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Permeable type I collagen membrane promotes glomerular epithelial cell growth in culture

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Interactions among different cell types are an important element of glomerular physiology and pathophysiology. The relative ease by which mesangial cells (MCs) can be cultured has been the major factor for recent advances in our knowledge of the pathobiology of MCs. However, difficulties in obtaining glomerular epithelial cells (GECs) in culture have hampered examination of this important cell type as well as its interaction with MCs. *In vitro* studies using defined cultured GECs, therefore, have been performed in few laboratories [1–6]. Generally cultured GECs show early growth from planted glomeruli on a plastic dish by forming domes, but they cease to proliferate before reaching confluence and detach from the dish. The reason why culture of GECs is difficult might be due to specific characteristics of GECs such as the functional relationship with extracellular matrices and polarity responsible for transcellular solute transport. Originally, Karnovsky indicated that GECs need collagen substrata in the form of gels [1, 2]. Since then, collagen gels have been extensively used as culture substrata for GECs [3–6]. Recently, Cybulsky et al indicated that types I and IV collagen support GEC growth [5]. It has also been shown that epithelial cells, including renal tubule cells such as MDCK [7–9], A6 [10] and epidermal cells [11], prefer permeable membrane supports, probably because of their polarity that are required for transcellular solute transport. These observations suggest that GECs may grow better in the presence of types I or IV collagen and on permeable supports. In this study, we tested a permeable type I collagen membrane as a culture support for GECs. The results show that GECs were comparatively easily propagated, compared with other culture substrata such as a plastic dish, nitrocellulose membrane, and the types I and IV collagen coated dish.

Methods

Materials

Permeable and translucent type I collagen membranes were obtained from Koken Co. (Tokyo, Japan). The membrane was made from purified type I collagen irradiated with ultraviolet lamp in order to produce cross-links among the collagen fibrils [11], and did not contain any type of different chemical composition.

Plastic culture dishes were obtained from Falcon (Becton Dickinson, Oxnard, California, USA). The nitrocellulose membrane filter was from Japan Millipore (Tokyo, Japan). Type I, type IV collagen coated dishes and 96 multi-well dishes were from Sumitomo Bakelite Co. (Tokyo, Japan). Acid soluble type I collagen was obtained from Koken Co. (Tokyo, Japan). Tissue culture media RPMI 1640 and fetal bovine serum (FBS) were obtained from GIBCO Laboratories (Grand Island, New York, USA). NuSerum was from Collaborative Research (Bedford, Massachusetts, USA). FITC conjugated rabbit anti-mouse immunoglobulins, goat anti-rabbit IgG, and peroxidase conjugated anti-mouse IgG were from Cappel Products (West Chester, Pennsylvania, USA). FITC conjugated anti-human factor VIII was from Binding Site (Birmingham, UK). Neuraminidase was purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). Wheat germ agglutinin (WGA) was from EY Laboratory (San Mateo, California, USA). All other chemicals were of analytical grade from commercial sources.

Culturing GECs and MCs

Isolated glomeruli were obtained by graded sieving [1] from female Sprague-Dawley rats weighing 180 to 220 g (Nippon Bio-Supply Center, Tokyo, Japan), and were suspended in RPMI 1640 supplemented with 5% FBS, 5% NuSerum, 0.66 U/ml insulin, 100 U/ml penicillin and 100 μg/ml streptomycin. Isolated glomeruli were explanted at the same density per cm^2 onto the following: (1) plastic dish; (2) 0.45 μm nitrocellulose membrane filter; (3) type I collagen coated dish; (4) type IV collagen coated dish; (5) type I collagen gel coated-nitrocellulose membrane filter; and (6) permeable type I collagen membrane. Mesangial cells (MCs) were propagated in RPMI 1640 supplemented with 20% FBS and 0.66 U/ml insulin from three to four weeks after the isolated glomeruli were placed on the plastic dish. They were then transferred to either the permeable collagen membrane, type I collagen coated dish, or the plastic dish, and cultured in the same medium used for primary culture of MCs.

Measuring proliferations of GECs and MCs

To measure GECs and MCs proliferation, the cell number was determined by visual counting of scraped and dissociated cells in sufficient volume of trypsin-EDTA solution prepared on

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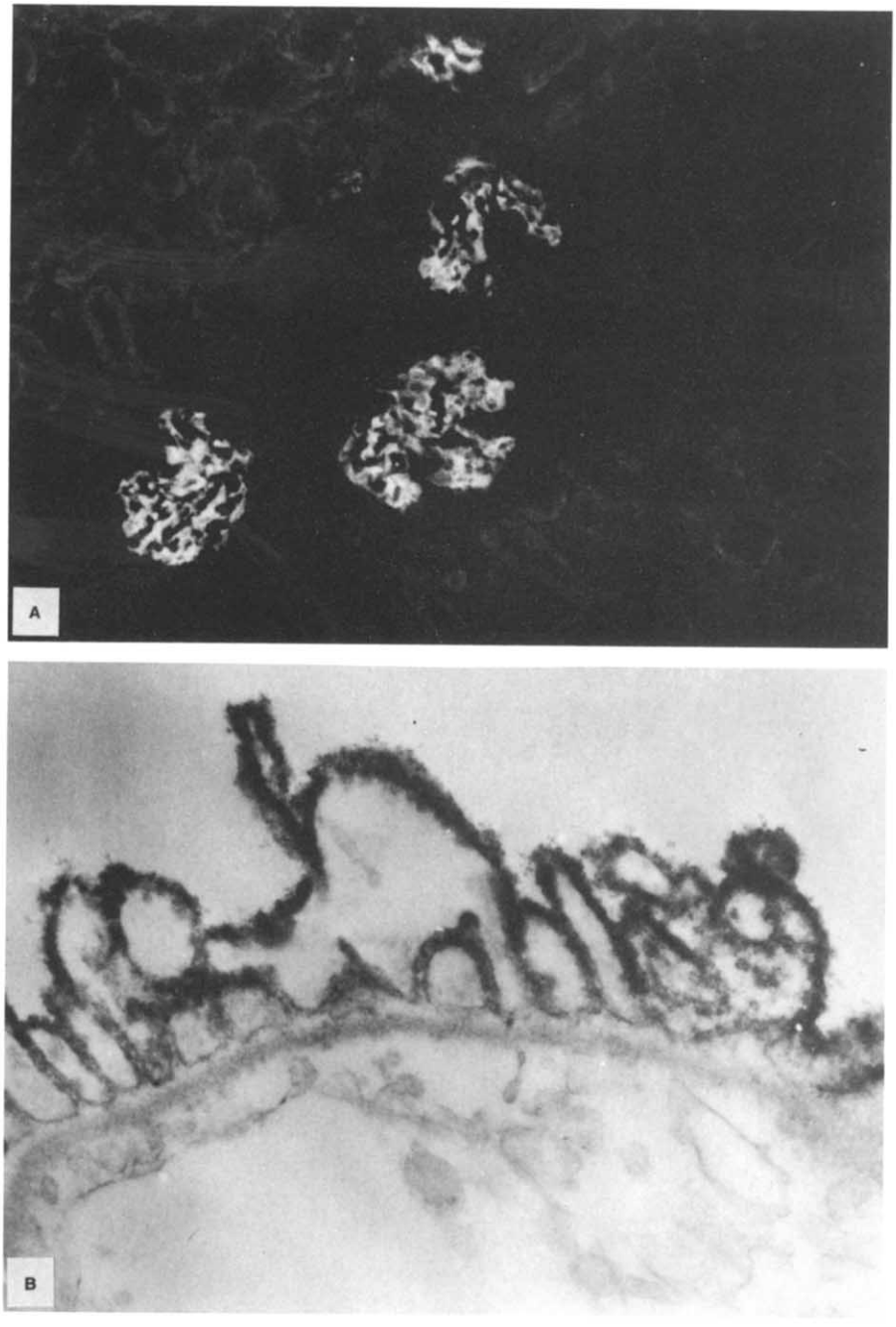

Fig. 1. *Binding sites of monoclonal antibody (GSA3) recognizing the GECs surface.* This monoclonal antibody (GSA3) binds to peripheral capillary walls of rat glomeruli, as determined by immunofluorescent microscopy (**A**, ×250) and the reaction products of peroxidase were visualized on the surface of GECs using immunoelectron microscopy (**B**, ×22,000).

days 7, 14, and 21 after initial planting for GECs, or on day 4 after the first passage for MCs. Cell numbers are expressed as counts per cm^2 of each culture substratum. Several separate experiments were performed in triplicate.

Preparation and characterization of monoclonal antibody (GSA3) recognizing the surface antigen of GECs

Spleen cells of balb/c mice (Nippon Bio-Supply Center) immunized with rat isolated glomeruli, were hybridized with a HGPRT deficient mouse myeloma cell line (P3-X-63-Ag8-U1), using polyethylene glycol 4000, according to the methods previously described by Köhler and Milstein [12]. A monoclonal antibody, designated GSA3, bound to the peripheral capillary walls of rat glomeruli and did not recognize other renal epithelial cells such as tubular and capsular epithelium as determined by indirect immunofluorescence microscopy using frozen sections (Figs. 1A, 2A). Additionally, immunoelectron microscopic study using a periodate-lesin-paraformaldehyde (PLP) fixed frozen section [13], revealed that the binding sites for GSA3 were located on the surface of GECs (Fig. 1B).

Furthermore, biochemical characteristics of the antigens

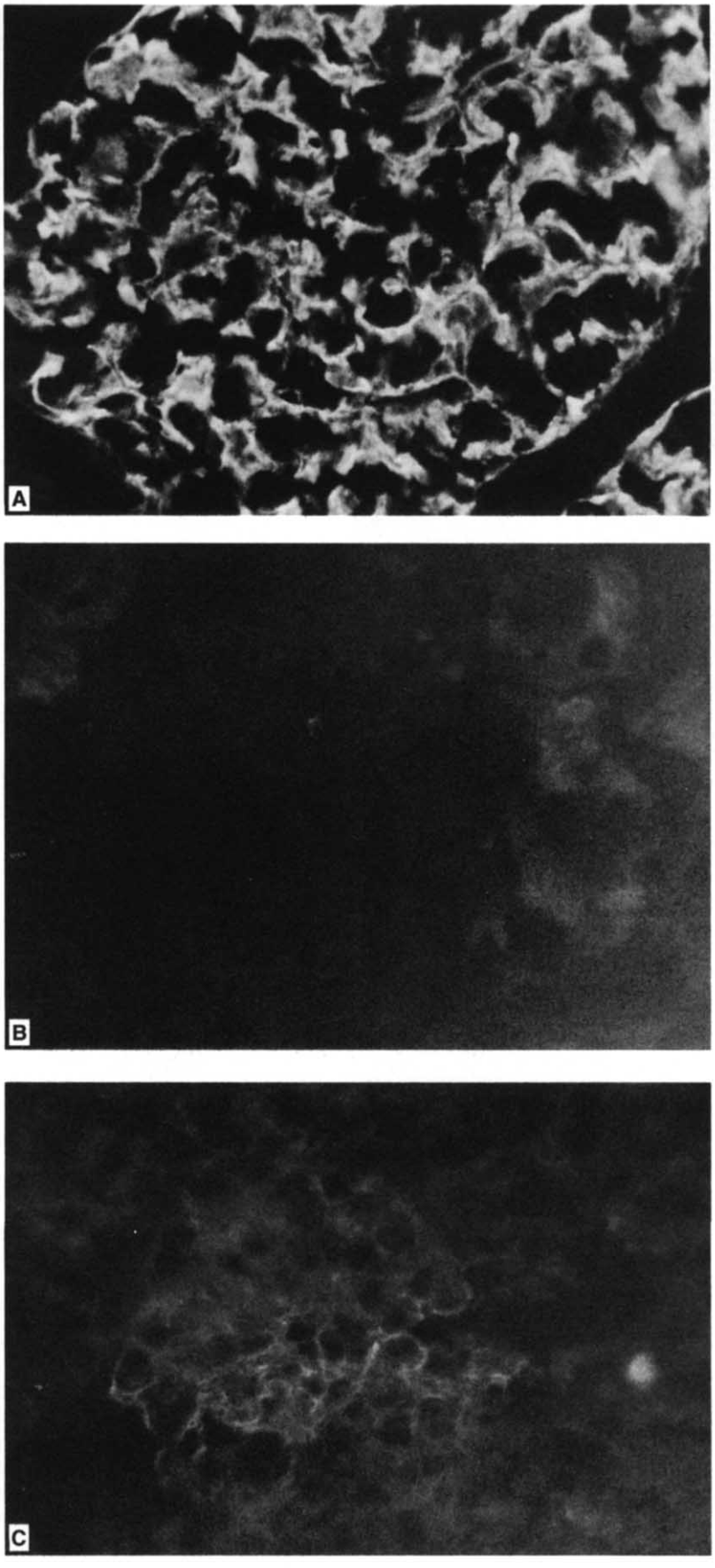

Fig. 2. *Chemical characteristics of GSA3 binding sites.* Pretreatment with neuraminidase (**B**, ×1,200) or WGA (**C**, ×1,200) blocked or removed the antigenicity in glomerular peripheral capillary walls recognized by GSA3 (**A**, ×1,200).

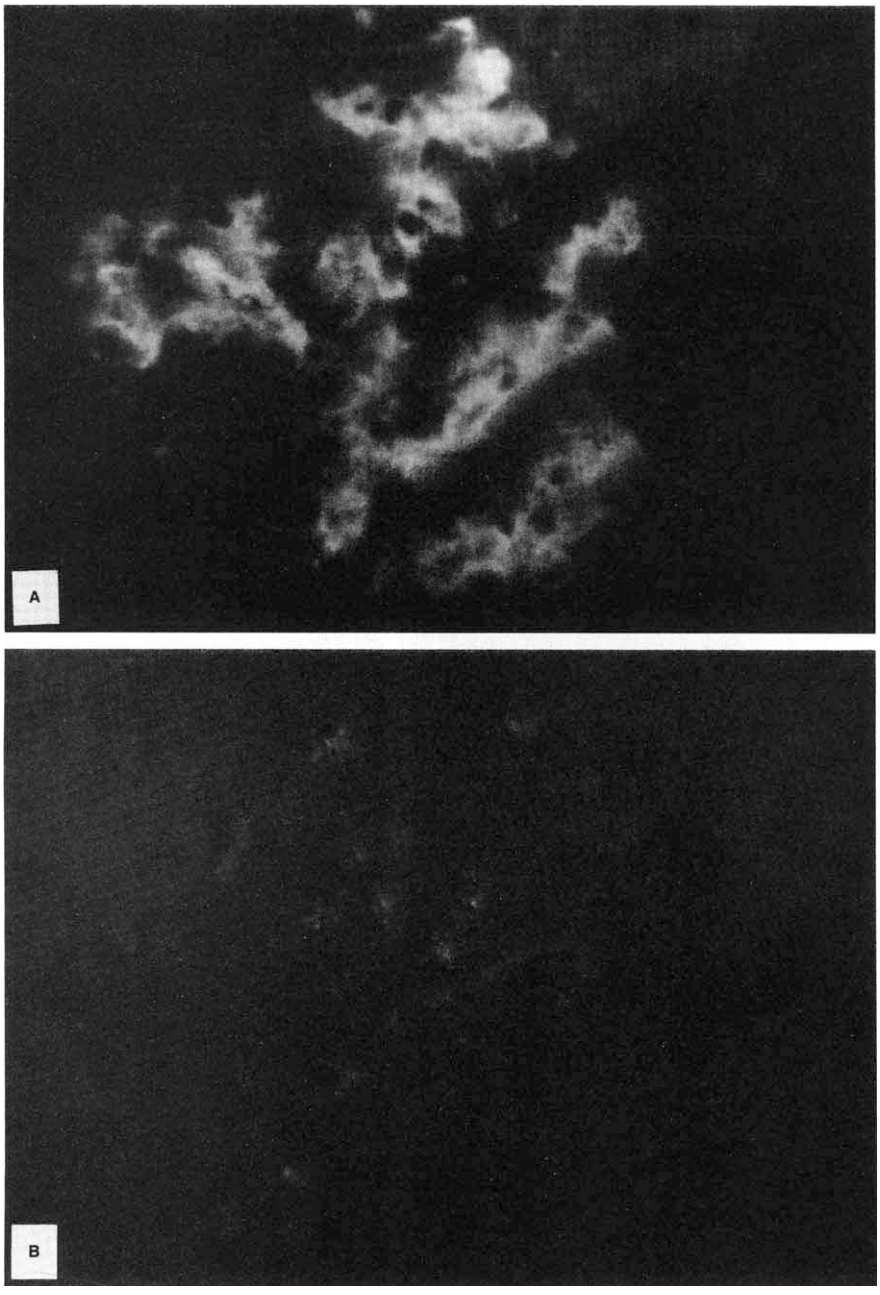

Fig. 3. *Binding sites of rabbit anti-thymocyte serum and sheep anti-factor VIII antibody.* Anti-thymocyte serum, which we prepared, bound to rat mesangial cells *in vivo* (**A**, ×2,000). Anti-human factor VIII antibody, which was known to cross react with rat factor VIII, recognized glomerular endothelial cells (**B**, ×1,200).

recognized by the monoclonal antibody were checked by examining the binding properties following exposure of frozen sections to neuraminidase and WGA [14, 15]. The antigenicity in capillary walls corresponding to GSA3 was removed by neuraminidase (Fig. 2B) and blocked by WGA (Fig. 2C) on frozen sections. Molecular weights of the antigens in the glomeruli were tested by gel electrophoresis and transblot, as follows: the isolated glomeruli were solubilized with 1% sodium dodecyl sulfate (SDS) and electrophoresed through 15% polyacrylamide gels. The bands were transblotted onto nitrocellulose membranes and incubated with the antibodies. The antigen-antibody complexes were visualized with 4-chloro-1-naphthol. The molecular weight of the antigen reacting with GSA3, determined by comparing the original electrophoretic mobility, was 130 kd.

Characterization of GECs

Cells cultured under the above conditions were observed under phase contrast microscopy and GECs were characterized immunocytochemically. On day 7 following initial plating of glomeruli, the collagen membranes were rinsed three times with phosphate buffered saline (PBS), and fixed with acetone. The membranes were cut and transferred to plastic tubes. Direct or indirect immunofluorescent reactions were performed using the following antibodies: (1) the monoclonal antibody (GSA3) directed against rat glomerular epithelial cell membranes; (2) a

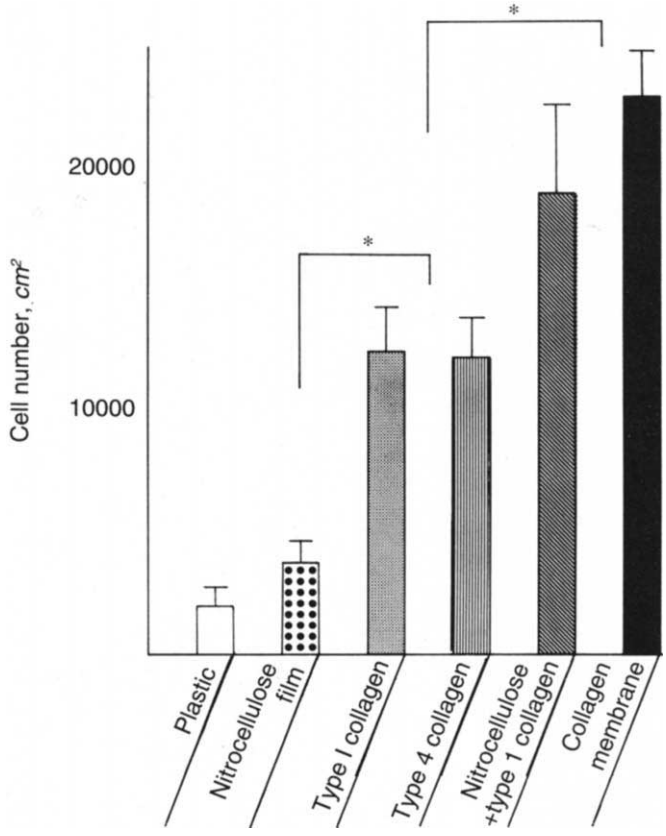

Fig. 4. *Growth characteristics of glomerular epithelial cells: Effects of different culture substrata.* Isolated rat glomeruli were explanted at the same density onto the following supports: (1) plastic dishes; (2) 0.45 μm nitrocellulose membrane filters; (3) type I collagen coated dishes; (4) type IV collagen coated dishes; (5) type I collagen gel coated-nitrocellulose membrane; and (6) permeable type I collagen membranes. Cell number was determined by visual counting of dissociated cells on day 7 after initial plating. Five separate experiments were done in triplicate. GEC growth on permeable type I collagen membranes was the highest among the 6 different culture substrata used. $*P < 0.01$.

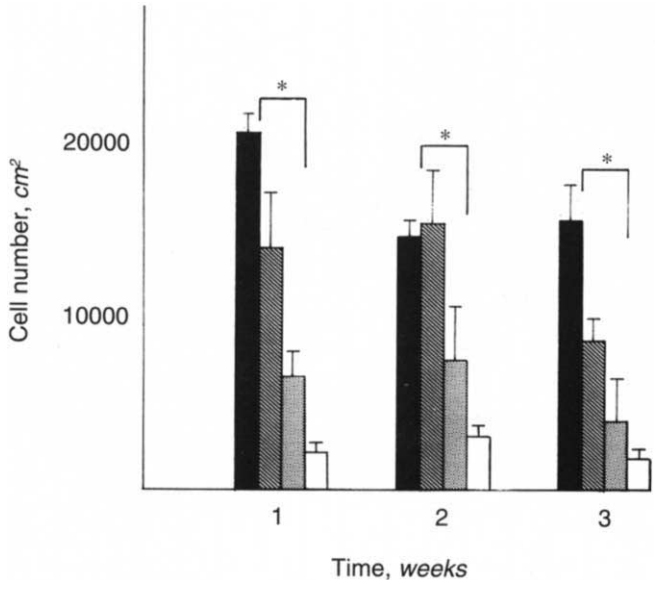

Fig. 5. *Time course of GEC growth on different culture supports.* Cell number was determined on days 7, 14 and 21 after initial glomerular plating on permeable collagen membrane (■), type I collagen gel coated-nitrocellulose membrane (▧), type I collagen coated dish (▥), and nitrocellulose membrane (□). Four separate experiments were performed in triplicate. GEC growth on type I collagen coated dish and simple nitrocellulose membrane did not reach the level of that on permeable collagen membrane. On the other hand, collagen gel coated-nitrocellulose membrane showed the additive effects of type I collagen coated dish and nitrocellulose membrane. $*P < 0.01$.

rabbit anti-rat thymocyte antibody that we prepared and recognized rat mesangial cells *in vivo* (Fig. 3A); and (3) FITC conjugated rabbit anti-human factor VIII antibody, which cross reacts with rat glomerular endothelial cells (Fig. 3B). FITC conjugated goat anti-rabbit IgG or rabbit anti-mouse immunoglobulins were used as second antibodies in indirect procedures.

We demonstrated the sites recognized by GSA3 more precisely, using immunoelectron microscopy. The samples were kept at 4°C until fixation. After three washes with cold PBS, the cells together with collagen membranes were incubated with GSA3 or the culture supernatants of non-productive myeloma cells as controls for 30 minutes. After three washes with PBS, the cells were incubated with peroxidase-labeled anti-mouse IgG for 30 minutes. After three washes with PBS, the samples were fixed with 2% glutaraldehyde for 20 minutes, then washed with 50 mM Tris-(hydroxymethyl) aminomethane-HCl buffer (pH 7.6). The samples were kept in diaminobenzidine for 5 to 10 minutes, washed three times with PBS, post-fixed in OsO_4 and embedded in Epon. Ultrathin sections were cut with glass knives and observed unstained using a Hitachi H-800 electron microscope.

Statistical analysis

Cell numbers were expressed as means ± SE. The differences among different culture substrata were evaluated statistically by means of the unpaired Student's *t*-test and analysis of variance. A P value of less than 0.01 was considered significant.

Results

Effect of different culture supports on GEC and MC proliferation

As shown in Figure 4, GEC growth on permeable type I collagen membranes was the highest among the six different culture substrata tested. On day 7 after initial glomerular plating, GEC growth on permeable collagen membranes was approximately 20-fold that on normal plastic dishes, sixfold that on membrane filters only and twofold that on types I or IV collagen coated dishes. GEC growth on type I collagen gel coated-nitrocellulose membrane was higher than that on simple nitrocellulose membrane or on types I and IV collagen coated dishes, but appeared to be lower than that on permeable collagen membrane (statistically insignificant). After that, on days 14 and 21, these differences were also observed, and the cells on other substrata did not reach the density of those on the permeable collagen membrane on day 7 (Fig. 5). In contrast, MC growth did not significantly differ among the three culture supports, although type I collagen coated dish and permeable

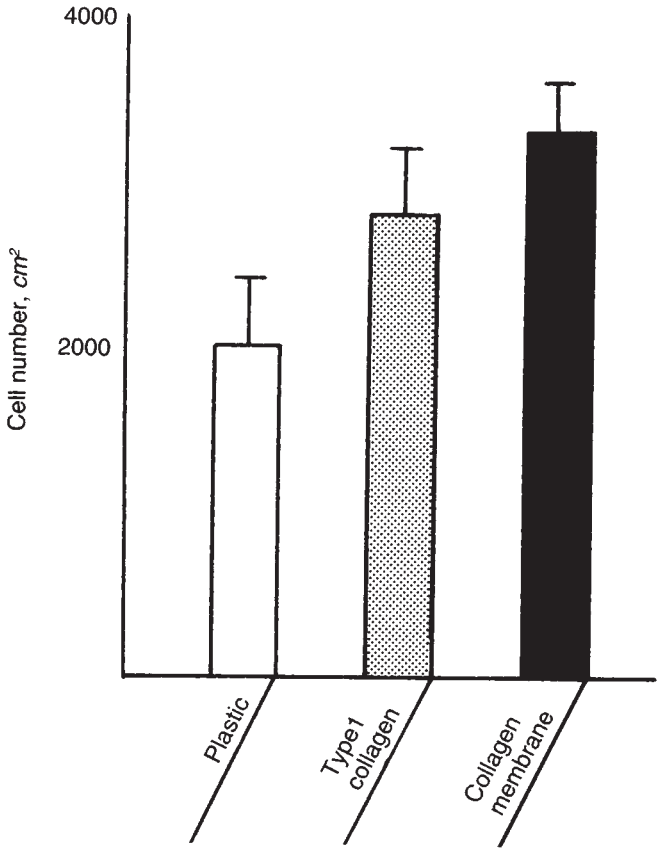

Fig. 6. *Growth characteristics of glomerular mesangial cells. Effects of different culture substrata.* Mesangial cells (MCS) were propagated in RPMI 1640 medium supplemented with 20% FBS and 0.66 U/ml insulin from 3 to 4 weeks after isolated glomeruli were placed on plastic dishes, then transferred to either permeable collagen membrane, type I collagen coated dishes, or plastic dishes. They were cultured in the same medium for an additional four days and the cell numbers were counted. Four separate experiments were performed in triplicate. MCs growths were not significantly different among the three culture supports.

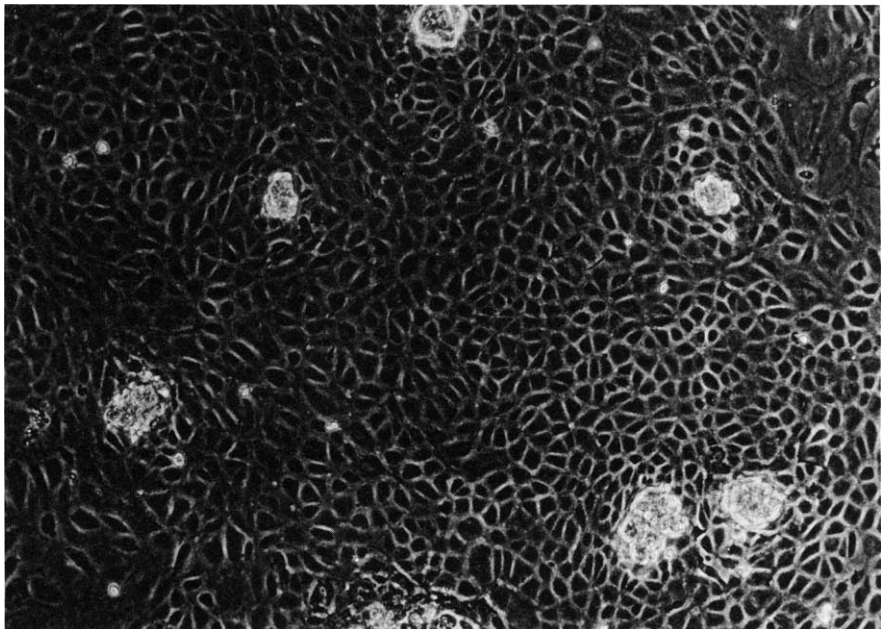

Fig. 7. *The cells grown on permeable type I collagen membrane 7 days after initial plating of glomeruli.* Under phase contrast microscopy, GECs propagated into a polygonal shape and with a cobblestone-like appearance, reaching confluence without detachment from the membrane.

collagen membranes appeared more suitable for MC growth than normal plastic dishes (Fig. 6).

Characterization of GECs

GECs appeared polygonal and cobblestone-like on day 3 after initial plating, and reached confluence without detachment from the membrane on day 7 under the culture conditions used in this study, that is, under a comparatively low concentration of FBS (5%) and 5% Nuserum, and on permeable type I collagen membranes (Fig. 7). On the other hand, GEC growth was extremely poor and limited on plastic dishes.

A monoclonal antibody (GSA3), which recognized GECs in

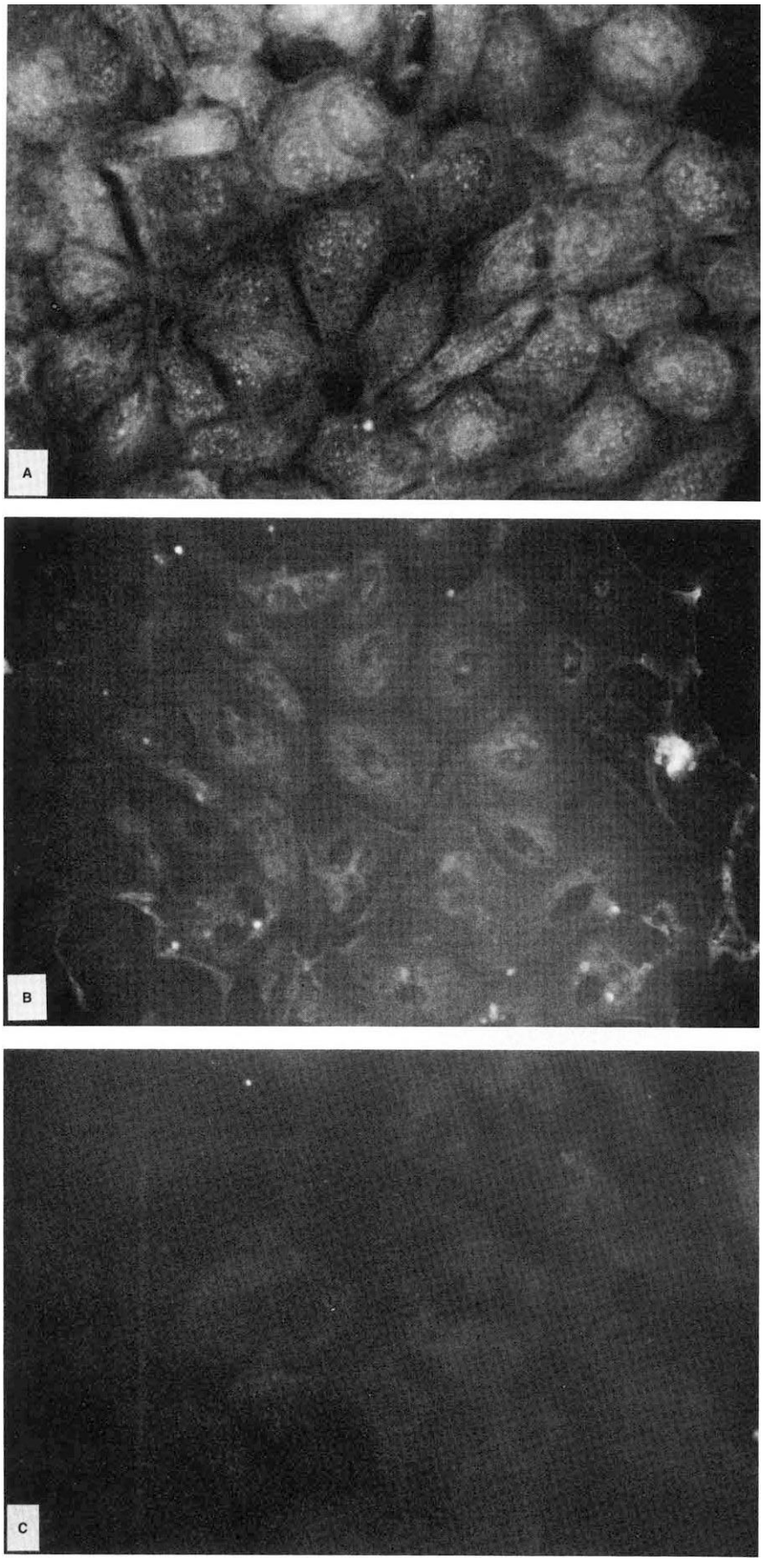

Fig. 8. *Binding of a monoclonal antibody (GSA3), anti-rat thymocyte antibody, and anti-factor VIII to cultured GECs.* A monoclonal antibody (GSA3), which recognized GEC surfaces in frozen sections of normal rat kidney, bound to cellular components in cultured GECs on permeable collagen membrane in diffuse and granular pattern (**A**, ×1,200). On the other hand, anti-thymocyte (**B**, ×1,200) and anti-factor VIII (**C**, ×1,200) antibodies did not bind any components of the cells grown on the permeable collagen membrane.

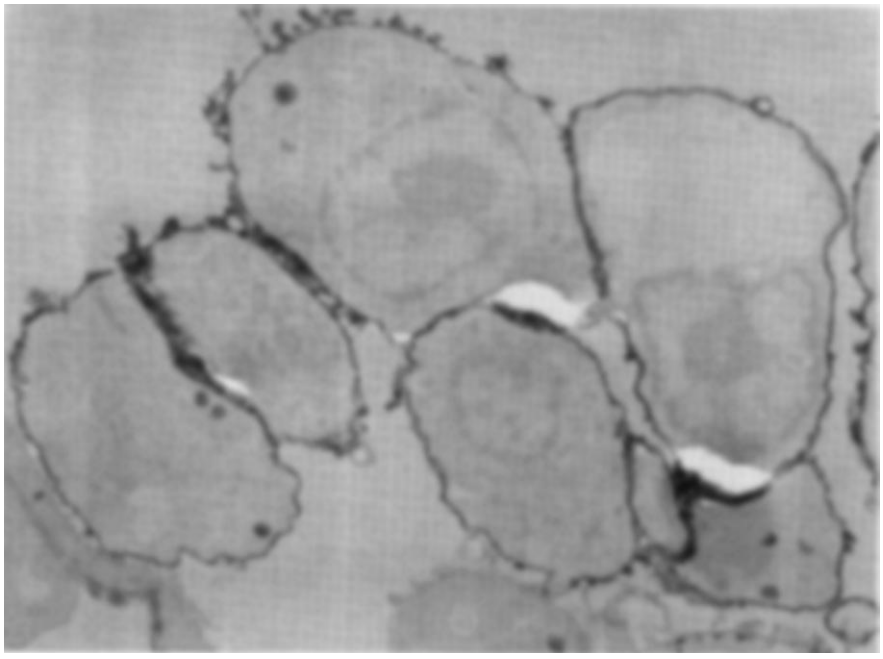

Fig. 9. *Binding sites of a monoclonal antibody (GSA3) to cultured GECs: Immunoelectron microscopy.* The specimen was cut in parallel with the collagen membrane. Reaction products were recognized on GEC surfaces that formed microprojections (unstained, ×3,800).

normal rat kidney on frozen sections, reacted diffusely to cellular components of cultured GECs on permeable collagen membrane, as determined by immunofluorescent microscopy (Fig. 8A), whereas GSA3 did not react with cultured MCs. On the other hand, anti-thymocyte antibodies, which recognized MCs in culture, did not react with any components of cultured GECs (Fig. 8B). Furthermore, anti-human factor VIII antibody, which was shown to bind the rat glomerular endothelium by immunofluorescent microscopy, also did not recognize cultured GECs (Fig. 8C). However, the binding sites of GSA3 to GECs were not clear in this light microscopic study, since whole cells were stained with GSA3 and observed straight down. Therefore, it was determined by immunoelectron microscopy (Fig. 9) that the binding sites of GSA3 to GEC were on the cell surface. The majority of the cells grown on the permeable collagen membrane were occupied by GSA3-positive, and anti-thymocyte antiserum- and anti-factor VIII antibody-negative cells.

These results show that permeable collagen membrane is the best choice for GEC culture.

Discussion

In contrast to glomerular mesangial cells (MCs), glomerular epithelial cells (GECs) have been difficult to obtain in culture. Investigations using cultured GECs, therefore, have been extremely limited [1–6]. GECs contact the glomerular basement membrane, which is composed of several extracellular matrix components such as type IV collagen, laminin, entactin, fibronectin and heparan sulfate proteoglycan [16–20] and might require the interaction with these substances *in vivo*. On the other hand, other types of epithelial cells such as MDCK [7–9], A6 [10] and epidermal cells [11] prefer permeable supports. This phenomenon can be explained by one characteristic specific to epithelial cells; the polarity, that participates in transepithelial solute transport in physiologic state. GECs should possess the polarity in common with the epithelial cells, and prefer permeable culture support to simple plastic dishes. The effects of different culture substrata on GECs growth indicated that both types I and IV collagen coated dishes are more effective than simple plastic dishes and nitrocellulose membranes, which agree with Cybulusky's results [5], whereas the permeable type I collagen membrane is more suitable than these collagen coated dishes. Furthermore, type I collagen gel coated-nitrocellulose membrane showed additive effects of simple nitrocellulose membrane and collagen gel on GEC growth, although the results were considerably dispersed. These results support the theory that the permeable collagen membrane as a culture substratum possesses both the characteristics of extracellular matrices and of a permeable support, which are necessary for GEC growth, and provides a suitable circumstance for constant GEC growth in culture.

Another important problem for culturing GECs is characterization of the cell type. Characteristic of cultured GECs include early growth, polygonal shape, proliferation to cobblestone appearance, the presence of junctional complex, and cytokeratin [1–6]. Furthermore, cultured cells should not have markers for MCs and glomerular endothelial cells. The cells predominantly grown on permeable collagen in the present study show these common epithelial characteristics, without the antigenicity specific to MCs and glomerular endothelial cells, that is, they don't react with anti-thymocyte antibody and anti-factor VIII, respectively. In addition, specific monoclonal or polyclonal antibodies bind to GECs surface, for example, anti-podocalyxin [21] and antibodies against other glycoproteins on the glomerular epithelial surface [22]. In this study, we used a monoclonal antibody (GSA3) which recognizes the glomerular epithelial surface, as shown in Figure 1 A and B. Loss of antigenicity in glomerular capillary walls after exposure to WGA or neuraminidase due to be masked or removed indicates

that this monoclonal antibody might recognize some of the sialoprotein which is prevalent in the epithelial cell surface [14, 15, 21] and constitutes some anionic sites in capillary walls. The molecular weight of the antigen, 130 kd, suggests that it might be "podocalyxin," a major component of the epithelial cell coat [21]. The cells grown on the permeable collagen membrane showed positive GSA3-binding and should therefore originate from glomerular visceral epithelial cells.

The method used here is an extremely simple and easy one to perform GEC culture. The membrane, which is translucent and permeable, is suitable for both morphological and physiological examinations on cultured cells. Permeable collagen membrane facilitates culture of GECs, and will be useful to investigate the role of GECs in various physiological and pathological states.

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