Brief Definitive Report

ISOLATION, CHARACTERIZATION, AND PROPAGATION IN VITRO OF HUMAN GLOMERULAR ENDOTHELIAL CELLS

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The isolation and characterization of human (1–7), and animal (see reference 8 for a review) glomerular cells has been reported. However, it has been difficult to obtain endothelial cells from the glomeruli in any species. Endothelial cells have been isolated from large blood vessels of many species, and recently, from small blood vessels of several vascular beds in humans and animals (9–12). Here we report the isolation, cloning, and characterization of endothelial cells from human glomeruli.

Materials and Methods

Isolation of Glomeruli

Human glomeruli were isolated from four kidneys by a method previously described (7). The separated cortex was diced and forced through a graded series of stainless steel meshes. A suspension was made and single, uncapsulated glomeruli were segregated using a pasteur pipette, and explanted onto a multichamber culture dish. Care was taken to exclude other structures or isolated cells.

Culture Conditions

The glomeruli were explanted in complete Waymouth's medium containing 20% fetal bovine serum (FBS), penicillin, streptomycin, and 2 ng/ml platelet-derived growth factor (PDGF), highly purified as previously described (2, 13, 14). The cultures were maintained in a 5% CO_2 humidified incubator at 37°C. Cells were passaged following brief exposure to 0.5% trypsin, EDTA, pH 7.4.

Isolation of Endothelial Cells

Under these conditions the first cellular outgrowth was seen at 5–7 d. The outgrowth was allowed to continue for several days, after which the culture was trypsinized and the cells dilute plated into a 96-well plate. Wells containing one cell were marked and were the source of eight clones from four kidneys.

Characterization of Endothelial Cells

Morphology. Phase photomicrographs were taken of cells at various stages of confluence, using an inverted microscope with ASA 400 film. For electron microscopy monolayers were fixed in one-half strength Karnovsky's fixative for 1 h and transferred to 0.1 M cacodylate buffer, pH 7.4. The cell layers were dehydrated in a graded series of alcohols and embedded in Epon. Sections were cut parallel and perpendicular to the

This work was supported in part by grants GM29853, GM24990, HL30542, and HL18645 from The National Institutes of Health and by a grant from R. J. Reynolds Industries, Inc. Reprint requests should be addressed to Dr. G. E. Striker, Dept. of Pathology SM-30, Univ. of Washington, Seattle, WA 98195.

monolayers, examined in a transmission electron microscope, and photographed. For scanning electron microscopy (SEM) cover slips were dehydrated in an ascending series of alcohols, and critical point dried with liquid CO_2. The segments were placed on planchets painted with silver-conducting paste. SEM specimens were then coated with gold palladium in a sputtering device. The specimens were viewed and photographed in a scanning electron microscope at 20 kV.

Immunofluorescence. For immunofluorescence studies, semi-confluent cells were transferred onto Meloy^R slides. Medium was removed, cells were rinsed in phosphate-buffered saline (PBS), pH 7.4, fixed in 100% methanol at room temperature, and then air dried. Immediately before staining, cells were rehydrated with PBS and immersed in 0.5% Triton for 5 min.

Indirect immunofluorescence for von Willebrand's factor (VWF) was performed using an affinity-purified rabbit antibody, a commercial preparation (Calbiochem-Behring Corp., La Jolla, CA) and a monoclonal antibody to VWF (a gift from Dr. Helene Sage, University of Washington). Controls included fluorescein-labeled goat anti-rabbit immunoglobulins and rabbit anti-mouse IgG. Indirect immunofluorescence was performed using monoclonal antibodies directed to vimentin, cytokeratin, and keratin (15) and polyclonal antibodies to actin and tubulin (Calbiochem-Behring).

Angiotensin-converting Enzyme (ACE). The medium was removed from confluent monolayers that were rinsed several times with complete medium. The cell layers were washed, frozen, and assayed for ACE. The activity of ACE was determined as that amount of hippurylglycylglycine hydrolyzed and which could be inhibited by SQ20881 (16). Liberated glycylglycine was assayed in a programmed amino acid analyzer. Enzyme activity was expressed as the amount of substrate cleaved in 1 min.

Proliferation Assay. Endothelial cells were plated at subconfluent density (5,000/cm^2) in complete medium containing 10% FBS. 16 h later the medium was replaced with that containing 0.5% plasma (17). 48 h later (0 time) 2 ng/ml PDGF was added to the medium. Cell numbers were counted electronically at 0, 1, 2, and 3 d. Cells were counted in triplicate. Controls included plates containing medium with 0.5% plasma without PDGF and medium containing 10% FBS.

Results

Phase Microscopy. The first outgrowth from glomeruli consisted of a monolayer of polygonal cells. They appeared earlier than epithelial cells, which were typically seen at 12–14 d. Following trypsinization and dilute plating, individual cells grew to form small clusters of cuboidal cells and then confluent monolayers (Fig. 1). When maintained at confluence, some clones formed overlapping cells in patches that resembled the "sprouts" typical of bovine aortic endothelial cells (18). The monolayer retained its cobblestone pattern for multiple passages. After more than 20 passages large cells with irregular contours appeared and increased in number with additional passages. The cells remained diploid.

Immunofluorescence Microscopy. Antibodies to VWF revealed cytoplasmic granules, which were both elongated and round (Fig. 2). The monoclonal antibody revealed considerably more heterogeneity in the size of the granules than did either the commercial or affinity-purified preparations. Human umbilical vein endothelial cells stained positively with the antibodies, but the granules were larger. A 549 cells, glomerular epithelial and mesangial cells, fibroblasts, and vascular smooth muscle cells were not stained by any of the VWF antibody preparations.

Monoclonal antibodies to vimentin that stained intermediate filaments in human umbilical vein endothelial cells, fibroblasts, and smooth muscle cells, did not stain either the initial outgrowth of cells or any of the clones thus far examined. Polyclonal antibodies to actin and tubulin stained the cytoskeleton of

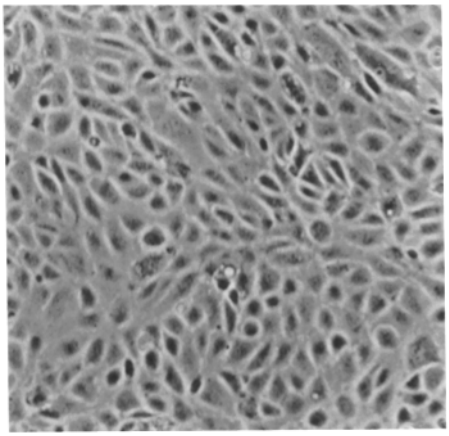

FIGURE 1. Phase photomicrograph of a confluent monolayer. Original magnification. × 100.

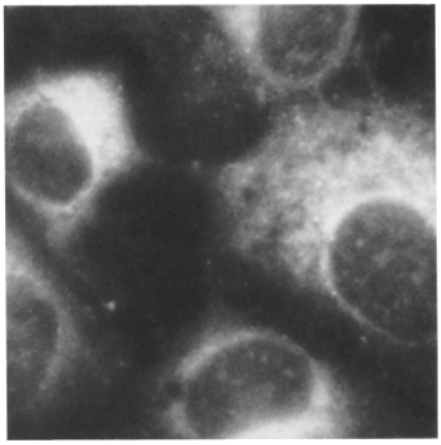

FIGURE 2. Immunofluroescence micrograph of cells stained with antibody to Von Willebrand's antigen: granules are scattered throughout the cytoplasm. Original magnification. × 630.

all cells examined. Antibodies to cytokeratin and keratin failed to stain these cells, whereas glomerular visceral epithelial cells were stained with the antibody to cytokeratin.

Electron Microscopy. Scanning electron micrographs revealed a monolayer of cells with closely apposed margins. The surfaces had a small, variable number of villi, but no other irregularities. Transmission electron micrographs revealed that microfilaments were more prominent near the cell surfaces, whereas intermediate filaments and microtubules were dispersed throughout the cytoplasm. The luminal surface of the cells had many subplasmalemmal vesicles as well as occasional coated pits and microvilli. Only one structure resembling a Weibel-Palade body was found (19).

Proliferation. Early outgrowth from isolated glomeruli was only seen with added PDGF (2 ng/ml). In the presence of PDGF there was approximately a five- and-one-half–fold increase in cell number by 3 d (Table I). Cultures without added PDGF had either a stable or slightly decreasing cell number over the same period of time.

TABLE I
Proliferative Response to Platelet-derived Growth Factor

Day	PDGF	Cell no.
0	−	6,037 ± 1,147
	+	6,119 ± 817
1	−	6,291 ± 1,175
	+	10,582 ± 1,464
2	−	6,182 ± 1,239
	+	17,555 ± 3,536
3	−	5,790 ± 1,189
	+	34,164 ± 4,413

* Mean ± 1 standard deviation using five clones from three different kidneys.

TABLE II
Angiotensin-converting Enzyme Content of Cell Layers

Cell type	Kidney no.	Specific activity nM/h/mg protein*	Passage no.
Endothelial clones	1	160 ± 140	6
	2	88.15 ± 50.85	7
	3	218.6 ± 56.4	11
	4	166.4 ± 14.9	11
Human umbilical vein		125 ± 5	Primary cultures
Human dermal fibroblasts		9 ± 3	Multiple

* Mean ± 1 standard deviation.

ACE Content. Assays of cell layers from the eight clones revealed that the specific activity of ACE was in the same range as that of umbilical vein endothelial cells (Table II). The values were considerably higher than those of dermal fibroblasts. The level of enzyme activity did not appear to decrease with increasing passage number, but was higher in confluent cultures. The range of activity within one clone was most likely related to the degree of confluency (20).

Discussion

Endothelial cells derived from individual, isolated human glomeruli were cloned and serially passaged. They represented the first outgrowth of cells from the glomeruli. Confluent cultures had the cobblestone, monolayer appearance characteristic of large vessel endothelium (9, 18). Cytoplasmic granules and elongated structures of varying size were found to contain VWF factor antigen by indirect immunofluorescence. High levels of ACE were consistently found in the eight endothelial cell clones studied. There were nearly undetectable levels of the enzyme in isolated visceral epithelial cells and low levels in mesangial cells from the same kidneys. The variation between different isolates of the same clones was thought to be a function of the degree of confluence of the cultures. Del Vecchio and Smith (20) showed that ACE activity increases sevenfold from near-confluent to post-confluent cell layers (20). The presence of ACE was an

unexpected finding, since previous studies by immunofluorescence (16) and immunoelectron microscopy (21) had concluded the enzyme was largely restricted to the brush border of the proximal tubule. In agreement with the current data, previous assays of ACE revealed that homogenates of glomeruli had nearly 10% of the activity found in proximal tubule brush border preparations (16). Since the glomerulus contains a relatively small volume of endothelial cell membrane per unit area compared to the proximal tubule brush border, it is not surprising that the enzyme has been undetectable within the glomerulus by morphologic techniques.

From the above data, it was concluded that glomerular endothelial cells shared the following features with endothelial cells from large blood vessels in humans and animals in vitro: morphology, presence of VWF antigen, and high levels of ACE. Glomerular endothelial cells differed from endothelial cells of large blood vessels in two aspects. First, they could not be stained by a monoclonal antibody directed against vimentin that decorated cells derived from both human umbilical veins and bovine aortae. Since these glomerular cells contained a number of intermediate filaments by electron microscopy, it was assumed that their cytoskeleton was different antigenically from that of large blood vessels. The second difference was the growth requirements of glomerular endothelial cells. The addition of PDGF to isolated glomeruli resulted in an early outgrowth of endothelial cells. Furthermore, these cells required the addition of PDGF for proliferation. They could be passaged and cloned in the presence of PDGF and this did not result in loss of VWF. It is not clear whether these growth requirements are unique to glomerular endothelial cells. Endothelial cells from adult human dermis require medium containing 50% human serum for optimal growth. Normal human serum contains PDGF in the range of 15–50 ng/ml (22–24). Thus it is possible that dermal endothelial cells may require PDGF.

Endothelial cells could be differentiated from the other two cell types present in glomeruli. While they resembled epithelial cells by phase-contrast microscopy, endothelial cells contained VWF antigen and the intermediate filaments of epithelial cells were stained by antibodies to cytokeratin. The stellate appearance of mesangial cells by phase-contrast microscopy allowed them to be recognized as a distinct cell type. In addition, mesangial cells have been shown to appear relatively late in cultures of human and animal glomeruli (2–8).

Summary

Human glomerular endothelial cells have been isolated, cloned, and characterized. They appeared as the first outgrowth from human glomeruli in the presence of platelet-derived growth factor, which was also a requirement for continuous growth. By phase microscopy they appeared as monolayers of polygonal cells. Von Willebrand's factor (VWF) was detected in the cytoplasm of all clones. Their intermediate filaments differed antigenically from that present in human umbilical vein endothelial cells. Like other endothelial cells, they demonstrated high levels of membrane-associated angiotensin-converting enzyme (ACE).

Received for publication 21 February 1984 and in revised form 11 April 1984.

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