with purified mouse fibronectin overnight at room temperature. Preabsorbed antisera to mouse fibronectin was similarly incubated with chicken plasma and with purified chicken fibronectin. Finally, the antisera were clarified by centrifugation. The antibodies against laminin and type IV collagen were kindly donated by Dr. Rupert Timpl (Max-Planck Institute, Munich).

Immunomicroscopy: Double-diffusion experiments were performed using 1% agarose plates. To test the cross-reactivity of the antisera, we used an enzyme-linked immunosorbent assay: wells of microtiter plates were coated with gelatin solution (1 mg/ml PBS) overnight at room temperature. After two washes with PBS the wells were saturated with PBS containing 1% (wt/vol) bovine serum albumin for 3 h at 37°C. The wells were then washed and treated with predetermined dilutions of either pooled mouse serum or chicken plasma for 3 h at 37°C. Finally, after the washings, the wells were incubated with various dilutions of the absorbed antisera. After a 3-h incubation at 37°C, the antibodies bound to the wells were detected by the double antibody combined with the alkaline phosphatase.

The enzyme reaction was completed by treating the wells with 0.2% phosphate substrate (p-nitrophenyl disodium phosphate; Sigma Chemical Co., St. Louis, MO) solution in commercial diethanolamine buffer (Orion Pharmaceuticals, Helsinki, Finland). After a 30-min incubation at room temperature, the optical densities were recorded at 450 nm.

RESULTS

Cross-reactivity of the Unabsorbed Antisera

In double-diffusion experiments, both unabsorbed antisera to mouse or chicken fibronectin produced a precipitation line against pooled mouse serum and chicken plasma (Fig. 1). Antiserum to mouse fibronectin revealed a spur in the precipitation line indicating a partial cross-reaction between mouse and chicken fibronectins. The antisera to chicken fibronectin showed a precipitation line indicating a complete cross-reactivity (Fig. 1).

Species Specificity of the Absorbed Antisera

Absorbed antisera to chicken or mouse fibronectin showed a good reactivity in an enzyme-linked immunosorbent assay system where the wells of microtiter plates were coated with the chicken and mouse fibronectin, respectively. Both antisera, however, exhibited a slight activity against the fibronectin used for absorption (Fig. 2, A and B).

In immunofluorescence staining of frozen sections, the absorbed antisera to chicken fibronectin reacted only with chicken and quail tissues and did not stain mouse tissues (Fig. 3, A and B). Similarly, the absorbed antisera to mouse fibronectin showed fluorescence staining in mouse but not in

1 Abbreviations used in this paper: CAM, choioallantoic membrane; TRITC, tetramethylrhodamine isothiocyanate; WGA, wheat germ agglutinin.
chicken or quail tissues (Fig. 3, c and d). Immunoperoxidase staining revealed essentially the same pattern of reactivity as was seen with immunofluorescence. In paraffin-embedded samples the staining pattern was similar except that the species-specific antiserum to mouse fibronectin did not stain the paraffin-embedded samples (data not shown).

**Chorioallantoic Grafts**

Embryonic mouse kidneys transplanted and cultured on avian CAM become vascularized by chorioallantoic vessels. Thus, interspecies glomeruli form with mouse podocytes and avian endothelial-mesangial cells (15). The distribution of fibronectin in these mouse/avian chimeric kidneys was studied by immunofluorescence techniques employing cross-reacting or species-specific antibodies to mouse or chicken fibronectins.

Fig. 4a demonstrates that the cross-reacting unabsorbed antibodies stained the CAMs, the interstitium of the kidney transplants, the vascular walls, and kidney tubules. In the hybrid glomeruli (Fig. 4b) the mesangial-endothelial area and the glomerular basement membrane were stained with the cross-reacting antibodies (Fig. 4c).

The absorbed antibodies, reacting with mouse but not with quail and chick fibronectin, did not stain the CAMs (Fig. 5a), but produced strong staining in the interstitial areas of mouse kidney explants. Fluorescence staining was also seen in the basement membrane area of the kidney tubules (Fig. 5b). To localize glomerular podocytes, TRITC-WGA was used. Double stainings with TRITC-WGA and antibodies specific for mouse fibronectin revealed that the glomeruli did not contain detectable mouse fibronectin (Fig. 5, c and d).

The absorbed antibodies, reacting with quail and chick but not with mouse fibronectin, clearly stained the CAMs, vessel walls, and the interstitial areas of the mouse kidney explant, but the tubular basement membranes remained unstained (Fig. 6a). The endothelial/mesangial area of glomeruli also stained brightly with these antibodies (Fig. 6, b and c). The distribution of fibronectin in the hybrid kidneys is summarized in Table I.

**Organ Culture Experiments**

To further clarify the distribution of fibronectin, we studied organ cultures of differentiating, experimentally induced, metanephric mesenchymes. In these cultures the glomeruli remain avascular (10). The interstitial areas and tubular basement membranes showed fluorescence for fibronectin, whereas the glomeruli, localized by WGA-lectin (Fig. 7a), remained unstained (Fig. 7b). Within the glomerular bodies, antibodies to type IV collagen did not reveal any fluorescence (data not shown), but antibodies to laminin showed a weak staining in a discontinuous pattern (Fig. 7c).

**Cellular Outgrowths from Embryonic Glomeruli**

In primary cell cultures of glomeruli from 15- to 18-d embryonic metanephric kidneys, all cells were strongly positive for laminin and bound WGA-lectin (Fig. 8, a and b). Staining with WGA-lectin could not be used to distinguish podocytes from other cells. Fibronectin was seen in most cells, as detected by cross-reacting antibodies to fibronectin. However, approximately 20–25% of the cells did not show any fluorescence for fibronectin at all (Fig. 8, c and d). In the stained cells fibronectin was seen on the cell surface as a fibrillar distribution and within the cytoplasm in a more granular pattern (Fig. 8d).

**DISCUSSION**

Three different experimental approaches were used to analyze the cellular source and distribution of fibronectin in chimeric embryonic kidneys. The data obtained suggest that glomerular epithelial cells, podocytes, do not produce fibronectin, which in the glomeruli appears to be solely produced by the endothelial and mesangial cells. In addition, we show that the intertubular stromal cells are also capable of fibronectin production.

Previous studies on the location of fibronectin in adult (2,
FIGURE 3 Tissue reactivity of absorbed fibronectin antiserum at a dilution of 1:200. Absorbed antiserum against chicken fibronectin stains chicken tissues (a), but not mouse tissues (b). Similarly, absorbed antiserum against mouse fibronectin stains only mouse tissues (c), and not chicken tissues (d). Indirect immunofluorescence staining of frozen sections of kidneys. (a and d) × 200. (b and c) × 250.
Expression of fibronectin in mouse kidney grafts grown for 7 d on quail CAM. (a) The unabsorbed cross-reacting antibodies to chicken fibronectin decorate CAM and, within kidney explants, interstitial areas, vascular walls, and kidney tubules. (b) The kidney grafts are vascularized by chorioallantoic vessels and form hybrid glomeruli with mouse podocytes (P) and quail endothelial cells (En). (c) Within these glomeruli (G), fibronectin is stained in the endothelial-mesangial area and a faint staining is also seen along the glomerular basement membrane. (a and c) Indirect immunofluorescence staining of frozen sections. (b) Feulgen. (a) × 200. (b) × 600. (c) × 450.

FIGURE 4 Expression of fibronectin in mouse kidney grafts grown for 7 d on quail CAM. (a) The unabsorbed cross-reacting antibodies to chicken fibronectin decorate CAM and, within kidney explants, interstitial areas, vascular walls, and kidney tubules. (b) The kidney grafts are vascularized by chorioallantoic vessels and form hybrid glomeruli with mouse podocytes (P) and quail endothelial cells (En). (c) Within these glomeruli (G), fibronectin is stained in the endothelial-mesangial area and a faint staining is also seen along the glomerular basement membrane. (a and c) Indirect immunofluorescence staining of frozen sections. (b) Feulgen. (a) × 200. (b) × 600. (c) × 450.
Figure 5 Staining for fibronectin in mouse/chick hybrid kidneys as analyzed by absorbed antiserum specific for mouse fibronectin at a dilution of 1:200. (a) CAM remains unstained, but the stromal areas (S) and tubules (T) are brightly positive. (b) Fluorescence is seen also in the lower crevice of the S-shaped body (arrow) which will form the glomerulus. (c) WGA binds to the polyanionic coat of glomeruli (G), where no staining for fibronectin can be seen (d). Note that the only mouse cells in the hybrid glomeruli are podocytes. Indirect immunofluorescence staining of frozen sections. (a) × 250. (b) × 550. (c and d) × 600.
Figure 6 Staining for fibronectin in mouse/chick hybrid kidneys as analyzed by absorbed antiserum specific for chicken and quail fibronectin at a dilution of 1:200. CAM and all interstitial areas of kidney explants, as well as vascular walls, are stained, but tubules remain unstained. That is clearly seen in the branches of the collecting ducts (arrows) surrounded by condensed mouse cells (a). Both by immunoperoxidase (b) and immunofluorescence techniques (c), the glomerular (G) endothelial–mesangial area is stained, but no staining is seen in podocytes. Note the strong interstitial staining also outside the vasculature (arrows). (a and b) Biotin–avidin peroxidase staining of paraffin-embedded samples. (c) Indirect immunofluorescence staining of frozen section. (a) × 180. (b) × 450. (c) × 600.

Table I

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* Staining was seen only in the basement membrane area, and not within the cytoplasm.

Fibronectin production. Fibronectin is also present in plasma, from where it can be deposited into tissues (26, 33). In in vitro organ cultures such a deposition from serum does not occur. Recently, we have postulated (34) that the deposition of plasma fibronectin into tissues requires an intact vascular bed and a hemodynamic pressure. In the chorioallantoic grafts there is both a normal vascular bed and a proper pressure. We now show that fibronectin under these conditions becomes deposited not only in the endothelial basement membrane area, but also widely in the mouse interstitial tissue outside the vessels. We conclude that exact localization studies of in vivo tissues using either light or electron microscopy cannot provide us with definite information on the actual cellular source of fibronectin, unless it is detected inside the cells in biosynthetic organelles (12). Use of interspecies hybrid tissues combined...
FIGURE 7 Staining for fibronectin and laminin in organ cultures of metanephric mesenchyme grown in vitro. The glomerulus-
like bodies (G) remain avascular and consist of podocytes only. They can be stained by WGA (a), and no fibronectin (unabsorbed
cross-reacting antibodies) is seen (b), although the podocytes express laminin in an irregular and discontinuous pattern (c). Indirect
immunofluorescence staining of frozen sections. (a and b) Double staining. (c) Adjacent section. $\times$ 500.
with stainings that use species-specific antibodies can, however, give information on the cellular source of extracellularly deposited fibronectin. We also conclude that fibronectin production by cultured cells does not necessarily reflect its in vivo production.

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REFERENCES


**Figure 8** Staining for laminin and fibronectin in primary glomerular outgrowths. Double staining with TRITC-WGA (a) and antibodies against laminin (b) shows that all cells in culture bind WGA and express laminin. Double staining with TRITC-WGA (c) and fibronectin (unabsorbed cross-reacting antibodies) (d) reveals a cell population negative for fibronectin. In stained cells fibronectin is seen extracellularly in a fibrillar pattern and intracellularly in a more granular pattern. Indirect immunofluorescence staining. × 850.