SELECTED KIDNEY CELL TYPES IN PRIMARY GLOMERULAR EXPLANT OUTGROWTHS BY IN VITRO CULTURE CONDITIONS

TERRY D. OBERLEY, AN-HANG YANG AND JEANINE GOULD-KOSTKA

Department of Pathology, University of Wisconsin Medical School, Madison, WI 53706, USA
Pathology Service, William S. Middleton Memorial Veterans Hospital, 2500 Overlook Terrace, Madison, WI 53705, USA

SUMMARY

Adult guinea pig glomeruli were grown in vitro either in serum or in a chemically defined medium. Glomeruli were plated either directly into plastic flasks or into plastic flasks that had been coated with the extracellular matrix produced by the PF-HR-9 mouse teratocarcinoma endodermal cell line. Both the composition of the medium and the nature of the culture substrate affected whole glomerular attachment and the type of cells produced in culture. Quantitative studies demonstrated selection of cell types by different culture conditions. Three colony types, each composed of distinctive cell types, could be identified by morphological features. The cells constituting two of these colony types were epithelial in nature, but they were identified as different epithelial types by both histochemical and ultrastructural criteria. Previous studies suggested that one epithelial cell type was derived from the glomerular visceral epithelial cell. This study demonstrates that this cell type could be selectively grown in defined medium on plastic. A second cell type showed several features of renal tubular epithelial cells, including histochemical staining for catalase, cell surface microvilli and cilia, and formation of hemicysts and structures that resembled tubules after prolonged periods in culture. To demonstrate that the 'glomerulus-derived' tubular cells were indeed tubular epithelium, we isolated purified renal cortical tubules (>99% pure) and cultured them on the HR-9 matrix in a serum-free chemically defined medium. The resultant outgrowths had morphological properties identical to those of the glomerulus-derived tubular cells. It seems likely that small tubular fragments attached to a minority of the glomeruli are the source of these glomerulus-derived tubular cells. Neither epithelial cell type could be subcultured on plastic, but both could be passaged on the HR-9 matrix. A third cell type, the spindle-shaped cell, was easily propagated on both plastic and the HR-9 matrix. The origin of this cell type is not clear. Our results demonstrate the important effect of culture conditions on the selection, growth and differentiation of kidney cell types in vitro.

INTRODUCTION

Study of kidney glomerular pathology depends upon the development of cell culture systems. Consequently, several laboratories have studied the growth of glomerular cells in vitro (Kreisberg & Karnovsky, 1983; Striker & Striker, 1985). The glomerulus is a complex structure composed of three major cell types: epithelial, mesangial and endothelial cells. These cell types are distinguishable because they

* Author for correspondence.

Key words: kidney, glomeruli, tubules, extracellular matrix.
occupy defined anatomical locations in vivo and have characteristic morphological features. However, this compartmentalization as well as several anatomical characteristics, such as endothelial cell fenestræ and epithelial cell foot processes, are lost in culture.

Because at least three cell types may arise from glomeruli in culture, several studies have been done to define culture conditions for the selective growth of different glomerular cell types. Harper et al. (1984) and Striker & Striker (1985) reported selective growth of mesangial cells from rat and human glomeruli, respectively, using medium containing high concentrations of calf serum. Their methodologies differed in that Harper et al. used trypsin-treated glomeruli while Striker & Striker explanted glomeruli without enzyme treatment. Furthermore, Harper et al. included insulin and conditioned medium from Swiss 3T3 fibroblasts in the growth medium in addition to serum. Harper et al. and Striker & Striker also reported selective growth of glomerular epithelial cells. Again, their methodologies were different. In Striker & Striker’s study, epithelial cells were grown from collagenase-digested human glomeruli using 0.5% serum, while Harper et al. grew epithelial cells from untreated glomeruli on collagen in a medium containing 5% serum and conditioned medium from Swiss 3T3 fibroblasts.

Since such widely disparate conditions appeared to stimulate the growth of glomerular cells, our study was designed to test the effect of medium conditions on the selective growth of glomerular cell types. We also decided to test the effect of a basement membrane on glomerular cell growth. Gospodarowicz et al. (1984) demonstrated that the basement membrane from the mouse teratocarcinoma cell line PF-HR-9 supported growth and differentiation of kidney epithelial cells derived from collecting tubules of adult bovine kidney. We have demonstrated that the outgrowth of glomerular cells from adult guinea-pig glomerular explants onto a plastic substrate depends on the extracellular matrix glycoprotein fibronectin (Oberley et al. 1982), whereas outgrowth of tubular cells requires a different extracellular matrix glycoprotein, laminin (Oberley & Steinert, 1983). Neither glomerular nor tubular cells could be subcultured to confluency after culture on plastic in the presence of isolated extracellular matrix molecules (unpublished observations). In this paper we report the successful culture and passage of several kidney cell types on the HR-9 matrix.

MATERIALS AND METHODS

Animals

Five-week-old randomly bred Hartley guinea pigs (300–400g) of either sex (O’Brien, Oregon, WI) were maintained ad libitum on laboratory chow and drinking water supplemented with ascorbic acid.

Glomerular culture

Glomeruli were isolated by a modification of a previously described screening technique (Oberley et al. 1979). Kidneys from anaesthetized animals were surgically removed under sterile conditions and rinsed twice with sterile phosphate-buffered saline (PBS) (10 mM-phosphate buffer,
Selection by culture conditions

0.133 M NaCl, pH 7.2) containing penicillin (100 units ml⁻¹) and streptomycin (100 μg ml⁻¹) (Gibco, St Louis, MO). Pericapsular fat and capsule were removed and the cortex was separated from the medulla. The isolated cortex was minced and ground with a pestle while washing with PBS through a 450-μm pore size nylon sieve (Tetko, Elmsford, NY). The homogenate was passed through one layer each of HC-3-132 (132 μm) and HD-3-73 (73 μm) nylon sieve (Tetko). Glomeruli were collected from the HD-3-73 sieve and centrifuged at 150 g in a model CL clinical centrifuge (International Equipment Co., Needham, MA) for 3 min. The supernatant was removed and the glomeruli were resuspended in sterile PBS and placed into culture vessels (25 cm² flasks, Costar) containing fresh medium.

Glomeruli were cultured in Waymouth's basic medium MB 752/1 with penicillin (100 units ml⁻¹) and streptomycin (100 μg ml⁻¹). This medium was supplemented with either 20% foetal bovine serum alone or with the following additives, from Collaborative Research, Waltham, MA: insulin (5 μg ml⁻¹), transferrin (5 μg ml⁻¹) and selenium (5 ng ml⁻¹); from Sigma, triiodothyronine (7 ng ml⁻¹) and hydrocortisone (0.18 μg ml⁻¹); and fibronectin (7.7 μg ml⁻¹), a gift from Dr Deane Mosher, University of Wisconsin.

Flasks initially contained 6 ml of medium. On days 6 and 10, half of the medium was carefully replaced, avoiding loss of unattached glomeruli. On days 12 and 16, all of the medium was replaced. On days 6-20, cells were fixed for analysis as described in the text. Glomeruli were seeded at a density of about 400 cm⁻² (Oberley et al. 1986). Cultures were maintained in a non-humidified atmosphere of 5% CO₂/95% air at 37°C.

Preparation of culture flasks coated with HR-9 basement membrane

The HR-9 matrix was prepared as described by Gospodarowicz et al. (1984). Cells from the mouse teratocarcinoma-derived endodermal cell line PF-HR-9 were obtained from Dr R. Kramer, University of San Francisco. The cells were grown in fibronectin-coated 25 cm² Costar flasks. Flasks were coated as previously described (Oberley & Steinert, 1983), and cells were grown in the presence of Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum. Once the cultures had been confluent for 5 days, the medium was removed and the confluent cultures were washed once with PBS. Cells were then exposed to 0.02 M ammonium hydroxide in distilled water for 5 min, followed by washing extensively with PBS (seven times; 20 ml PBS per wash). This resulted in denudation of the basement membrane produced by the PF-HR-9 cells.

Enzyme histochemistry

Peroxidase staining. Cells were histochemically stained for peroxidase as described by Herzog & Fahimi (1976). For peroxidase staining, cells were fixed in 1:25% glutaraldehyde, 5% sucrose in 0.1 M cacodylate buffer (pH 7.4), for 5 min at 20°C. Cells were rinsed in 0.1 M cacodylate buffer containing 5% sucrose (pH 7.4), and then preincubated in 5 mM-diaminobenzidine·HCl in 0.1 M cacodylate containing 5% sucrose (pH 6.5), for 60 min at 20°C. For staining, cells were incubated in 2 mM H₂O₂ in preincubation buffer for 90 min at 20°C in the dark. Cells were finally washed and photographed in situ with a bright-field microscope without the use of a counterstain. None of the control cells, in which the H₂O₂ was omitted, showed staining.

Catalase staining. Cells were histochemically stained for catalase as described by Herzog & Fahimi (1976). Cells were fixed in 1:25% glutaraldehyde, 0.05% calcium chloride (w/v) in 0.1 M cacodylate buffer (pH 7.4), for 30 min at 20°C. Cells were then washed with cacodylate buffer and preincubated with 5 mM-DAB·HCl, 5% sucrose, in citrate–phosphate–borate/HCl buffer (pH 10.5), for 60 min at 20°C. For staining, cells were incubated with 0.1 M H₂O₂ in preincubation buffer, 5% sucrose (pH 10.5), for 2 h at 20°C. Cells were finally washed and photographed in situ with a bright-field microscope without the use of a counterstain. None of the control cells, in which the H₂O₂ reaction was omitted, showed staining.

Scanning electron microscopy

A previously described protocol (Oberley et al. 1979) was used to prepare cells for scanning electron microscopy (SEM). Cells were grown in 25 cm² Costar flasks and areas of interest were cut from the plastic surface. Cells were washed several times with PBS, fixed in 0.1 M PBS/1%
glutaraldehyde (pH 7.4) for 24 h, and dehydrated via an ethanol series for 30 min each in 30, 50, 75, 85, 90 and 95% ethanol and for two 30-min periods in 100% ethanol. Cells were critical-point dried with 100% ethanol as the intermediate fluid and liquid CO2 as the transitional fluid. The plastic substrates containing cells were cemented onto metal stubs and coated with a 74-nm gold layer. Cells were viewed at 10 kV accelerating voltage with a JEOL 356 scanning electron microscope.

**Transmission electron microscopy**

Transmission electron microscopy (TEM) was performed as previously described (Oberley et al. 1981a,b). Glomerular cells were washed in situ with PBS and then fixed with 2.5% glutaraldehyde in PBS. Cells were then fixed in 1% osmium tetroxide in veronal buffer, dehydrated in an ethanol series, and then embedded in Epon. Sections of interest were cut from the plastic with a small hack-saw and sectioned for microscopy. Ultrathin sections were double-stained with lead citrate and uranyl acetate, and examined at 75 kV with a Hitachi H-500 electron microscope.

**Morphometry**

Glomeruli were grown in 25 cm² flasks in duplicate under different culture conditions and separate flasks were fixed with 3% paraformaldehyde on days 6, 8, 10, 12 and 20. Cells were stained with Giemsa stain and air dried. Surface areas covered by different cell types were morphometrically assessed using the point-counting technique (Aherne & Dunnill, 1982). Flasks were placed in a modified photographic projector (Dust, Latico 200) and projected onto a point-counting grid with the points arranged in a square pattern. The total number of points overlaying the colonies of each type of cell (n) and the total number of points overlaying the cell surface covered by cells (N) were counted. The surface fraction (Aa) was estimated by p = n/N. Binomial statistics were used to determine the adequacy of sampling; the relative standard error (R.S.E.*) of a proportion is given by R.S.E.* = \sqrt{\frac{1}{N}}. R.S.E.* is derived from the standard error of proportions (Sp): Sp = \sqrt{\frac{p(1-p)}{N(N-1)}}. When N is large, the difference between N and N-1 is slight. Sp can be rewritten as: Sp = \sqrt{\frac{p(1-p)}{N}} since p = n/N = Aa, Sp/Aa = \sqrt{\frac{1-n/N}{n}} = \sqrt{\frac{1-Aa}{n}} = R.S.E.* \times (100\%)

**Glomerular attachment**

For determining glomerular attachment with different combinations of medium and matrix, attached glomeruli were counted from day 6 cultures. Glomerular density was determined by morphometry as described above. The bottom surface of the flask was subjected to 45-162 point-counts, giving 200-300 counts per glomerular colony. This limited the relative standard error to less than 7%. Four random colonies were counted from each of the flasks and data (glomerular density of colony) were converted to the number of glomeruli per unit area of flask bottom surface. Final densities were obtained by:

\[
\text{Glomeruli/cm}^2 = \frac{\text{Average glomerular density in colony} \times \text{total colony area}}{25 \text{ cm}^2}
\]

Analysis of variance (ANOVA) and the Tukey test were used to compare the effect of different combinations of medium and substratum on glomerular attachment (Scheffler, 1984; Sachs, 1982).

**Hemicyst formation**

Hemicyst formation has been recognized as a sign of functional and morphological differentiation of tubular cells (Misfeldt et al. 1976). Hemicysts were counted under a phase-contrast microscope (Leitz) and data were expressed as the number of hemicyst per tubular colony.
Selection by culture conditions

Isolation of renal cortical tubules

Kidneys were decapsulated and cortical tissue isolated. Cortical tissue was minced with a scalpel and disrupted with a sterile tissue homogenizer (Wheaton, 7 ml, type B) containing PBS. This procedure released glomeruli and retained tubules as small aggregates. Homogenized tissue was transferred to a beaker and diluted to 1000 ml with PBS. The suspension was passed through a 132 μm nylon sieve (Tetko), resulting in the filtration of free glomeruli and tubules and the retention of tubular fragment aggregates. Tubular fragment aggregates were transferred to a 25 cm² tissue culture flask that contained 20 ml Waymouth's medium supplemented with 10% foetal bovine serum and 250 units ml⁻¹ collagenase (Cooper, 170 units mg⁻¹). The flask was kept in a 37°C incubator and occasionally agitated. After 6–8 h, tissue aggregates were digested to form single tubules; digestion also released a small number of glomeruli. The enzyme-digested tissue was then passed through an 88 μm nylon sieve (Tetko); tubules were retained in the sieve while glomeruli passed through. Purity of the isolated tubules was assessed by using a counting eyepiece. Ten random areas (4-8 mm × 4-8 mm) were counted using a phase-contrast microscope (Leitz). Purity of the tubules was greater than 99.5%.

Subculture techniques

Both epithelial cell types and the spindle-shaped cell were subcultured by brief treatment with trypsin and ethylenediaminetetraacetic acid (EDTA). The proliferative potential of the spindle-shaped cell on different culture substrates was assessed by serial passage. Cells were isolated from a primary culture (day 18) that had been grown in Waymouth's medium plus foetal bovine serum. The culture was first exposed to 0.25% trypsin and 0.1% EDTA to remove epithelial cells between the spindle-shaped islands and then treated with trypsin and EDTA for a longer period to detach the spindle-shaped cells. Cells were initially plated at a concentration of 1.8 × 10⁸ cells/flask and then passaged every 5–7 days on either plastic or the HR-9 matrix.

RESULTS

Cell type and colony morphology

Previous studies and the present study demonstrate that glomeruli grown on plastic in a chemically defined medium produced a single type of colony composed of cells with epithelial morphology (flat, polygonal cells; Fig. 1A). Further, electron-microscopic studies demonstrated that these cells arose from the epithelial side of the glomerular basement membrane, suggesting that the cell of origin was the glomerular epithelial cell (Oberley et al. 1986). Although there are no biochemical markers that could be used to prove conclusively that these cells are derived from the glomerular epithelial cells, we shall nevertheless call these cells 'glomerular epithelial cells' for the purpose of simplifying our discussion.

In previous studies of glomerular cultures, we found a second colony type; very small numbers of this cell type were observed in glomerular explants that had been grown on plastic in high concentrations of foetal bovine serum (Oberley et al. 1981a). In this study we demonstrate that this cell type grows in colonies with sharply circumscribed edges (Fig. 1B), in contrast to the glomerular epithelial cell colonies in which cells at the edge of the colony often separate from the central colony. Again, although we have not conducted marker studies to identify this cell type, a number of features of this cell type (see below) indicate that it is derived from tubular epithelium. Hence, we have labelled these cells 'tubular cells'.

Under certain conditions (see below), a third cell type (a spindle-shaped cell) was seen in glomerular culture (Fig. 1C). Although most authors believe this is a
Fig. 1. Light microscopy of cell types in glomerulus. A. Glomerular epithelial cells, flat and polygonal; cells at edge of colony tend to separate from the main colony. B. Tubular epithelial cells (t), are tightly packed, although there is space between individual cells. Colony is well circumscribed. C. Spindle cells (s) are elongated and tend to overlap. Giemsa stain; A–C, ×45.
mesangial cell (Harper et al. 1984; Striker & Striker, 1983), we are not certain of the origin of this cell type and shall therefore call these cells 'spindle cells'.

**Effect of medium and substrate on colony type**

To assay the effect of culture conditions on colony selection, glomeruli were grown either on plastic or on the HR-9 matrix. The HR-9 matrix resembles authentic basement membrane (Fig. 2). The media used were a chemically defined medium containing Waymouth's MB 752/1 medium plus insulin, transferrin, selenium, hydrocortisone and fibronectin, or Waymouth's MB 752/1 medium plus 20% foetal bovine serum. Glomeruli were grown for 6, 8, 10, 12 or 20 days and then stained with Giemsa stain.

Glomeruli grown in defined medium on plastic showed only one type of colony morphology (Fig. 3C). These cells were shown by light-microscopic examination to be glomerular epithelial cells (Fig. 3G). In contrast, glomeruli grown on plastic in the presence of foetal bovine serum showed two colony types, one that resembled that seen in defined medium plus a second type that stained very darkly with Giemsa.

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Fig. 2. TEM of PF-HR-9 cells. A basement membrane is deposited beneath cells (ecm). Cells were removed and the denuded matrix was used for studies described in this paper. p, plastic. ×6665.
Fig. 3. A–D. Bright-field microscopy of colony type in glomerular culture. Cells grown in: A, defined medium on HR-9 matrix; B, foetal bovine serum on HR-9 matrix; C, defined medium on plastic; and D, foetal bovine serum on plastic. The glomerular epithelial colony is seen in C (arrows). The spindle colony is best seen in D (arrow). The tubular colony is best seen in A (arrow). Giemsa stain. ×18. E–H. Light microscopy of colony types in glomerular cultures. Cells grown in: E, defined medium on HR-9 matrix; F, foetal bovine serum on HR-9 matrix; G, defined medium on plastic; and H, foetal bovine serum on plastic. g, glomerular cells; t, tubular colony; s, spindle colony. Giemsa stain; E–H, ×45.
Fig. 4A, B. For legend see p. 80
Fig. 4C, D. For legend see p. 80

stain (Fig. 3D). Cells constituting the latter colony type were shown by light-microscopic examination to be spindle cells (Fig. 3H).

Glomeruli grown in a defined medium on an HR-9 matrix showed colonies similar to those grown on plastic (glomerular epithelial cells) plus a colony type identifiable by its well-circumscribed edges (tubular cell) (Fig. 3A,E). Glomeruli grown in foetal bovine serum on an HR-9 matrix showed glomerular epithelial, spindle and tubular colony types (Fig. 3B,F).

*Enzyme histochemical analysis of cells*

Histochemical analysis indicated that glomerulus-derived tubular cells stained more intensely for peroxidase and catalase than glomerular cells (Fig. 4A,B). In the control culture (absence of H₂O₂) neither cell type showed staining. To date, we have not completed histochemical analysis of the spindle cell.

*Ultrastructure of cell types*

Ultrastructural analysis of the glomerular epithelial cell in culture has been previously reported. SEM demonstrated that glomerular epithelial cells in culture have a smooth cell surface (Oberley et al. 1982), while TEM demonstrated relatively small, tight junctions between cells and a cytoplasm containing a large amount of microfilaments but only a few mitochondria (Oberley et al. 1986). Tubular cells were ultrastructurally examined and found to be distinct from the glomerular epithelial cells. SEM showed numerous microvilli and cilia (Fig. 5A). TEM showed large nuclei with prominent nucleoli, numerous mitochondria, microfilaments just beneath the cell membrane, cell surface microvilli, and prominent, large tight junctions (Fig. 5B,C). In contrast, spindle cells had a smooth cell surface (Fig. 6A); TEM revealed elongated nuclei, prominent rough endoplasmic reticulum, prominent microfilaments, cytoplasm containing amorphous granules, and a large extracellular matrix (Fig. 6B,C).

*Morphometry of surface area occupied by colony types*

Since morphological and histochemical analyses indicated that the three colony types were composed of distinctive cell types, we decided to quantify the area occupied by each colony type. Quantitative results (Fig. 7) confirmed the gross observations (Fig. 3). Glomeruli grown on plastic in chemically defined medium predominantly showed glomerular epithelial cell colonies (Fig. 7A), while glomeruli grown on plastic in foetal bovine serum showed both glomerular epithelial and

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Fig. 4. Histochemical analysis of glomerular epithelial cell types. Glomerular epithelial cell cultures maintained on plastic and grown in serum-supplemented medium were processed through peroxidase and catalase-staining procedures as described in Materials and Methods. Compared with controls (B, peroxidase control; D, catalase control), all tubular colonies (t) were strongly positive for peroxidase (A) and catalase (C), while glomerular epithelial cells (g) show only background staining for both peroxidase and catalase. These photographs were taken in situ with a bright-field microscope; the cells were not counterstained. ×6000.
Fig. 5. Ultrastructural features of tubular cells in culture. A. SEM of tubular cells; cells have numerous microvilli (arrow). ×6000. B. TEM of tubular cells; cells have large nuclei with prominent nucleoli, microvilli (arrowheads), rough endoplasmic reticulum, and microfilaments just beneath the cell membrane (arrow) (parallel section). ×3240. C. TEM of tubular cells; prominent junctional complexes are seen between cells (arrows) (parallel section). ×8160.
Fig. 6. Ultrastructural features of spindle cells in culture. A. SEM of spindle cells; cells have a completely smooth cell surface. ×3138. B. TEM of spindle cells (parallel section); cells are elongated and have an elongated nucleus (n), prominent rough endoplasmic reticulum (rer), intracellular granules (g), and a prominent extracellular matrix (m). ×12334. C. TEM of spindle cells (perpendicular section). Occasional intracellular microfilaments (arrow) and amorphous cytoplasmic granules are seen (g). rer is prominent and extracellular matrix (m) is present. ×12334.
Fig. 7. Morphometry of surface area occupied by different glomerular colony types. Glomeruli grown in: A, defined medium on plastic; B, foetal bovine serum on plastic; C, defined medium on HR-9 matrix; D, foetal bovine serum on HR-9 matrix. Asterisks indicate relative standard error of <5%. (■) Tubular; (●) spindle; (○) glomerular epithelial.

Table 1. Influence of extracellular matrix and culture medium on glomerular attachment to substratum

<table>
<thead>
<tr>
<th>Substratum</th>
<th>Medium</th>
<th>Glomeruli/cm²</th>
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<tbody>
<tr>
<td>Plastic</td>
<td>Chemically defined medium*</td>
<td>62±3±2.9†</td>
</tr>
<tr>
<td>Plastic</td>
<td>+20% foetal bovine serum†</td>
<td>161±6±18.5</td>
</tr>
<tr>
<td>HR-9</td>
<td>Chemically defined medium</td>
<td>183±2±24.6</td>
</tr>
<tr>
<td>HR-9</td>
<td>+20% foetal bovine serum</td>
<td>231±6±41.5</td>
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*Chemically defined medium: Waymouth’s medium supplemented with insulin (5 μg ml⁻¹), transferrin (5 μg ml⁻¹), selenium (5 ng ml⁻¹), triiodothyronine (7 ng ml⁻¹), hydrocortisone (0.18 μg ml⁻¹), and fibronectin (7.7 μg ml⁻¹).

†Data expressed as mean ± s.e.m. Day 6 cultures were fixed in 3% paraformaldehyde and stained with Giemsa. Attached glomeruli were counted from four random colonies. A photographic projector and morphometry were used to determine colony-covering areas (see Materials and Methods). Final density = density in colony × total colony area/25 cm².

‡Waymouth’s medium supplemented with 20% foetal bovine serum.
spindle cell colonies (Fig. 7B). On day 20, cells grown in chemically defined medium on plastic were almost 100% glomerular epithelial cells, while cells grown on plastic in the presence of foetal bovine serum showed approximately 50% each of glomerular epithelial and spindle cells. In contrast, glomeruli grown on the HR-9 matrix in defined medium showed both epithelial cell types (Fig. 7C); on day 20, the flasks contained approximately 80% glomerular epithelial cells and 20% tubular cells. Glomeruli grown on the HR-9 matrix in the presence of foetal bovine serum showed epithelial, spindle and tubular cells (Fig. 7D); on day 20, morphometric analysis showed approximately 50% tubular cells, 30% spindle cells and 20% glomerular epithelial cells.

Effect of medium and substrate on glomerular attachment

Attachment of glomeruli was quantitatively assessed by morphometric techniques. Attachment of whole glomeruli was clearly influenced by culture conditions. The least attachment occurred when glomeruli were grown on plastic in chemically defined medium, and the greatest attachment occurred on the HR-9 matrix when the glomeruli were grown in foetal bovine serum (Table 1). Statistical analysis using ANOVA and the Tukey test indicated that greater attachment occurred on plastic in medium containing serum than in chemically defined medium. There was no significant difference in glomerular attachment on the HR-9 matrix in medium containing serum compared to chemically defined medium. Glomerular attachment was greater on the HR-9 matrix than on plastic in both serum and chemically defined medium.

Source of glomerulus-derived tubular cells

Quantitative studies indicated that our glomerular preparations were greater than 98% pure (unpublished observations). However, we occasionally found glomeruli attached to a fragment of proximal tubule in all our preparations (Fig. 8). Since these contaminants seemed to be the source of tubular cells in glomerular culture, we decided to study the morphology of cells in culture derived from isolated purified renal tubules (>99.5% pure). These tubular explants gave rise to cell outgrowths on the HR-9 matrix, either in serum or in chemically defined medium, which were identical to the tubular cells seen in glomerular culture. The cells formed well-circumscribed colonies (Fig. 9); SEM showed cells with microvilli and cilia, and TEM showed a morphology identical to that of tubular cells in glomerular culture (prominent tight junctions between cells). It is important to emphasize that this tubular morphology occurred in the absence of glomeruli.

Tubular cell differentiation: hemicyst and tubule formation

Tubular colonies from both glomerular and tubular explants formed prominent hemicysts on the HR-9 matrix but not on plastic (Fig. 10A). As shown by TEM, hemicysts were composed of tubular cells and lined with a basement membrane (Fig. 10B). Hemicyst formation was greater in foetal bovine serum than in defined medium (Table 2).
Selection by culture conditions

Fig. 8. Phase-contrast microscopy of initial glomerular preparation. A minority (<2%) of glomeruli (g) were attached to a small fragment of proximal tubule (t). X169.

Fig. 9. Light microscopy of outgrowth from tubular explants. Purified renal cortical cells (>99.5% pure) were cultured in foetal bovine serum on HR-9 matrix. Colonies of cells identical to the tubular cell type present in glomerular culture were seen. Giemsa stain; X101.
Table 2. Hemicyst formation in glomerular culture on HR-9 matrix

<table>
<thead>
<tr>
<th>Day</th>
<th>Hemicysts/flask (number)</th>
<th>Hemicysts/cm² tubular colony † (number)</th>
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<tbody>
<tr>
<td></td>
<td>Defined medium*</td>
<td>WY medium†</td>
</tr>
<tr>
<td></td>
<td>Defined medium</td>
<td>WY medium†</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
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<tr>
<td>10</td>
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<td>13</td>
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</tr>
<tr>
<td>20</td>
<td>33</td>
<td>17·9</td>
</tr>
</tbody>
</table>

Data expressed as mean of duplicate cultures.
*Chemically defined medium: Waymouth's medium supplemented with insulin (5 μg ml⁻¹), transferrin (5 μg ml⁻¹), selenium (5 ng ml⁻¹), triiodothyronine (7 ng ml⁻¹), and hydrocortisone (0·18 μg ml⁻¹).
†Waymouth's medium supplemented with 20% foetal bovine serum.
‡Area of tubular colony determined by morphometry (described in Materials and Methods).

Subculture of glomerular cells

Both glomerular and tubular epithelial cells were subcultured several times on the HR-9 matrix, but could not be subcultured on plastic (data not shown). In contrast, the spindle-shaped cell was subcultured both on the HR-9 matrix and on plastic in the presence of foetal bovine serum (Fig. 12). The cells survived for at least 14 generations (cell doublings) until senescence. The exact number of generations that occurred is greater than 14 but we could not calculate the number of cell divisions that occurred before the first subculture. (We do not know the number of viable cells that can undergo division in primary glomerular explants.)

Discussion

Previous studies in our laboratory demonstrated the growth of adult kidney glomerular cells on plastic in a chemically defined medium containing insulin, transferrin, triiodothyronine, selenium and fibronectin. Although this medium was selective in that it resulted in growth predominantly of one cell type (the glomerular epithelial cell; Oberley et al. 1983, 1986), these cells could not be subcultured. Similarly, although we were able to grow tubular cells on plastic in the same chemically...
Fig. 11. Morphology of tubule formation. A. Phase-contrast microscopy of tubule formation. Tubule-like structures (arrow) extend between tubular colonies. ×160. B. TEM of tubular structures (perpendicular section). The monolayer on the HR-9 substrate is composed of flattened cells (pe). More cuboidal cells (te) are seen above the monolayer. Most tubules (t) are 'inside-out', with microvilli (m) on the outside. p, plastic. ×1350.
defined medium, with the exception that laminin was substituted for fibronectin (Oberley & Steinert, 1983), these cells also did not grow well in subculture.

In the present study, we tested the effect of an authentic basement membrane on kidney cell growth. An intact basement membrane scaffold is required in vivo for the maintenance of orderly tissue structure: the basement membrane scaffold defines the spatial relationships between different tissue cell types. The cell substrate plays an important role not only in cell attachment and migration (Hay, 1981; Kleinman et al. 1981; Yamada, 1983), but also in the cell response to various growth-promoting agents present in plasma, lymph or interstitial fluid (Gospodarowicz & Tauber, 1980). During embryonic development, when different tissues are formed as a result of strictly timed and spatially interrelated proliferative and differentiating events, interaction of cells with newly formed basement membrane has been shown to result in cell proliferation and the expression of new phenotypes (Hay, 1978; Saxen et al. 1982). A well-documented example of the role of basement membrane in development is the formation of the kidney nephron, which results from migration of the ureter bud into the metanephric mesenchyme (Ekblom et al. 1980, 1981a,b; Ekblom, 1981). Metanephric mesenchymal cells are embedded in an extracellular matrix composed of collagen types I and III, and fibronectin. Under the influence of the invading ureter bud, mesenchymal cells synthesize a basement membrane composed of type IV collagen, heparan sulphate proteoglycan and laminin. Creation of this new basement membrane closely correlates with the development of the epithelial part of the glomerulus and the associated nephron tubule. Induction of the
ureter bud therefore stimulates the production of a basement membrane containing type IV collagen, heparan sulphate proteoglycan and laminin, resulting in the formation of the proper substrate for epithelial cell attachment and further differentiation. The basement membrane used in this study, the HR-9 matrix, is an epithelial type of basement membrane and is composed of type IV collagen, laminin and heparan sulphate proteoglycan (Lewo et al. 1982; Strickland et al. 1980; Hogan et al. 1982). Thus, its composition is very similar to that of kidney epithelial basement membrane and would be expected to support the growth and differentiation of kidney epithelial cells.

The present study demonstrates dramatic effects of the HR-9 extracellular matrix on the biology of cultured kidney cell types, which include: (1) the ability to subculture epithelial cells; (2) selection of cell types; (3) differentiation, including formation of multicellular domes (hemicysts), and structures resembling renal tubules. The formation of multicellular domes is a phenomenon of renal tubular epithelium associated with transepithelial salt transport (Misfeldt et al. 1976; Taub & Sato, 1980). This phenomenon and the formation of structures resembling tubules suggest that the HR-9 matrix promotes differentiation in vitro.

The nature of glomerular cells in culture remains a subject of controversy. Harper et al. (1984) concluded that the glomerulus-derived cell that we describe as the tubular cell is actually the glomerular epithelial cell. This conclusion was based on the assumption that the initial glomerular preparation was 'pure'. However, morphological studies in our laboratory suggest that even the purest preparations may contain tubular contaminants. The fact that nearly pure (>99.5% pure) tubular preparations gave rise to cells that were morphologically identical to glomerulus-derived tubular cells (characterized by microvilli, cilia and tight junctions) is a strong argument in favour of the tubular origin of these cells. Using SEM, both Cade-Trayer & Tsuji (1975) and Oberley & Steinert (1983) demonstrated that isolated cortical tubular cells display prominent microvilli and cilia on their cell surface just after cellular isolation and retain these features in primary culture. Further, in the present study isolated renal cortical tubules gave rise to tubular cell outgrowths in 4 days (unpublished observations), in contrast to the glomerulus-derived tubular cells, which required many days to become a significant fraction of the total cellular population, even on the HR-9 matrix. The formation of hemicysts and structures resembling tubules strongly indicates that glomerulus-derived tubular cells were derived from tubular epithelium. Finally, the histochemical studies performed in the study reported here are consistent with the tubular origin of these cells. In vivo analysis showed stronger tubular than glomerular staining for catalase and peroxidase (unpublished observations). In vitro histochemical studies conducted in the present investigation demonstrated that the tubular colonies stained more heavily than the glomerular colonies for both catalase and peroxidase.

The origin of the spindle cell is uncertain. Most investigators believe this cell to be derived from the mesangial cell (Harper et al. 1984; Striker & Striker, 1985). Indeed, the presence of microfilaments with dense bodies observed in the present study is consistent with the proposed smooth muscle origin of this cell type.
However, it is possible that the spindle cell represents a primitive cell type present in the glomerulus (a stem cell?), and we therefore hesitate to identify this cell conclusively until biochemical markers are available. Certainly, this cell type is unique in that it grows equally well on plastic and on the HR-9 matrix.

The molecular basis for the selection of cell types by the extracellular matrix is not clear. However, understanding the culture conditions that determine selection has enabled us to isolate homogeneous populations of glomerular epithelial, tubular epithelial, and spindle cells (unpublished observations). These cell types can be used for studies of the biochemical mechanisms involved in glomerular pathology. Such studies should prove more informative than studies in which mixed cell populations grown in serum have been used, a medium that contains many unknown factors.

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REFERENCES


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