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TECHNICAL NOTE

Improved methods for culturing rat glomerular cells

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Several investigators [1] have outlined techniques for culturing glomerular cells but many of these methods resulted in mixed cell populations and an inability to passage the cells consistently. There is also a lack of good criteria for cell identification. Previously, Kreisberg, Hoover, and Karnovsky [2] devised techniques in which they were able to isolate and grow pure, homogeneous cultures of rat glomerular cells. They characterized these as (A) a renin producing cell, (B) a contractile spindle-shaped cell containing numerous filamentous bundles, characterized as a mesangial cell, and (C) a flat polygonal-shaped cell possessing C3 receptors and the occasional cilium. The latter of these cell types was considered to be epithelial in origin. This procedure, however, is not thoroughly consistent, particularly with regards to the epithelial cell, in both the maintenance of good morphology over several passages and the ability to passage the cells successfully. In this study, we describe methods modified from those of Kreisberg, Hoover, and Karnovsky [2], in which we can obtain relatively homogenous cultures of epithelial and/or mesangial cells, which can be easily passaged, and in which the cells maintain the characteristics found in the explants.

Methods. Animals. Male or female Sprague-Dawley (CD strain, Charles River Breeding Labs, Wellesley, Massachusetts) weighing 50 to 60 g were used. Animals were anesthetized with ether then given lethal doses of sodium pentobarbiturate.

Isolation of glomeruli. The kidneys were excised and the cortices cut away from the medullae. These cortical pieces were chopped into millimeter square pieces and passed through a series of steel sieves (W. S. Tyler, Inc.) with decreasing pore sizes—200 μ-pore (60 mesh), 150 μ-pore (150 mesh), and 75 μ-pore (200 mesh)—with the glomeruli resulting on top of the 200-mesh sieve [3]. Previous studies using the same isolation method [2] have shown by scanning electron microscopy that the glomeruli are stripped of their capsules and that the preparations are almost 100% free of any tubular tissue. The capsules and the tubular material become stuck on a larger pore sieve through which the glomeruli pass.

Cell culture. The isolated glomeruli were rinsed twice in Hanks' balanced salt solution, buffered with HEPES, pH 7.4 (HBSS), containing antibiotics (penicillin, 100 U/ml, streptomycin, 100 μg/ml, and amphotericin, 0.25 μg/ml), and incubated with trypsin (0.2%) for 20 min at 37°C followed by an incubation with 0.1% collagenase (189 U/ml, Worthington Diagnostics Systems, Inc.) for 40 min at 37°C. This procedure loosens up the glomeruli but gives few single cells. After it was washed once in buffered HBSS and antibiotics, the pellet was divided, resuspended in the appropriate media, and plated under the appropriate conditions to permit proliferation of either mesangial or epithelial cells.

Polygonal cells were obtained by resuspending the enzyme-treated glomeruli in four 60-mm Petri dishes in a medium (K1-3T3) consisting of equal parts of: *(1),* a defined medium (K1) [4] containing 5% NuSerum (Collaborative Research, Waltham, Massachusetts) and *(2),* 24-hr conditioned medium taken from cultures of Swiss 3T3 fibroblasts grown in Dulbecco's minimum essential medium plus 10% fetal calf serum. After a week, the predominantly polygonal cells were passaged at low density (10^2/ml) by mild trypsinization. The dishes were washed twice in calcium-magnesium free HBSS, followed by a trypsin (0.025%)/EDTA (0.5 mM) solution which was immediately poured off. After 5 min at 37°C, the cells were detached by agitation and plated at a concentration of 10^2/ml onto collagen gel-coated 100 mm Petri plates in K1-3T3 medium.The collagen (Flow Lab., Inc.) gel-coated tissue culture plates were prepared as follows: The acid soluble collagen was mixed 8:1:1 with 10X RPMI-1640 medium and 0.1 N NaOH. Three to four milliliters of this solution were added per 100 mm Petri dish and allowed to gel at 37°C for 60 min. Once colonies appeared (~7 days), some subsequently reaching a diameter of ~1 cm, their position was marked on the underside of the dish. The cloning efficiency was approximately 10%; however, only about ten clones of varying sizes were picked per dish. This was done by cutting around each of the marked colonies with a hypodermic needle, and placing each in a tube containing 1 ml of 0.2% collagenase. This was incubated at 37°C until the collagen was completely solubilized (about 30 min). Enough K1-3T3 medium was then added to fill the tube and the cells were pelleted by centrifugation ($\times 100g$ for 5 min). Each cell pellet was resuspended in 1 ml of the K1-3T3 medium and plated into one well of a 24-well cluster dish coated with collagen gel. Once the cells reached confluency, they could be passaged into larger dishes—always using the K1-3T3 medium and collagen-coated dishes.

In some experiments, we used a new medium developed by Hybridoma Sciences, Inc. (Atlanta, Georgia), called HSI-LoSm, plus 5% NuSerum in place of the K1-3T3 mixture. In other experiments, we tried plating our primary cells onto a

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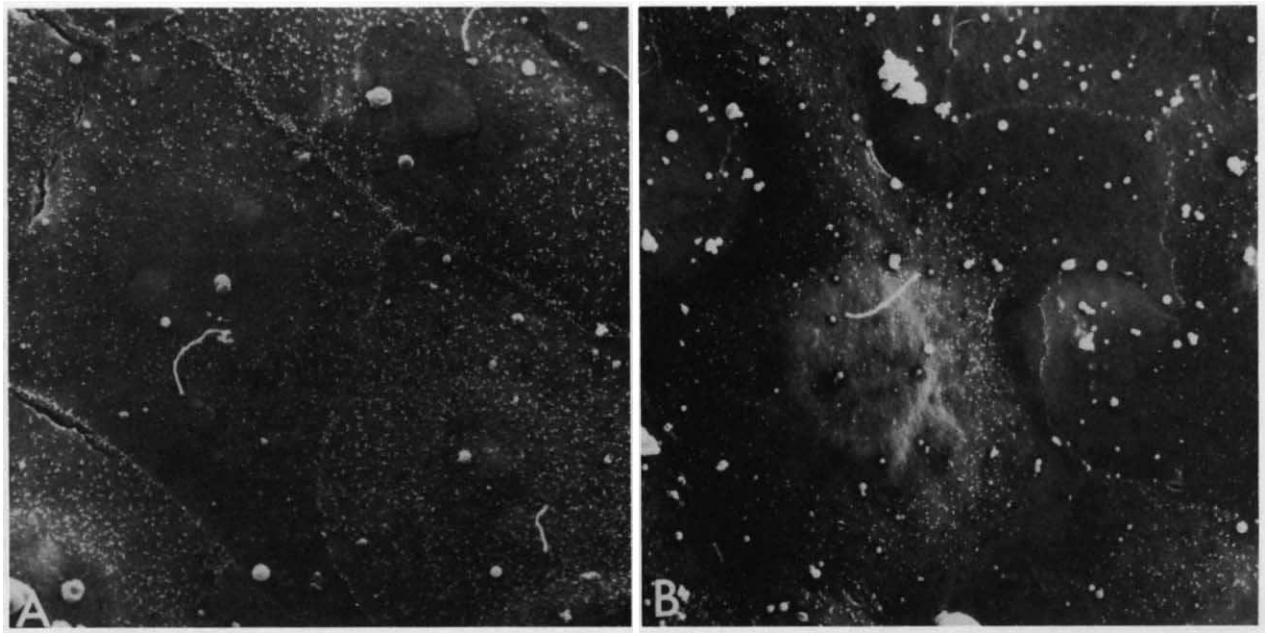

Fig. 1. A *SEM, cellular outgrowth from explanted glomeruli showing typical epithelial morphology in primary cultures.* The cells are polygonal, closely packed, flat, bear numerous microvilli, and one or two cilia. (×1700) **B** *SEM, less common appearance of cellular outgrowth from explanted glomeruli, showing greater irregularity and overlapping margins.* (×1700)

new tissue culture plastic, Primaria (Falcon Products), which was designed to inhibit fibroblast growth.

Spindle-shaped cells were obtained by suspending the enzyme-treated glomeruli in a 1:1 mixture (RPMI-3T3) of RPMI-1640 medium plus 20% fetal calf serum plus antibiotics plus insulin (0.66 U/ml) and conditioned medium from Swiss 3T3 fibroblasts and plated into untreated tissue culture plastic dishes. When the colonies developed, their positions were marked and removed by mild trypsinization. The growth medium was decanted and the cells washed twice in calcium-magnesium free HBSS. Each clone was isolated on the dish using glass cloning rings (6 to 10 mm, Bellco Glass, Inc.) into which one drop of trypsin (0.025%)/EDTA (0.5 mM) was added. After 5 min at 37°C, the trypsin action was stopped by the addition of 100 μl of growth medium containing serum. The clones were removed by gentle tituration with a Pasteur pipette and plated in one well of a 24-well cluster dish containing 1 ml of the RPMI-3T3 medium. After the culture reached confluency, and in subsequent passages, the cells were maintained in RPMI-1640 medium plus 20% fetal calf serum plus antibiotics. The 3T3 conditioned medium was no longer needed.

Immunofluorescence. Cells were plated onto coverslips (16 mm diameter) and allowed to grow to the desired density. The coverslips were rinsed twice in buffered HBSS and fixed in a 1:10 dilution of formalin (37%)/buffered HBSS for 1 hr at 22°C. The coverslips were rinsed twice with buffered HBSS, permeabilized with cold acetone (−20°C) for 5 min and immediately air-dried. Rabbit anti-sera raised against myosin from either smooth muscle (human uterus) or non-muscle (platelet) tissues (gifts from Dr. K. Fujiwara, Department of Anatomy, Harvard Medical School, Boston, Massachusetts) were then used to stain the coverslips. The antiserum raised against human platelet myosin has been described previously [5] and shown by immunodiffusion to react with one component in human platelet extracts and in purified platelet myosin. The antiserum stains a number of different cell types in culture consistent with their myosin distributions, including endothelium [6], smooth muscle [7], and HeLa cells [5]. The antiserum prepared against smooth muscle (human uterus) has also been described previously [8] and shown to react with a single component in extracts of human uterus. Under similar conditions it does not react with cardiac or skeletal muscle extracts or an extract from human platelets but does stain smooth muscle cells both in vivo and in vitro [8]. The dried coverslips were stained for 60 min at 22°C, rinsed twice with buffered HBSS, stained with either rhodamine- or fluorescein-conjugated goat anti-rabbit IgG (Cappel) for 1 hr at 22°C, then rinsed twice with buffered HBSS, and once with distilled water. The coverslips were mounted on slides with 50% glycerol in buffered HBSS and examined with a Leitz Ortholux II microscope equipped for epifluorescence with K2 (fluorescein observation) and N2 (rhodamine observation) filter blocks.

Scanning electron microscopy. Cells grown on coverslips were prepared for scanning electron microscopy (SEM) by first fixing in 2% glutaraldehyde and 4% formaldehyde in 0.1 M phosphate buffer (pH 7.0) for 48 hr at 4°C. After rinsing several times in phosphate buffer, the samples were treated with 1% tannic acid in 0.1 M cacodylate buffer (pH 7.2) for 30 min at 22°C, rinsed extensively with 0.1 M cacodylate buffer and postfixed in 1% osmium tetroxide in cacodylate buffer for 60 min at 22°C. The samples were then rinsed several times and dehydrated through a graded series of acetones. At the critical point they were dried out of carbon dioxide using a Balzers Union critical point drier and coated with gold/palladium using a Technics sputtering device. Samples were examined with an ETEC Autoscan electron microscope.

Transmission electron microscopy of domes. Cells grown to confluency on glass collagen-coated coverslips were fixed and

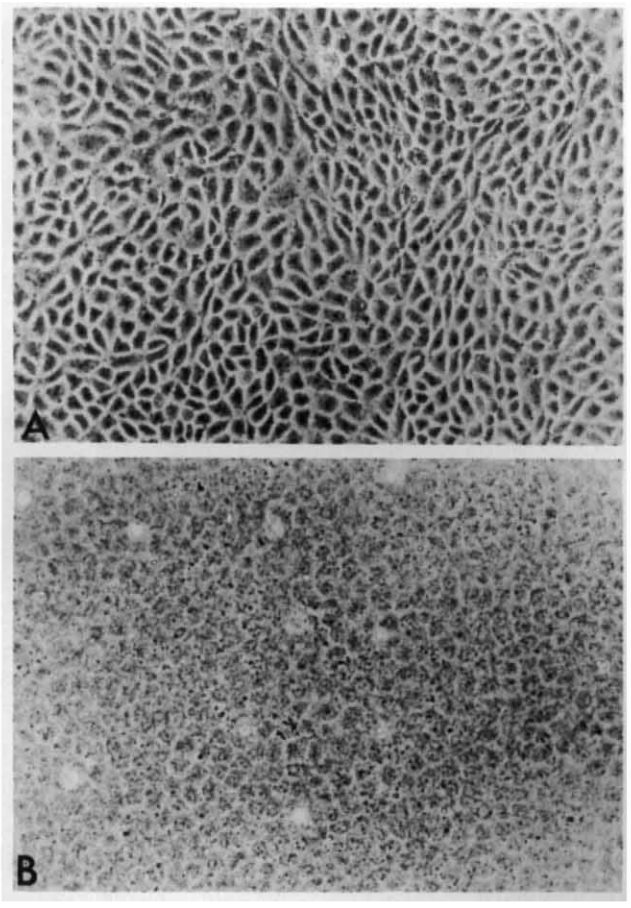

Fig. 2. *LM. Epithelial cells on collagen substratum in cloning medium.* **A** *Center of clone.* (×400) **B** *Dense packing of cells in extended cultures.* (×400)

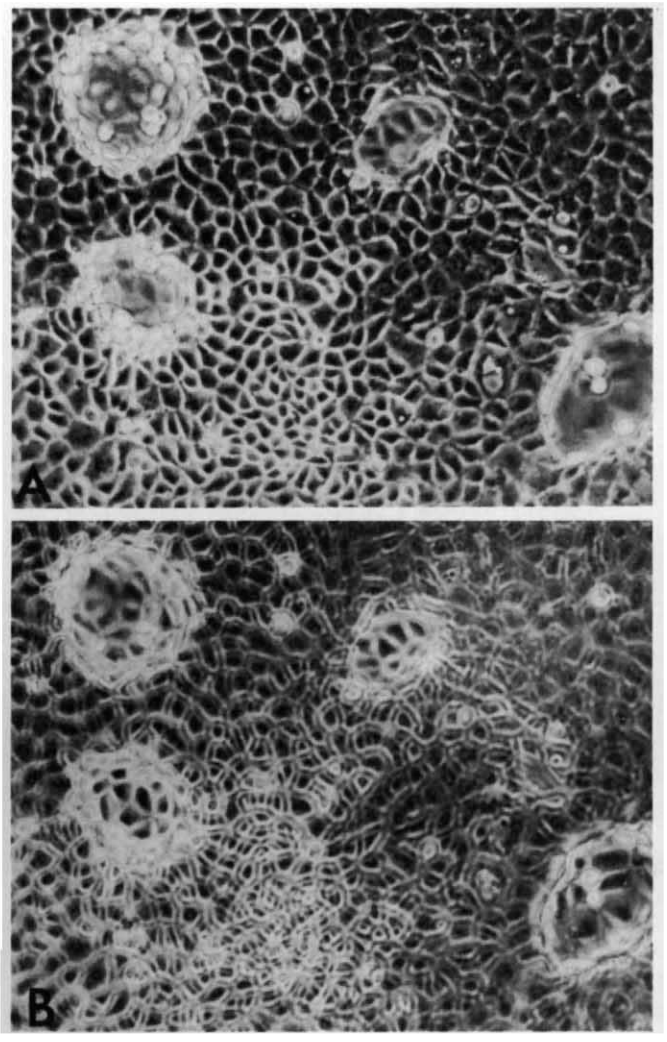

Fig. 3. *LM. Epithelial cells on solid substratum (glass/plastic), showing "doming."* **A** *Plane of focus on the monolayer.* (×400) **B** *Plane of focus above the monolayer.* (×400)

prepared for electron microscopy and sectioning perpendicular to the substrate as previously described [9]. Serial "thick sections" (5 μm) were cut and mounted on glass slides coated with silicon release spray (Rocol Ltd., Leeds, United Kingdom) as described by Schabtach and Parkening [10]. Serial thick sections were photographed with Nomarski optics, after which selected thick sections were re-embedded. Serial thin sections were then cut from these 5-μm sections, collected on formvar-coated single slot grids, stained with lead citrate, and examined in a Phillips 200 electron microscope.

Results. The glomeruli collected from the 200-mesh screen were devoid of Bowman's capsule and contained little if any contaminating tubular fragments as previously shown [2]. When placed in tissue culture, the glomeruli attached to the plastic dish within 24 hr, and rapid outgrowth ensued in all media tested. The cells that migrated from the glomeruli were generally oval and tightly packed together. Electron microscopy revealed that any podocytes still associated with the glomerular cells underwent a number of morphological changes. The major foot processes were no longer apparent and the cells were rounder in appearance. There were numerous microvilli present with cilia, usually only one, on the apical surface. By SEM two apparent cell morphologies were seen in the 4-day outgrowth explants in K1-3T3 or RPMI-3T3 media. The cells (podocytes) showed extensive surface ruffling with large bleb-like structures at the cell margins, and in some areas there were long parallel projections arising from the cell. The podocytes in the glomerulus, however, possessed numerous small microvilli with prominent cilia, all of which has been shown previously by us [2] as well as others [3, 11, 12].

Polygonal cells. The initial outgrowth of cells from the glomeruli in K1-3T3 medium was closely packed forming a "cobblestone" appearance. The cells were polygonal and quite flat with prominent nuclei. There were numerous microvilli on the cell surface and usually one or two cilia (Fig. 1**A**). A minority of the cells have a more variable morphology (Fig. 1**B**). These cells were irregularly shaped and gave off large cellular extensions which occasionally overlapped neighboring cells. Cilia were present less frequently and there were fewer microvilli.

After about a week following a low density passage, five to ten colonies became apparent. After transfer to collagen-coated 16 mm wells of a 24-well cluster dish, approximately 75% of the colonies could be successfully established in long-term cultures. The morphology of these isolated cells was very similar

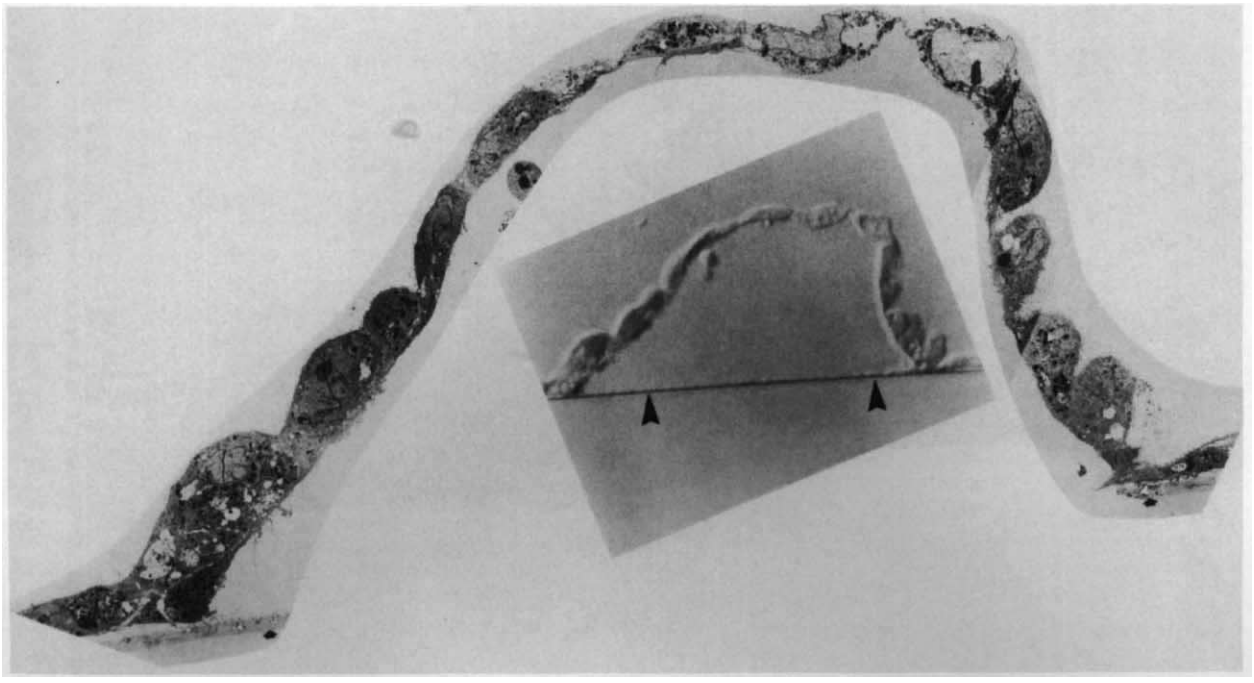

Fig. 4. *Composite of electron micrographs and a light micrograph of the same dome.* The *center area* is a Nomarski interference contrast image of a 5-μm thick Epon section of a dome cut perpendicular to the substratum *(arrowheads).* (×550) The *outer portion* is a montage of low-power electron micrographs of the same dome which has been reembedded as described in the **Methods.** The substratum is indicated with small *arrowheads.* (×1900)

to those seen in primary cultures. Figures 2 **A** and **B** are light micrographs of a typical colony. If these cultures are maintained for extended periods of time without subculturing, the cells become densely packed and individual cells become obscured (Fig. 2**B**). The use of the collagen together with K1-3T3 medium greatly facilitated the plating efficiency and subsequent maintenance of the polygonal cells. We also found that the HSI-LoSm medium could be substituted successfully for the K1-3T3 medium; however, the Primaria culture dishes were not as helpful. In fact, they inhibited the cellular outgrowth.

Colonies could not be established easily on a plastic substratum so that for electron microscopy, a different approach was used, that is, subculturing at higher passage numbers (6 or >) at a high-split ratio. The general morphology of these cells was identical to that of the majority of cells seen in the primary culture except that the passaged cells were somewhat smaller than the cells growing out of the glomerulus. None, however, displayed the classical in vivo podocyte morphology. Because of the similarity in the general morphology of these cells to vascular endothelial cells, we tested for two endothelial markers (staining of Factor VIII and angiotensin converting enzyme activity). Both were negative for the polygonal cells, strongly suggesting that these cells are not endothelial in origin.

The polygonal cells grown on a solid substratum, however, displayed a distinct characteristic not seen in those cells grown on collagen gel. Once the cells became confluent, areas of the culture form hemi-cysts, that is, small areas of 4 to 20 cells which appear to be lifted off the bottom of the dish in a blister-like manner. This can be seen in Figure 3 where the plane of focus is on the monolayer (Fig. 3**A**) and in Figure 3**B** where it is above the monolayer, revealing individual cells in the dome. By electron microscopy one can see more clearly the individual cells comprising the top of the cysts and the complete absence of any cells within the cysts (Fig. 4). Occasionally, these structures can be seen in primary cultures which have been maintained for longer than usual and allowed to approach confluency.

Spindle-shaped cells. The spindle-shaped cells in culture require a relatively high concentration of fetal calf serum (20%) for growth. The K1-3T3 medium which consists of a final concentration of 2.5% NuSerum and 5% fetal calf serum is insufficient to support growth of the spindle cells. This medium combined with a collagen-gel substrate causes any cells which do arise to be extremely attenuated. Discrete colonies cannot be established and often the cells appear to migrate into the collagen gel (Fig. 5**A**). When these cells are grown in K1 with only 10% FCS on a collagen gel, the cells grow to a confluent monolayer then migrate together to form ridges consisting of multilayers of cells. This never occurs when the cells are grown in the presence of NuSerum, even when plated at high concentrations. In comparison, cells grown on plastic in RPMI-1640 plus 20% FCS spread, overlap to a certain degree but do not form the ridges seen when grown on collagen (Fig. 5**B**). This medium can also be used successfully to grow and isolate colonies of the spindle-shaped cells.

Because of the striking similarity between vascular smooth muscle cells and mesangial cells (for example, morphology [13], contractility [14], and heparin-inhibition of proliferation (Harper, Castellot, Hoover, and Karnovsky, work in preparation) [15]) we examined the distribution of myosin in the

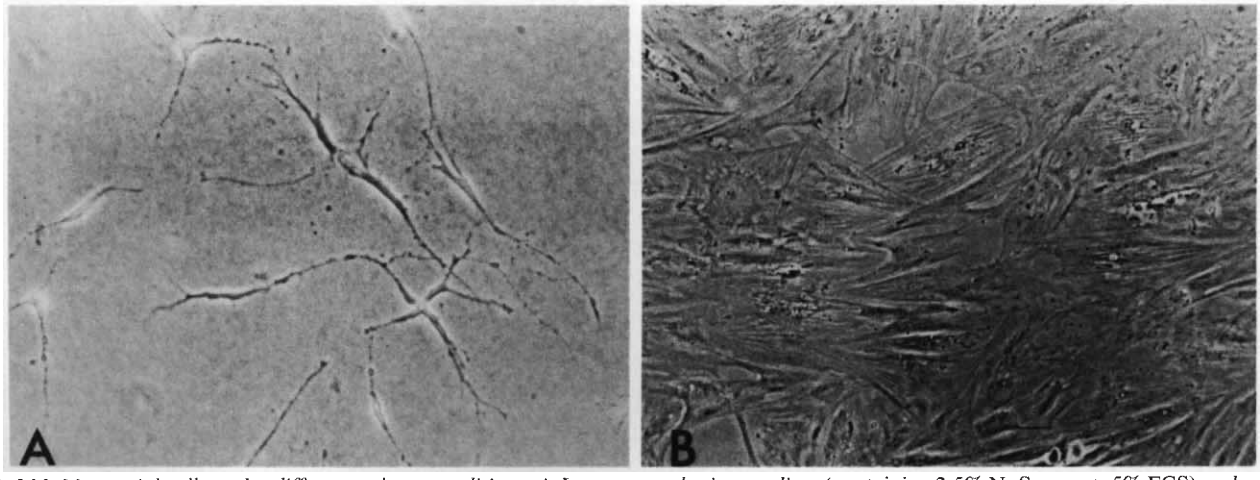

Fig. 5. *LM. Mesangial cells under different culture conditions.* **A** *Low serum cloning medium* (containing 2.5% NuSerum + 5% FCS) *and collagen gel.* Note the attenuation of the cells. (×400) **B** *Cells in high serum* (20% FCS) *on plastic form a confluent monolayer.* Note the strap-like morphology. (×400)

spindle-shaped cells, the polygonal-shaped cells and rat aortic smooth muscle cells using antibodies prepared against myosin from both muscle and non-muscle sources. Rat vascular smooth muscle cells and the spindle-shaped cells stain brightly along filaments and around the nuclei with antibodies from both muscle and non-muscle tissues; however, the staining of the glomerular epithelial cells is diffuse with both antibodies and less intense with the anti-smooth muscle myosin.

Discussion. The technique for isolation of rat glomerular cells has now been improved so that one can obtain consistently (at least in 90% of our isolations) pure cultures of two distinct cell types. Through several passages, the cells maintain their particular cellular characteristics. The one cell type is polygonal, forms domes in culture (characteristic of some epithelial cells [4, 16]), has cilia, is sensitive to the aminonucleoside of puromycin (unpublished data), and stains predominantly and diffusely with the anti-platelet myosin antibody, while the other cell type is spindle-shaped, has numerous filaments, and stains along its filaments with antibodies raised against myosin from both muscle and non-muscle sources. It should also be noted that other investigators have reported contractile proteins in mesangial cells [11, 17, 18]. Since the first set of characteristics is common to epithelial cells and the latter to smooth muscle-like cells, we believe that we have isolated homogenous cultures of glomerular *epithelial* and *mesangial* cells.

The ability to select and grow cultures of kidney cells is based on the fastidious requirements of the cells for a particular medium, substratum, and serum concentration. The epithelial cells grew best on a collagen gel-coated substratum and in low serum while the mesangial cells did best on plastic and in a high concentration of serum (20%). Indeed, mesangial cells would not proliferate on collagen gel-coated dishes in the presence of our defined medium (K1) plus low serum; however, when grown on collagen in the presence of 10% FCS but lacking NuSerum, the cultures formed ridges (multilayers of cells), reminiscent of confluent vascular smooth muscle cells in culture [19]. Similarly, the epithelial cells could be selected against because they did poorly on a plastic substratum and in high serum. In primary cultures, the polygonal cells grew out of the glomerulus onto tissue culture plastic and looked epithelial-like; however, they would not attach to the plastic in subsequent passages. In addition, we [2] previously found that we can also not select fibroblasts and promote epithelial and mesangial outgrowth by culturing in a medium in which L-valine has been replaced with D-valine [20].

By virtue of these techniques one can further study in vitro the biology and pathobiology of the glomerulus. Hopefully, it will now be possible to expand early morphological studies to include biochemical studies and look for specific effects on particular cells and examine the interactions between them.

Acknowledgments

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