Virchows Archiv (1994) 425:391–398

ORIGINAL ARTICLE

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Cell culture from rat renal glomeruli

Received: 5 April 1994 / Accepted: 26 July 1994

Abstract The primary culture of rat renal glomeruli was found to result in the ready outgrowth of two cells types. One type designated c-cells were cytokeratin positive and exhibited microvilli and cilia. The second type designated f-cells were vimentin positive and showed rugose surfaces. C-cells were polygonal in culture on plastic surfaces and were derived from cells of parietal epithelial origin. F-cells assumed a more extended form on plastic and were judged to be a sub-set of parietal epithelial cells. Neither cell type was derived from the visceral epithelium which was found to have been destroyed during isolation of the glomeruli. When cultured on isolated glomerular basement membrane both the c-cells and f-cells assumed a polygonal morphology but when grown on Matrigel the cells assumed the form of long strands interconnecting the outgrowths between the glomeruli. The appearance of the cells in the strands, judged from scanning electron microscopy, suggested that these were formed from f-cells but other cell types were entrained in the structures. Glomeruli subjected to vigorous proteinase digestion of the basement membrane allowed culture of a wider variety of cells. These included endothelial cells, judged by OX-43 antibody and anti-von Willebrand Factor staining, and mesangial cells. In cultures from glomeruli polygonal cells are often assumed to be visceral epithelial cells, the results from this study indicate that this assumption is unsound. The very different behaviour of cells grown on isolated basement membrane as compared with cells grown on Matrigel suggests that Matrigel may not faithfully mimic basement membrane with respect to cell response in culture.

Key words Kidney · Renal · Glomeruli · Endothelial cells · Mesangial cell

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Introduction

The culture of renal glomerular cells has attracted interest since the technique promises to provide new insights regarding normal and abnormal renal function [16]. Four major cells types are present in the glomerulus, parietal epithelial cells (PEC) lining the Bowman's capsule, visceral epithelial cells (VEC) on the external surfaces of capillaries, endothelial cells (EC) and mesangial cells (MC). Techniques for glomerular cell culture have been developed but, except for mesangial cells, the characterisation of outgrowing cells has been uncertain. Mesangial cells grow rapidly at high (20%) serum concentrations, exhibit a characteristic appearance in culture and have been studied in some detail regarding their physiological and pathological roles in the glomerulus [19]. Epithelial-like cells, in contrast, grow at low serum concentration (10%), form "cobblestone" monolayers, and have commonly been observed in culture and in subculture [11, 17, 27]. Their lineage is uncertain; both visceral [11, 27] or parietal [14, 22] origins have been proposed. Culture of glomerular endothelial cells has proved more difficult; their culture from man [26], cow [3] and mouse [17] has been reported though outgrowth of EC in primary cultures seems an uncommon event.

Comparisons between published findings are difficult; different methods of cell culture, the use of different, often idiosyncratic, antibodies for cell characterisation, the possible loss of cell antigens, and changes in cell morphology in culture render comparisons uncertain. Holthofer and co-workers [13, 14] have systematically studied glomerular cell culture using a range of cytological markers and have shown at least four different cell types in primary cultures of rat glomeruli. Here the outgrowth of cells from rat glomeruli has been studied initially with the intention of isolating capillary endothelial cells. This aim was not achieved but the findings illustrated how constraints imposed by the structure of the glomerulus determined cell outgrowth.

Methods

Male wistar rats (200–250 g) were obtained from Olac, Oxon. Plates (24 well) and NUNC 25 cm^2 flasks for cell culture were from Becton and Dickinson (New Jersey, U.S.A.). Hank's balanced salt solution, RPMI 1640 medium, fetal calf serum (FCS), fungizone, penicillin/streptomycin, were from ICN (High Wycombe, U.K.). SF1 supplement was from Northumbria Biologicals (Northumberland, U.K.). Bovine serum albumin, collagenase (Types II, V and VII), gelatin (Type III), heparin and hexamethyldisilizane were from Sigma (Poole, U.K.).

Glomerular basement membrane (GBM) was isolated from rat kidneys and coated onto 24 well plates as described by Bray et al. [4]. Matrigel was obtained from ICN.

Rabbit anti-human von Willebrand factor IgG fraction, normal rabbit IgG fraction, rabbit anti-mouse fluorescein conjugate, swine anti-rabbit rhodamine conjugate and mouse anti-human desmin monoclonal were from DAKO (High Wycombe, U.K.). The monoclonal OX-43 [24, 25] was from Serotec (Oxford, U.K.). Anti-vimentin monoclonal (clone V9) was from Sigma, anti-cytokeratin monoclonal KT 8.13 (cytokeratins 1, 5, 6, 7, 8, 10, 11, 19) was from ICN. The control monoclonal JAB-1 (against slime mould glycoprotein) was a gift from Dr. Newell, Dept. of Biochemistry, Oxford. 5-1-6 monoclonal [23] was a gift from Dr. Shimizu, Department of Immunology, Niigata University School of Medicine. Purified fibroblast growth factor (FGF) from bovine brain was a gift from Dr. Heath, Dept. of Biochemistry, Oxford.

For isolation of glomeruli kidneys were removed from freshly killed rats without perfusion, and placed in Hanks buffered salt solution (HBSS). The cortex was removed, washed with HBSS, and sequentially sieved by pressing with a beaker through 250 μm and 180 μm mesh sieves with the glomeruli finally recovered at 75 μm. The glomerular suspension in HBSS was left at room temperature for 15 min to sediment only the glomeruli which were then centrifugally washed twice at 500 g. Preparations consistently contained <5% tubules which failed to adhere and grow when explanted. The majority of the glomeruli recovered from the final sedimentation were encapsulated though capsules often seemed to be split and were sometimes folded back from the capillary tuft.

In some experiments glomeruli were treated with collagenase by incubation with Type VII (1500 U/ml), Type V (1 mg/ml), or Type II (1 mg/ml) enzyme for 30 min at 37° C in HBSS and the glomeruli recovered after being allowed to settle as before. Under these conditions only partial digestion of the glomeruli was achieved. Complete digestion of the glomerular structure was achieved as described by Cook et al. [7]. Glomeruli were incubated in HBSS containing trypsin (0.5 mg/ml), collagenase type II (1 mg/ml) and DNAase (0.1 mg/ml) for 20 minutes at 37° C followed by a second incubation with collagenase type II (1 mg/ml). Cell clumps and debris were removed by allowing them to settle for 5 min at 4° C in 2 mM EDTA in calcium and magnesium free phosphate buffered saline (PBS) (0.15 M sodium chloride, 0.05 M sodium phosphate pH 7.4).

For cell culture glomeruli were routinely plated out at 10000 per 25 cm^2 flask or 1000 per well of 24 well plates in RPMI-FCS medium containing 15% FCS, supplemented with heparin (10 μg/ml), penicillin (final concentration 50 IU/ml), streptomycin (50 IU/ml), fungizone (250 ng/ml) and the supplement SF1. Cultures were left undisturbed for 5–6 days for the glomeruli to adhere; outgrowths were then examined by microscopy.

For scanning electron microscopy (SEM) samples grown on coverslips in RPMI-FCS were washed for 30 s with PBS at 4° C and fixed by immersion in 5% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.2 for 30 min. After 30 min at 4° C in 7% sucrose in cacodylate buffer, cover-slips were washed twice with distilled water at room temperature, dehydrated through a graded series of ethanol and water, washed twice with absolute ethanol and incubated for 5 min at room temperature with hexamethyldisilazane [21]. Samples were mounted on aluminium stubs, sputter coated and viewed using a Jeol JSM T20 scanning electron microscope operated at 20 kV.

For transmission electron microscopy (TEM) glomeruli were isolated from primary cultures by trypsin-treatment (0.1% w/v in 0.15 M sodium chloride, 0.05 M sodium phosphate buffer pH 7.4 at 4° C) followed by gravitational sedimentation. Glomeruli were fixed in 1% glutaraldehyde for 60 min at −20° C, washed twice in acetate buffer and then embedded in LR Gold resin overnight at −20° C. Thin sections were cut (100 nm), stained with uranyl acetate and lead citrate and examined using a Philips 410M electron microscope operated at 80 kV.

Fluorescent microscopy: for in situ studies of glomerular antigens, kidneys were removed immediately from freshly-killed rats, frozen in Tissue-Tec and sections (4–6 μm) cut. Primary cultures for immunofluorescence were grown on gelatin-coated or GBM-coated glass cover-slips in RPMI-FCS. Samples briefly washed in PBS were fixed in acetone for 20 min at −20° C (for staining of the intermediate filaments) or ethanol for 10 min at 4° C (all other antigens), followed by blocking with 10% serum of the same species as that of the secondary antibody for 60 min. Samples were stained with primary antibody diluted in PBS containing 10% bovine serum albumin (BSA) for 60 min, washed and incubated with secondary antibody diluted in PBS containing 10% BSA and 10% serum for 30 min. All incubations were at 37° C. Coverslips were then washed three times with PBS, left to stand in PBS for 10 min, dipped briefly in distilled water and mounted in 90% glycerol/10% PBS (v/v).

Samples were viewed using an Olympus OH2 fluorescent microscope and photographed with Ektachrome P800/1600 film.

Results

Cell outgrowth was readily obtained from isolated glomeruli when plated out as described under methods provided the glomeruli were allowed to adhere and grow without disturbance for 5–6 days. In RPMI-15% FCS outgrowths developed progressively over this period but their composition was influenced by the treatment accorded the glomeruli prior to plating as is to be described below.

When untreated or Type VII collagenase-treated glomeruli were cultured on plastic the glomeruli became encompassed by compact outgrowths of polygonal cells approximately 30–40 μm across; termed compact cells (c-cells). These were interspersed and usually surrounded by a second cell type with fibroblastic morphology (f-cells) which appeared to be more motile, illustrated together in Fig. 1. Occasionally flattened cells were found which contained one or two nuclei and were up to 150 μm across; these were never observed to develop as colonies.

Examination of the outgrowing cells by SEM revealed vigorous cell outgrowths apparently originating from within the capsules and confirmed the presence of two separate cell types. These showed distinctly different surface morphologies, one presenting a corrugated exterior while the other possessed abundant microvilli and were ciliated (Fig. 2). Immunofluorescence further confirmed this duality; the f-cells stained for vimentin and the c-cells for cytokeratin using the KT 8.13 mab (Fig. 3).

When tissue sections were reacted with KT 8.13 mab the only reactive cells in the glomeruli were crescents within the periphery of the Bowmans capsule (Fig. 4). Thus the c-cells appeared to be derived from these cyto-

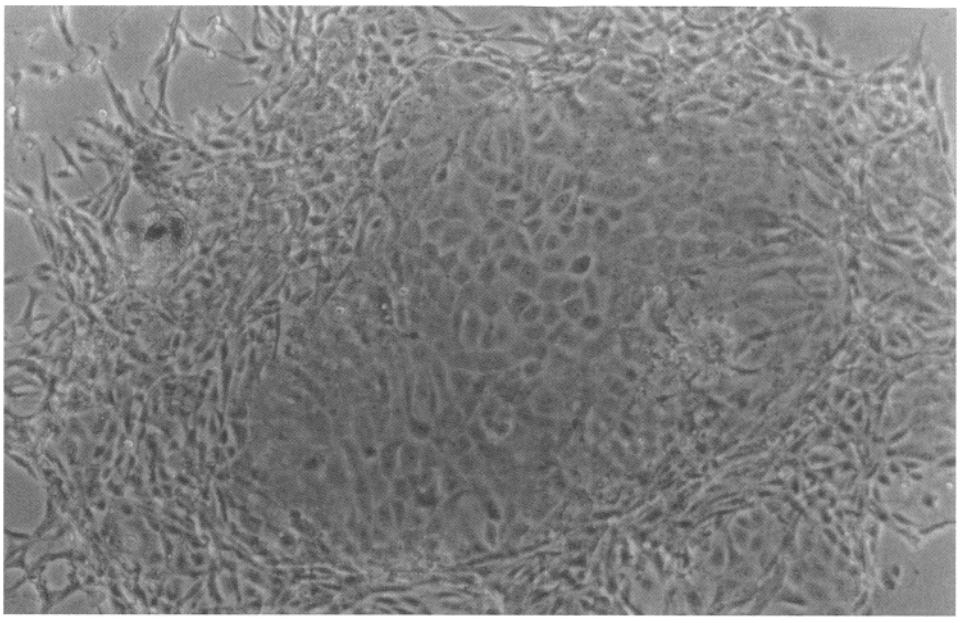

Fig. 1 Sixth day primary outgrowth from explanted glomeruli showing two different cell morphologies. ×130

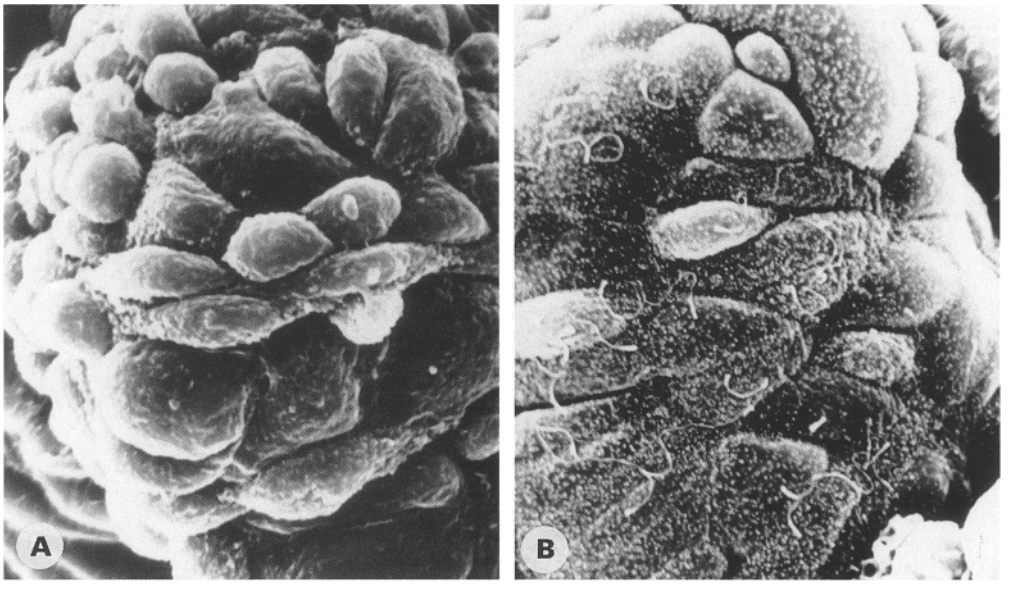

Fig. 2 Scanning electron micrographs (SEM) of glomeruli after five days in culture showing (**A**) rugose cells and (**B**) ciliated cells, ×3000

keratin-positive parietal epithelial cells. Vimentin staining in sections was most obvious within the glomeruli where the capillaries were highlighted (Fig. 4) which suggested that the f-cells might be endothelial or visceral epithelial in origin. Two immunofluorescence markers were used to identify endothelial cells. Anti-von Willebrand Factor was found to stain glomerular capillary endothelium in a linear pattern which was distinct from the punctate staining seen in arteriolar endothelial cells. OX-43 mab stained the endothelium of all the blood vessels in sections and gave strong staining with the glomerular capillaries (Fig. 4). However neither antibody cross-reacted with the cells in outgrowths. The 5-1-6 mab was confirmed in tissue sections as being reactive to the foot processes of the visceral epithelial cells [23]. This reagent also did not react with cells in outgrowths though it did stain the glomerular cores. It seemed therefore that the outgrowing f-cells were neither endothelial nor visceral epithelial in origin.

Examination of glomeruli by TEM both immediately after isolation from kidney and 5–6 days after incubation provided clearer insight. In newly isolated glomeruli the visceral epithelial cell bodies were found to have been destroyed although the foot processes were still associated with the capillary walls (Fig. 5); the VEC are presumably very fragile. This explained why the 5-1-6 mab reacted with isolated glomeruli but not with outgrowing cells. At this stage the parietal epithelial cells and the endothelial cells appeared normal (Fig. 5). After 5 days in culture the open structure of the glomeruli had collapsed.

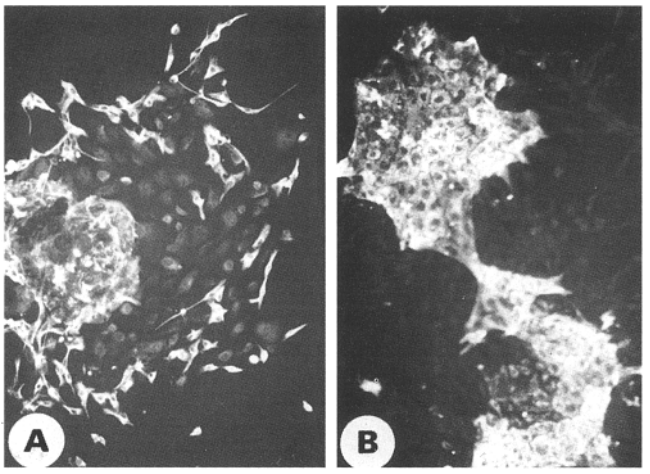

Fig. 3 Immunofluorescence staining of sixth day primary outgrowths from type VII collagenase treated glomeruli. (**A**) Anti-vimentin, (**B**) anti-cytokeratin KT 8.13. ×115

The parietal epithelial cells were dividing and becoming numerous while the endothelial cells, though intact, remained trapped within the capillary lumens (Fig. 5).

This entrapment seemed to have prevented their growing out from the glomeruli. The ready growth of the parietal epithelial cells accorded with the conclusion that the c-cells were of parietal origin. Further inspection of glomeruli stained for vimentin in tissue sections revealed segments of weak staining around the periphery of the glomerulus (Fig. 4). In view of the ready growth of the PEC it was concluded that the f-cells were also derived from the parietal epithelium, from a sub-set of cells which were vimentin and not cytokeratin positive.

Since the endothelial cells appeared trapped within the lumens of the glomerular capillaries different collagenase treatments were employed to disrupt the glomerular architecture. Glomeruli treated with type II or type V collagenase yielded few c-cells in outgrowths but f-cells were seen together with scattered cells of mesangial

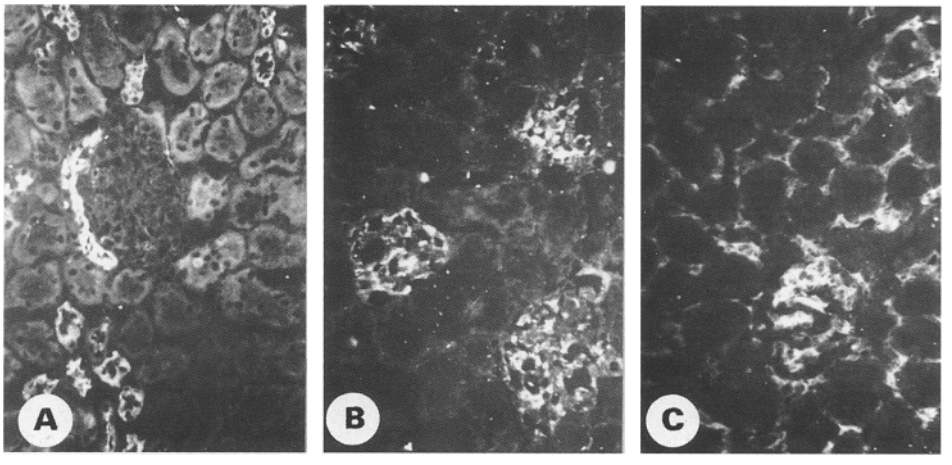

Fig. 4 Immunofluorescence staining of rat renal cortical sections (**A**) anti-cytokeratin, KT 8.13, ×67; (**B**) anti-vimentin, ×67; (**C**) OX-43 antigen, ×67

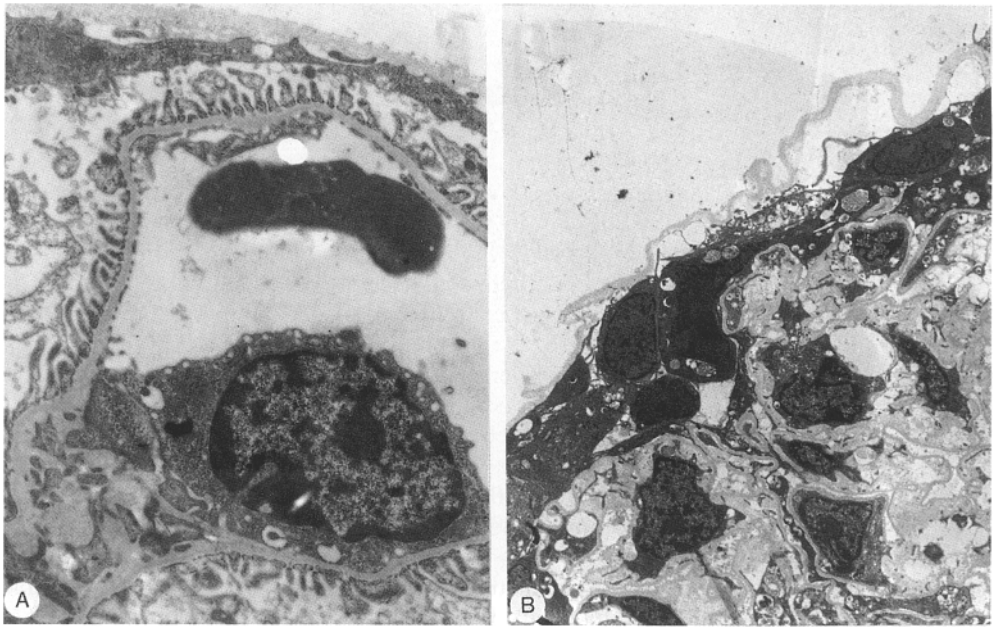

Fig. 5 Transmission electronmicrographs of glomeruli following isolation by sieving. (**A**) Freshly isolated glomerulus showing well-preserved endothelial and parietal epithelial cells but damaged visceral epithelial cells ×25500; (**B**) glomerulus after 5 days in culture showing collapsed capillaries, no visceral epithelial cells but numerous cells below the Bowmans capsule, × 7160

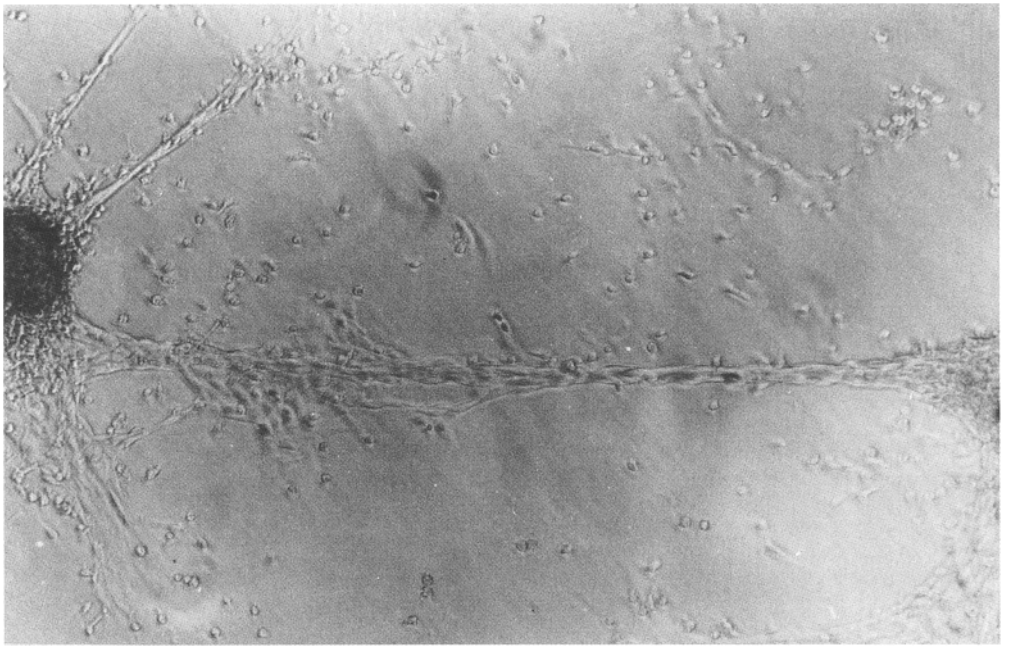

Fig. 6 Strand formed after 14 days by primary outgrowths of glomeruli cultured in serum free medium containing fibroblast growth factor, ×130

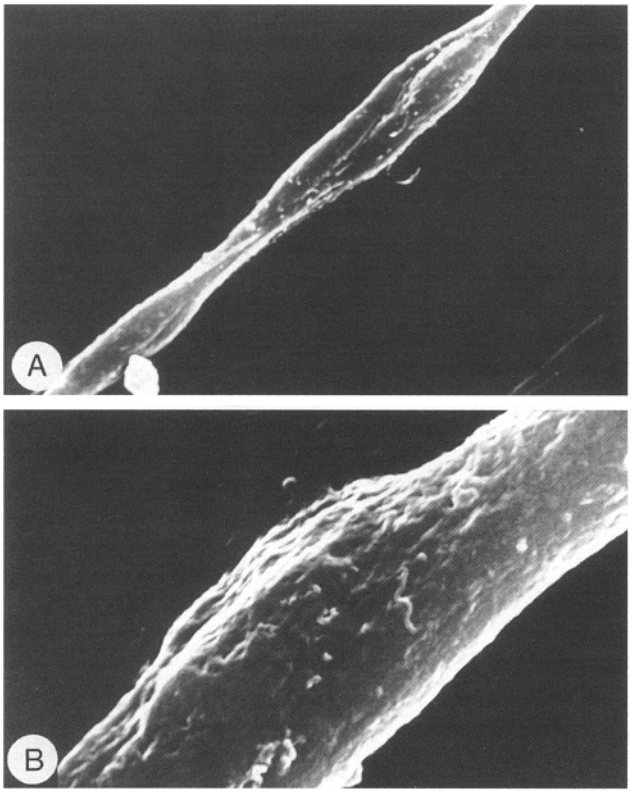

Fig. 7 SEM of strands formed after 5 days culture of glomeruli on Matrigel (**A**) thin strand of intertwined cells, ×3600 (**B**) strand showing pitted cell surfaces, ×11500

morphology which stained for desmin. The mesangial cells exhibited a characteristic morphology, flattened cells of irregular shape, which were readily distinguished from the f- and c-cells as neither stained for desmin. Endothelial cells were rarely seen as judged by anti-vWF or OX-43 staining though media were supplemented with bFGF (10 nM) which had been shown to support the growth of bovine aortic endothelial cells.

Disruption of the glomeruli with trypsin and type II collagenase produced mixed outgrowths of cells including mesangial cells but small, scattered islands of endothelial cells were now found which stained weakly with anti-vWF and with OX-43. To retard the vigorous mesangial growth, trypsin/type II collagenase treated glomeruli were grown on gelatin films in RPMI-15% plasma derived calf serum with endothelium growth supplement added. Distinct islands of endothelial cells were observed but surrounded still by large outgrowths of f-cells and c-cells. Attempts to isolate endothelial cells for onward culture proved impossible in our hands despite exploring a range of methods for sub-culture.

Attempts to encourage the growth of endothelial cells using growth factors and/or different growth matrices offered no advantage, nevertheless they did promote unexpected responses. When outgrowths from untreated or type IV collagenase treated cells were switched after 5 days into serum-free medium (RPMI with SF1 supplement) cell morphology changed, the cells becoming bipolar and migratory. Some 8–10 days following the switch the cells had formed networks of thin strands interconnecting the glomeruli (Fig. 6). Under SEM the strands were seen to be composed of intertwined, elongated cells (Fig. 7). This behaviour was reproducible in NUNC flasks but was difficult to reproduce in other containers, e.g. 28 well plates. Time-lapse photography showed elongated motile cells vigorously migrating over the flask surface, aggregating into strands which might then dissolve again or might remain and grow. Strands of the networks interconnecting the glomeruli formed and persisted, grew thicker and eventually snapped like elastic bands indicating that the strands were under tension.

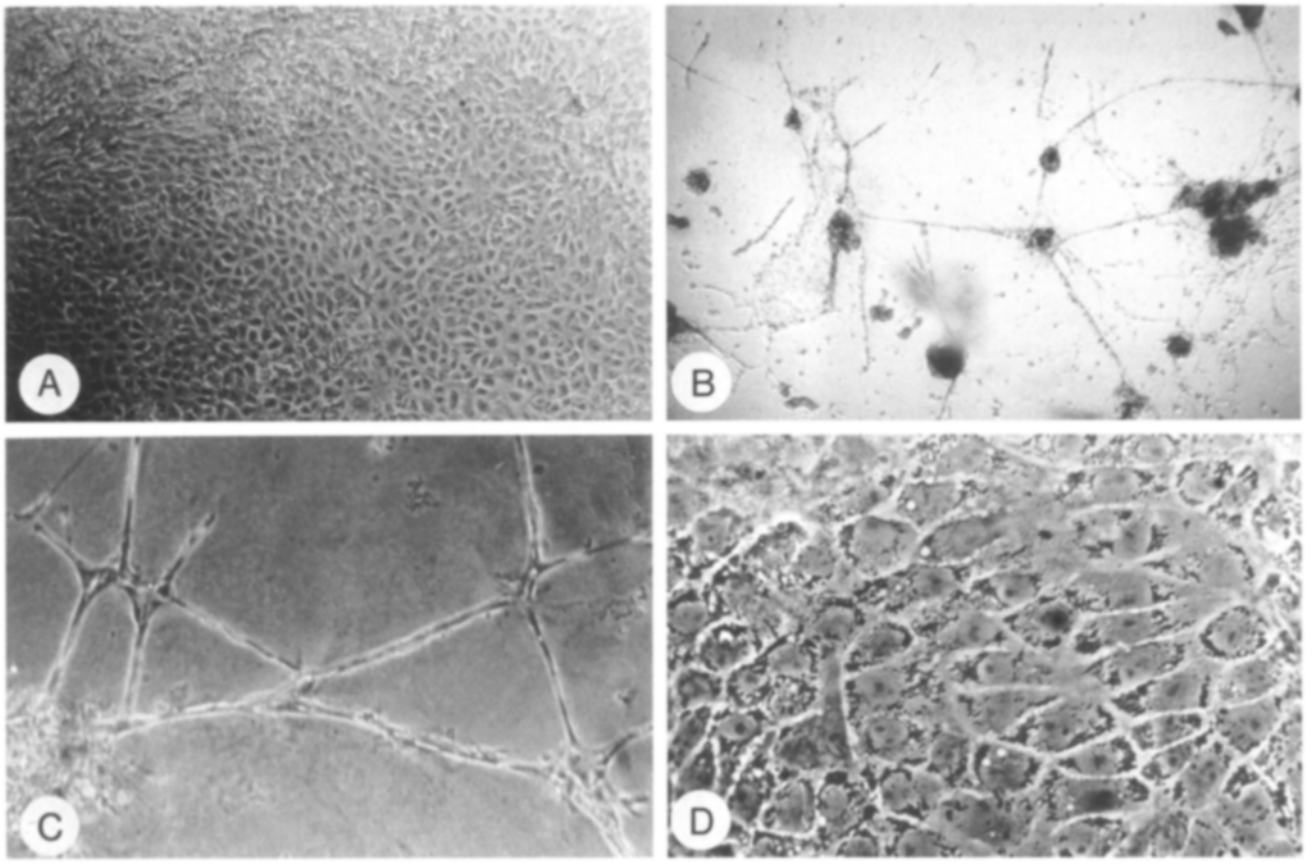

Fig. 8 Six day cultures of rat glomeruli (**A**) on GBM, ×100 (**B**) on Matrigel, ×50 (**C**) on Matrigel, ×120 (**D**) novel cell morphology seen only on Matrigel, ×490

The cells then clustered around the glomeruli and seemed to lose the potential to grow out.

Growing glomeruli (untreated and type VII collagenase treated) on Matrigel, a putative basement membrane analogue, resulted in strand formation within 5–6 days; strands could be up to 0.5 mm long (Fig. 8). These strands could be formed on cover slips for immunofluorescent analysis which allowed attempts to identify the cells within the strands. There was no staining with anti-vWF, OX-43, or anti-desmin.

Immunostaining for vimentin, cytokeratin or other proteins was complicated by background binding of antibody to the matrix making it difficult to judge whether the cells in the thin strands were staining. Possibly both f-cells and c-cells were present. On SEM some of the cells in the strands seemed to possess sparse microvilli while others were smooth surfaced which might support this interpretation. Matrigel also supported the growth of small colonies of granulated cells which were not seen under any other culture conditions (Fig. 8).

In attempting to resolve the question of the identity of the strand forming cells glomeruli were cultured on isolated GBM as an alternative physiological matrix. The cells did not now form strands but grew to confluence in 6/7 days as sheets of polygonal cells (Fig. 8). Immunostaining showed both c-cells and f-cells as judged by cytokeratin and vimentin staining. The f-cells had assumed polygonal form. No staining was found with anti-vWF, OX-43 mab, or anti-desmin. The cells behaved very differently on GBM as compared with Matrigel so that this approach did not fulfil the aim of identifying the strand-forming cells.

Attempts to isolate and passage the strand forming cells were frustrated by the tendency of the strands to retract into the main cell mass when severed. Passaging on Matrigel after dissociating cultures with Dispase produced clumps of cells with isolated cells assuming ringlet form. The cells did not revert to monolayers on many of the substrates.

During these studies a range of potential immunofluorescent markers were used for characterisation: OX-42 mab for the C3 receptor, Ulex and Bandeiraea (isoform B4 and BS4) lectins, anti-cytokeratin mab AE3 (basic cytokeratins) and PAN (cytokeratins 1–19). None proved usefully reactive though protein expression by cells may have changed during growth in vitro.

Discussion

The growth of cells from isolated glomeruli is easily achieved but establishing the identities of cells in the outgrowths is problematical. It is clear that there are two prominent cell types in 5–6 day primary cultures from

untreated glomeruli or from glomeruli after treatment with collagenase type VII. These were (Figs. 1 and 3) the compact cytokeratin positive cells (c-cells) and the more extended vimentin positive cells (f-cells). Their different intermediate filament proteins provided a positive means for discrimination. Though cell morphology did appear distinctive in primary culture on plastic, morphology was changeable and was an unreliable criterion for identification. When grown on basement membrane all the cells appeared polygonal (Fig. 8a) but on Matrigel the strand forming morphology was most obvious (Fig. 8b). Clearly morphology was critically dependent upon substrate.

The capacity of cells in primary outgrowths to form strands was striking (Figs. 6, 7b, 7c). This behaviour was invariably elicited when the cells were grown on Matrigel but never when the cells were grown on isolated basement membrane. Thus the assumption that Matrigel mimics basement membrane as a cell culture substrate [15, 28] must be viewed with caution. SEM showed the strands originating from rugose cells in the outgrowths and the cells in the strands showed no microvilli though they sometimes exhibited cilia-like protrusions (Fig. 7); commonly the cells appeared to possess pitted surfaces (Fig. 7b). Occasionally cells along the strands were found to be positive for vWF. It seemed that cells of different origins could become entrained in the strands. The technical difficulties noted earlier in trying to immunostain the strands for cytoskeletal proteins prevented more detailed study though the f-cells, which were able to assume an elongated morphology (Fig. 1), were judged to be the predominant cell in the strands. The strands were different in structure from the tubules formed by MDCK epithelial cells [28].

The origins of the major two cell types in outgrowths remain to be decided. They are not VEC since they did not stain for 5-1-6 antigen which was observed in tissue sections and is found in the podocytes [23]; this supports the observations of Holthofer et al. [14]. Absence of staining may well be explained by loss of phenotypic protein expression as the cells transferred to culture conditions. The more persuasive finding was the apparently complete destruction of the VEC seen by TEM (Fig. 5) which occurred during the isolation of the glomeruli (Fig. 5) and was seen even in glomeruli which had retained intact capsules; the VEC appear to be very sensitive to mechanical trauma. Norgaard [22] has proposed that large cells showing low proliferative capacity in rat glomerular cultures were VEC. Such cells were observed only rarely in our studies and their paucity is consonant with their having been destroyed during preparation of the glomeruli. Studies of human VEC cells identified by mab PHM5 [1, 10] have shown that these are large, 200 μm, vacuolated cells which are progressively lost from culture. These findings support Norgaard's view that the large cells seen in outgrowths from rat glomeruli are VEC. Neither are the cells EC since no staining was obtained with anti-vWF or OX-43 though glomeruli in situ and in vitro reacted positively. Cells reactive to both antibodies were most commonly observed when the glomerular capillary basement membrane had been extensively digested. Others have reported that EC in glomerular cultures show punctate staining for vWF [3, 18, 26]. Our observations suggest that in kidney such staining denotes cells of arterial rather than capillary origin.

Since the c-cells stained for cytokeratin their origin is most probably the parietal epithelium which stained for cytokeratin in situ where the staining was localised to a crescentic region of the capsule (Fig. 4a) as previously noted by Bachmann et al. [2]. This suggests that cytokeratin, which has been used as a marker for VEC [18, 26, 27], in fact delineates a sub-set of PEC; these cells persist in longer term cultures and were characterised by Holthofer et al. [14] as type I cells. Expression of cytokeratin by cells in culture may be variable, being lost during rapid proliferation [6] or when the appropriate substrate, basement membrane, is not provided [18]. However neither cytokeratin nor vimentin expression seemed to be influenced by the varied conditions of growth used here; growing cells on Matrigel or basement membrane did not seem to promote or diminish cytokeratin expression. The f-cells present a more difficult puzzle. Since they readily grew out from untreated or collagenase type VII treated glomeruli along with the c-cells they too are likely to be derived from the PEC. Against this PEC in situ seem not to show prominent staining for vimentin [(see Fig. 4b) and [2, 12]] but since they form but a thin layer within the Bowmans capsule staining in tissue sections may not be easily discernable. Other origins are though less likely. As argued above these cells cannot be endothelial or VEC which are either entrapped or destroyed. Their morphology, staining characteristics and behaviour are unlike those of MC. The cells were capable of forming ringlets on Matrigel, a capacity ascribed both to mesothelial cells [5] and to endothelial cells [9] but the f-cells do not possess the characteristics of either cell type. The capacity of cells to form networks and strands on some matrices is reported as a characteristic of EC [5, 8, 15, 20] but there was no evidence that the cells from glomeruli were endothelial in origin; thus such behaviour cannot be used for definitive identification. A further possibility is that these cells represent a "reserve" or "undifferentiated" cell type [2, 12] which may proliferate under in vitro conditions. However too little is known of these cells to be confident that they represent a distinct sub-set of glomerular cells. Weinstein et al. [30] have also judged glomerular cell outgrowths to be derived from parietal epithelium; in their studies the outgrowing cells were not recorded as being comprised of two separate sub-populations suggesting that differing culture conditions may encourage outgrowth of differing parietal cell types.

Glomerular cell cultures are increasingly used in studying the properties of individual cell types and of their response to inflammatory stimuli but identification of the cells used is sometimes cursory. Cells with polygonal morphology have been assumed to be VEC [11, 18, 27, 29] but the results reported here demonstrate that polygonal form is not an adequate criterion for the identifi-

cation of VEC. Indeed the VEC seem mechanically fragile and easily destroyed; their successful culture will require the development of less harsh methods for the isolation of glomeruli. In contrast EC culture seems to require vigorous disruption of the glomeruli to allow escape of the cells from the confines of the capillary basement membrane.

A strikingly reproducible effect was the difference in behaviour between cells grown on Matrigel and on GBM. On the former cells formed strands while on the latter only polygonal monolayers were formed. Indeed f-cell morphology became indistinguishable from that of c-cells on basement membrane. Matrigel is often used as a basement membrane analogue for cell culture. These results show that this substrate evokes very different cell behaviour from that induced by mature basement membrane. Hence cell behaviour on Matrigel may not reflect behaviour in vivo.

Acknowledgements We would like to thank Martin Barker for excellent technical help with both transmission and scanning electron-microscopy work and Miss J. C. Byrne for her skilled technical support. This work was supported by a grant (SP2028) from the Cancer Research Campaign. We are grateful to Dr. J. Heath and Dr. P. C. Newell of the Department of Biochemistry, and to Dr. Shimizu of Niigata University School of Medicine for generous gifts of materials.

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