Glomerular Endothelial Cells Secrete a Heparinlike Inhibitor and a Peptide Stimulator of Mesangial Cell Proliferation

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The regulation of cell growth in the kidney glomerulus plays a key role in many physiologic and pathologic processes. In this communication the authors have examined the possible role of glomerular endothelial cells as potential regulators of mesangial cell proliferation. Conditioned medium was collected from confluent cultures of glomerular endothelial cells and tested for its effects on glomerular mesangial cell and vascular smooth muscle cell growth. When glomerular endothelial cell-conditioned medium was mixed 1:1 with normal growth medium, the growth of these two closely related cell types was inhibited by 60–70%. If the conditioned medium was diluted to 1:9, a stimulation of mesangial and smooth muscle cells growth was seen. Approximately 70% of the antiproliferative activity was destroyed by a highly purified heparinase; the other 30% was sensitive to trypsin. Approximately 90% of the mitogenic activity was protease-sensitive. These results suggest that glomerular endothelial cells may participate in part in mesangial cell growth regulation via a heparin-mediated mechanism. (Am J Pathol 1986, 125:493–500)

THE REGULATION of cell growth in the glomerulus may play a key role in a number of important physiologic and pathologic processes, including differentiation, hypertrophy, aging, and the proliferation of mesangial cells (MCs) in some types of glomerulonephritides. The cellular and molecular mechanisms governing growth regulation in the glomerulus have not been resolved, in part because homogenous cultures of the glomerular cell types were not available until recently.[1] Platelet-derived growth factor has been reported to stimulate MC proliferation,[2] as have macrophage-derived products.[3,4] However, Ooi and co-workers[5] found that macrophage products inhibited MC growth, as did prostaglandin E_2. Very little is known about growth control of the other cell types in the glomerulus (epithelial cells and endothelial cells).

We recently demonstrated[6] that cultured glomerular epithelial cells (GECs) secrete both a heparinlike species and a protein which inhibit the proliferation of MCs. In addition, a polypeptide mitogen for MCs was detected in culture medium collected from GECs. Furthermore, purified preparations of anticoagulant and nonanticoagulant heparin inhibited MC growth *in vitro* and *in vivo*.[6]

In this communication, we demonstrate that glomerular endothelial cells (GEnCs) secrete both inhibitors and stimulators of MC growth into the culture medium, and that approximately 70% of the inhibitory activity is due to a heparinlike molecule. These data, when combined with the observations of others, suggest that GEnCs, GECs, and heparin may participate in MC growth regulation in the glomerulus.

Materials and Methods

Materials

Chondroitin ABS lyase and *Streptomyces* hyaluronidase were purchased from Miles Laboratories. Highly purified heparinase was the kind gift of Dr. Robert Langer (Childrens' Hospital Medical Center, Boston,

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Mass). All other chemicals were obtained from Sigma Chemical Co., unless otherwise noted.

Cell Culture

All cells were cultured at 37 C in a humidified 5% CO_2/95% air atmosphere. All growth media contained 4 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Glomerular Cells

The initial steps in the isolation of cells from rat kidneys were a modification[7] of the procedure of Kreisberg et al.[1] Male Sprague–Dawley rats (Charles River Breeding Laboratories, CD strain) weighing 100–150 g were lightly anesthetized with ether and then given a lethal dose of sodium pentobarbitol. The kidneys were excised and the cortexes cut away from the medullae. These cortical pieces were chopped into 2-mm-square pieces and passed through a series of sieves of decreasing pore size—200 μ, 150 μ, and 75 μ—with the glomeruli ending up on top of the 75-μ sieve.[8] These glomeruli have been shown to be stripped of their capsules, and the preparation is virtually free of tubular tissue.[1] The glomeruli are rinsed twice in Hank's balanced salt solution buffered with HEPES to pH 7.4 (buffered HBSS), incubated with 0.2% trypsin for 20 minutes at 37 C, and then incubated in 0.1% collagenase (189 U/mg) for 40 minutes at 37 C. These treatments "loosen up" the glomeruli but yield few single cells. The glomeruli are then centrifuged gently (300*g* for 5 minutes) and plated under the appropriate conditions, as described below.

Endothelial cells were obtained by passing the dissociated glomeruli through a 15 μ Nitex filter to yield a single cell suspension. The cells were sparsely plated (1000 cells/dish) in 100-mm culture dishes in a 1:1 mixture of Taub and Sato[9] defined medium K1 containing 5% NuSerum (Collaborative Research) and conditioned medium containing 10% fetal calf serum (FCS) taken from exponentially growing cultures of Swiss 3T3 fibroblasts (K1-3T3). This medium selects for endothelial and epithelial cells and against mesangial cells. Colonies that morphologically resemble endothelial cells were selected and removed by trypsinization. Each clone was transferred to one well of a 24-well cluster dish in the K1-3T3 medium described above. GEnCs were identified by their cobblestone appearance when confluent (Figure 1) and by the presence of angiotensin-converting enzyme activity. All clones that were identified as GEnCs had 30–60 U/10^6 cells of angiotensin-converting enzyme activity, compared with 19 U/10^6 bovine aortic endothelial cells. Mesangial cells and epithelial cells had <1 U/10^6 cells of angiotensin-converting enzyme activity. Two other markers for identifying endothelial cells were examined—the presence of Factor VIII antigen and uptake of acetyl-low-density lipoprotein (LDL). Our results, in accord with our previous experience and that of other laboratories, indicate that rat endothelial cells are negative for Factor VIII antigen and do not take up acetyl-LDL, which indicates that these particular endothelial markers are not useful for identifying rat endothelial cell cultures.

The appearance of endothelial colonies in a glomerular cell isolation experiment is a relatively rare event. In addition, GEnCs are quite difficult to maintain in

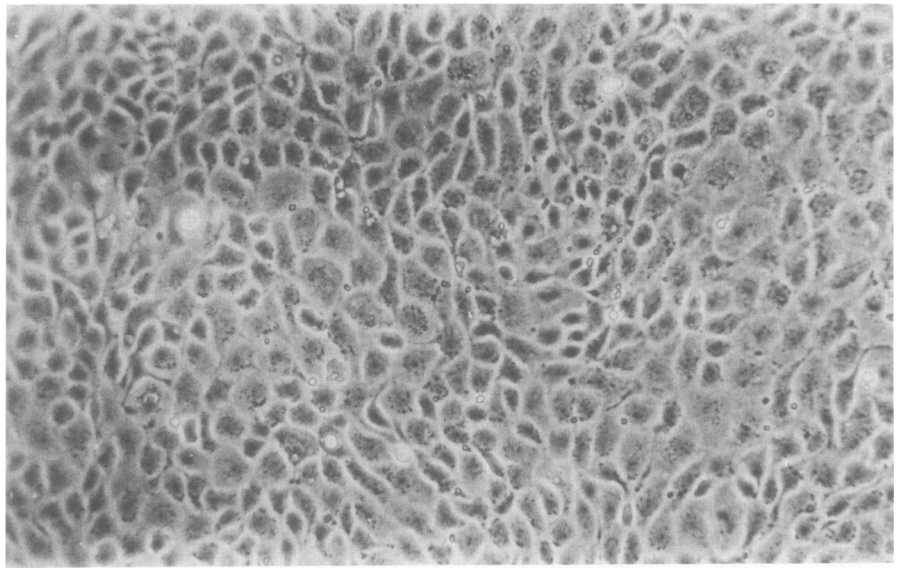

Figure 1—Phase-contrast photomicrograph of cloned rat glomerular endothelial cells. (×200)

culture long enough to generate the large numbers of cells needed for biochemical studies. We were able to propagate five colonies to the point where angiotensin-converting enzyme activity could be measured (approximately 10^5 cells). Of these, one colony was able to be continuously subcultured to provide sufficient cells for measurements of growth inhibitory and promoting effects (approximately 10^7 cells). This GEnC culture was used for the experiments described in this report. It was used at Passages 7–12; little change was observed in the degree or ratio of growth effectors secreted by this culture.

Mesangial cells were obtained by plating the glomeruli onto 100-mm culture dishes in a 1:1 mixture of RPMI-1640 medium containing 20% FCS and 0.66 U/ml insulin, and 3T3 conditioned medium. When colonies developed, they were removed by trypsinization and placed into 24-well cluster dishes in RPMI + 20% FCS-3T3 medium. After the cells reach confluency, they can be subcultured into larger dishes using RPMI + 20% FCS, without the 3T3 conditioned medium. MCs were identified by their characteristic morphologic features, the presence of numerous filament bundles in the cytoplasm, and the presence of smooth muscle cell myosin, as revealed by indirect immunofluorescence. MCs between Passages 3 and 6 were used in the growth assays.

Smooth Muscle Cells

Rat aortic smooth muscle cells (SMCs) from Sprague–Dawley rats (Charles River Breeding Laboratories, CD strain) were isolated, cultured, and characterized as previously described.[10,11] Briefly, the abdominal segment of the aorta was removed and the fascia cleaned away under a dissecting microscope. The aorta was cut longitudinally, and small pieces of media were carefully stripped from the vessel wall. Two or three such strips were placed in 60-mm tissue culture dishes. Within 1–2 weeks, SMCs migrate from the explants; they were capable of being subcultured about a week after the first appearance of cells. They were grown in RPMI-1640 medium containing 20% FCS. The cells were identified as SMCs by the presence of 1) numerous myofilament bundles in the cytoplasm, 2) numerous vesicles near the plasma membrane, 3) SMC-specific myosin, as revealed by indirect immunofluorescence,[12] and 4) the characteristic "hillocks and valleys" appearance of confluent cultures. They were used between Passages 2 and 6 in the growth assays.

Conditioned Medium

Conditioned medium (CM) was collected from post-confluent cultures of GEnC as previously described for vascular endothelial cells[13] and GECs.[6] Briefly, cultures were grown to confluence as described above and were allowed to remain confluent for 5–7 days. The cells were then washed once with serum-free medium and incubated for 48 hours in RPMI + 0.5% FCS (6 ml/100-mm dish). At the end of this time, the medium was collected and centrifuged at 2500*g* for 15 minutes for removal of any floating cells and cell debris. Cultures with noticeable debris or floating cells were discarded to minimize the possibility of lysed cell contents contributing to the inhibitory activity. Aliquots of this conditioned medium were subjected to further enzymatic treatments, as described below.

Enzyme Treatments of Conditioned Medium

Trypsin treatment of CM was accomplished by adding 30 μg/ml trypsin for 2 hours at 37 C. A tenfold excess of soybean trypsin inhibitor in a small volume was added 30 minutes before mixing the CM with RPMI + 20% FCS. *Chondroitinase* treatment was carried out by incubating CM with 1 U/ml chondroitin ABC lyase for 90 minutes at 37 C. *Hyaluronidase* treatment was done by adding 15 U/ml *Streptomyces* hyaluronidase to CM for 90 minutes at 37 C. *Heparinase* digestion was carried out by exposing CM to 10 U/ml *Flavobacterium* heparinase for 90 minutes at 37 C. This preparation has no detectable protease activity and does not degrade chondroitin sulfates, dermatan sulfate, or hyaluronate.[13] Enzyme-treated samples were boiled for 5 minutes, a treatment that kills all the above enzyme activities but does not affect the inhibitory activity. All of the glycosaminoglycan-degrading enzymes used in these experiments were from the same batch of enzymes used in our recent report on growth effectors secreted by GECs.[6]

Growth-arrest of Cells

To growth-arrest cultures, cells were sparsely plated ($5–8 \times 10^3$/2-sq cm well) in RPMI + 20% FCS. After approximately 24 hours, the cells were washed and placed in RPMI + 2% platelet-poor plasma or in RPMI + 0.4% FCS for 72 hours. Flow microfluorimetry indicated that approximately 90% of the cells were arrested in $G_0(G_1)$.

Growth Assays

For assay of the growth effects of GEnC secreted products, the growth of MCs was arrested as described above. Control cultures were released from G_0 block by placing them in RPMI + 20% FCS. Other cultures were exposed to RPMI + 20% FCS containing vary-

ing concentrations of GEnC-CM. Conditioned medium was mixed 1:1, 1:3, or 1:9 with RPMI containing the appropriate concentration of FCS to bring the final FCS concentration to 20%. Cell numbers were measured in duplicate samples with the use of a Coulter counter. We routinely checked trypsinized cultures by direct microscopic examination to ascertain that the trypsinization procedure had not lysed the cells and to ensure that all cells were removed from the multiwell. The cells were not fed during the experiment. The net growth of cells in control and GEnC-CM-treated cultures was obtained by subtracting the starting cell number (at the time the cells are released from G_0) from the cell number at the end of the experiment. The standard error in the growth assays is $< \pm 10\%$ unless otherwise indicated.

Results

Effect of Glomerular Endothelial Cell Secreted Products on Mesangial Cell Growth

We have proposed that cell–cell interactions are important in growth regulation in the kidney glomerulus, in part via the synthesis and secretion of heparin-like species.[6] To test this hypothesis further, we examined the effect of secreted products from a culture of GEnCs on MC proliferation (Figure 2). When GEnC-CM is tested at a 1:1 dilution (ie, 50% CM: final concentration of fresh FCS, 20%), MC growth is inhibited by 73%. Concentrations of CM greater than 50% were not tested because of problems with maintaining adequate levels of fresh medium and nutrients. We have previously shown for aortic SMCs that simple nutrient depletion was not causing growth inhibition, because a 1:1 mixture of CM and medium containing twice the normal nutrient concentration with a final FCS concentration of 20% had the same growth inhibitory activity.[13] In the present system, GEn-CM dialyzed against fresh medium yields the same inhibitory activity as undialyzed CM. When GEnC-CM is tested at 1:3, there is no net effect on MC proliferation. However, if CM is tested at a 1:9 dilution, a marked stimulation (240%) of MC growth is observed. The data suggest that both positive and negative growth effectors of MCs are elaborated by GEnCs. For comparison, GEnC-CM was also tested for its effects on vascular SMCs, close relatives of MCs[7,14–16]; the growth of these cells is also inhibited at 1:1 and stimulated at 1:9 dilutions (Figure 2). It is noteworthy that the growth stimulatory activity was observed even in the presence of serum concentrations (20%) that are optimal for MC and SMC growth. This suggests that GEnCs are producing a high level of mitogenic activity.

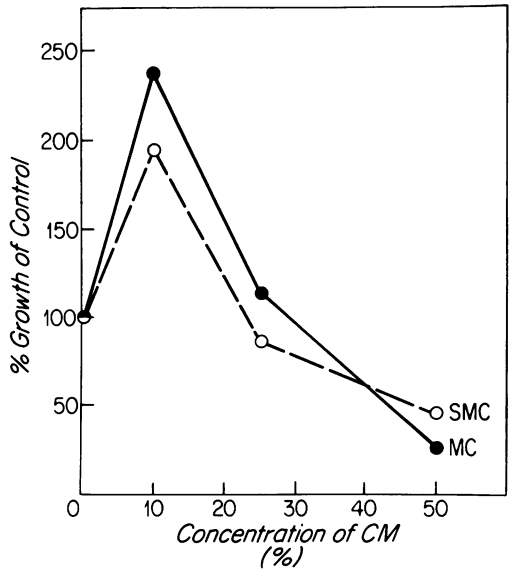

Figure 2—Effect of GEnC-secreted products on MC and vascular SMC growth. Growth-arrested MCs and SMCs were exposed to the indicated concentrations of GEnC-CM mixed with RPMI + FCS (final concentrations FCS, 20%). After 5 days, cell number was determined and compared with the growth of cells in normal growth medium (RPMI + 20% FCS) in the absence of CM. The data is expressed as the percent of growth in CM-containing medium, compared with growth in normal medium. These results are the average of duplicate determinations in three separate experiments. The average starting cell number in control cultures was 1.1×10^4 cells; the average final cell number was 21×10^4 cells, which represents approximately four doublings (see Figure 4).

Biochemical Characterization of the Inhibitory Activity

We subjected GEnC-CM to a series of enzyme treatments to characterize the biochemical nature of the inhibitory activity (Table 1; Figure 3). When GEnC-CM was incubated with 10 U/ml of highly purified *Flavobacterium* heparinase for 90 minutes at 37 C, 70% of the inhibitory activity was lost when tested at a 1:1 dilution. To eliminate the possibility of the heparinase directly affecting growth, enzyme-treated CM was boiled for 5 minutes, a treatment which kills the enzyme activity but does not affect the inhibitory activity. Boiled heparinase added to RPMI + 20% FCS or the 1:1 CM-RPMI + 20% FCS mixture also did not affect MC growth (not shown). This heparinase preparation has no detectable protease activity. It is specific for a heparin or heparinlike substrate, in that it requires a $1 \rightarrow 4$ linkage between sugar residues and also requires sulfated iduronic acid and N-sulfated glucosamine as a substrate.[13] Thus, it will not degrade chondroitins, dermatan sulfate, or hyaluronic acid.

GEnC-CM was incubated with 1 U/ml chondroitin ABC lyase for 90 minutes at 37 C. Separate aliquots were treated with 15 U/ml hyaluronidase for 90 minutes at 37 C. The samples were also boiled for 5 minutes for destruction of the enzyme activities. These enzyme

Table 1—Biochemical Properties of Glomerular Endothelial Cell Inhibitory Activities

Treatment	Percent inhibition
None	73 ± 12
Hyalurondiase (15 U/ml, 90 minutes 37 C)	67 ± 14
Chondroitin Sulfate ABC Lyase (1 U/ml, 90 minutes, 37 C)	66 ± 6
Heparinase (10 U/ml, 90 minutes, 37 C)	22 ± 5
Trypsin (30 μg/ml, 2 hours, 37 C)	54 ± 3
Trypsin followed by heparinase	3 ± 2
Heparinase followed by trypsin	6 ± 4

Glomerular endothelial cell conditioned medium was subjected to the indicated treatments and then assayed for its ability to inhibit the proliferation of growth-arrested mesangial cells when mixed 1:1 with RPMI + FCS (final concentration, 20% FCS). The data are expressed as percent inhibition ± the standard error.

treatments of GEnC-CM had little effect on MC growth (Table 1). Boiled chondroitin ABC lyase or hyaluronidase added to RPMI + 20% FCS did not affect SMC growth.

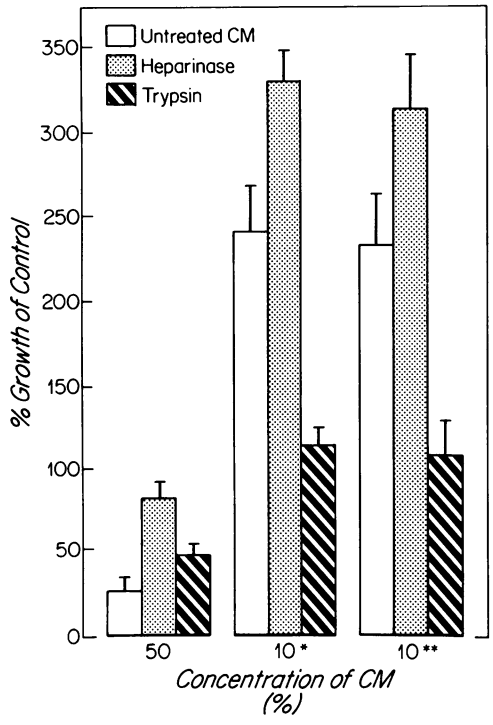

Figure 3—Biochemical characterization of the mitogenic activity. The GEnC-CM was treated with trypsin or heparinase as described in Materials and Methods. In experiments using 10% concentrations of GEnC-CM, the CM was either treated with enzymes and then diluted (*), or was diluted 1:9 and then enzyme-treated (**). The enzymes were neutralized or inactivated, and the conditioned media tested for their ability to affect MC proliferation. The net growth of cells in untreated (control) medium, ie, RPMI + 20% FCS, is set at 100%. Data shown are the average of duplicate determinations in three separate experiments. In these experiments, as in those shown in Figures 2 and 4, the control cells went through at least four population doublings during the experiment.

Treatment of GEnC-CM with 30 μg/ml trypsin for 2 hours at 37 C resulted in a 26% loss of inhibitory activity when tested at a 1:1 dilution (Table 1, Figure 3). The above data indicate that the antiproliferative activity in GEnC-CM is due to a combination of a heparin-like species and protein(s). When trypsin and heparinase treatments were performed sequentially (Table 1), 94% of the growth inhibitory activity was destroyed. Reversing the order of the enzyme treatments made no difference; ie, >90% of the antiproliferative activity was lost. These results indicate that glomerular endothelial cells secrete two independent inhibitory activities. Whether the heparinlike species and protein(s) are separate molecules or part of a heparin-proteoglycan complex in which both the carbohydrate and peptide portions are capable of inhibiting growth is not known.

To control for nonspecific enzyme effects, a premixed solution of trypsin and a tenfold excess of soybean trypsin inhibitor was added to RPMI + 20% FCS. No effect on MC proliferation was observed.

Biochemical Characterization of the Mitogenic Activity

When GEnC-CM is treated with trypsin as described above and then diluted 1:9, >90% of the mitogenic activity is destroyed (Figure 3). Diluting the GEnC-CM 1:9 and then treating with trypsin also resulted in a >90% reduction in the ability to stimulate MC proliferation. These data suggest that the mitogen secreted by GEnCs is a polypeptide. Heparinase digestion of a 1:9 dilution of CM resulted in a slightly greater stimulation of growth, probably due to the inactivation of the inhibitory heparin component. As described in the previous section, nonspecific enzyme effects were not present, as judged by the lack of effect on MC growth of a premixed solution of trypsin and soybean trypsin inhibitor.

Reversibility of the Glomerular Endothelial Cell Effect

The inhibitory effect of GEC-CM on MCs was found to be reversible[6]; in fact, this is the most important evidence that the inhibitory activity is cytostatic, rather than cytotoxic.[6,13] To test whether the antiproliferative effect of GEnC-CM on MCs was also reversible, growth-arrested MCs were released from the G_0 block with RPMI + 20% FCS in the presence or absence of a 1:1 dilution of CM (Figure 4). At 2 and 4 days after exposure to GEnC-CM, the CM-containing medium was removed and replaced with RPMI + 20% FCS. MCs that were exposed to heparin were capable of prolifer-

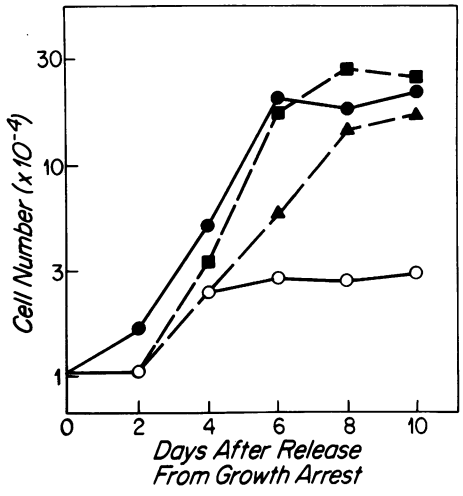

Figure 4—Reversibility of the GEnC-CM effect on MCs. Growth-arrested MCs were released from the growth block by placing them in RPMI + 20% FCS in the presence (○) or absence (●) of 100 μg/ml heparin. At 2 (■) *and 4* (▲) days after exposure to heparin, the heparin-containing medium was removed and replaced with RPMI + 20% FCS. Cell number was determined at the indicated times. The data shown are the average of duplicate determinations in two separate experiments.

ating to confluence after a very short lag period. The growth-inhibitory effect of heparin on MCs thus appears to be cytostatic in nature, and not a result of killing MCs. Several other direct microscopic observations support a cytostatic mechanism. First, the medium of CM-treated cultures does not contain detached cells. Second, the daughter cells are able to spread normally after cytokinesis. Finally, heparin-treated MCs do not take up trypan blue.

Discussion

We have examined cell-cell regulatory interactions which may be operating within the glomerulus. We have recently reported that heparin inhibits the proliferation of MCs and that GEC-derived heparin may be a physiologic regulator of growth in the glomerulus. The successful serial propagation of a cloned rat GEnC culture has allowed us to determine the ability of these cells to modulate mesangial cell growth.

When GEnC-CM was tested at a 1:1 dilution for its effect on MC growth, a marked inhibition of proliferation was observed. Enzymatic digestions demonstrated that heparin or heparinlike species accounted for 70% of the antiproliferative activity, with polypeptide(s) accounting for the rest. Sequential digestion with trypsin and heparinase indicate that there are two independent inhibitory activities. To our knowledge, this is the first demonstration and identification of a specific growth-inhibitory molecule produced by glomerular endothelial cells. These observations correlate well with previous findings that another glomerular cell type, the epithelial cells, produce antiproliferative heparin species *in vitro*.[6] The major difference between the production of growth effectors by these two glomerular cell types is that the predominant inhibitory activity secreted by the GEnC culture is a heparinlike species (as is the case for aortic endothelial cells,[13] whereas the GECs produce approximately equal amounts of heparinlike and peptide inhibitory factors. Other observtions also fit with the concept of heparinlike growth regulators in the glomerulus. For example, Striker and co-workers have shown that cultured GECs produce heparin.[3] In addition, Kanwar and Farquhar have demonstrated that heparan sulfate proteoglycan is associated with the lamina interna and externa of the glomerular basement membrane.[17]

When GEnC-CM was diluted to 1:9 and tested for its effect on MCs, a strong stimulation of growth was found. Protease digestion indicates that >90% of the mitogenic activity is polypeptide in nature. These results demonstrate that GEnCs produce both positive and negative effectors of MC growth, and parallel our observations with GECs and nonglomerular vascular endothelial cells, in which both inhibitors and stimulators of MC and SMC proliferation are observed.[6,13]

It is interesting to speculate on the possible interplay between glomerular mitogens and inhibitors. For example, the situation in the glomerulus may be similar to cultured vascular endothelial cells[13] and smooth muscle cells,[18] in which the secretion of antiproliferative heparinlike species dramatically increases when the cells are in a quiescent (and presumably differentiated) growth state. In contrast to this, exponentially growing endothelial cells produce more mitogenic activity than quiescent cultures.[13] Vascular endothelial cells are also known to secrete at least two different mitogens for SMCs,[19-22] one of which is platelet-derived growth factor. Whether or not the GEnC-derived mesangial cell mitogen is related to either platelet-derived growth factor or another vascular endothelial cell-derived mitogen is not known. The answers to these questions, as well elucidating the relationship, if any, between production of growth effectors in culture and their physiologic roles await further experimental analysis.

The GEnCs used in these experiments were obtained by a modification of our previous methods for culturing GECs and MCs.[1,7] GEnCs are very difficult to propagate, an experience shared by other laboratories as well as our own. Of the five GEnC colonies derived from the original glomerular cultures, we were able to continuously subculture one to generate the relatively large number of cells needed for the experiments reported here. The availability of all three major glomerular cell types in clonal form provides a homotypic culture system in which cell-cell interactions and kidney cell func-

Table 2—Characteristics of Cultured Rat Glomerular Cells

	Endothelial cells	Epithelial cells	Mesangial cells
Medium	K1-3T3 + 5% NuSerum or DMEM + 5% FCS + 5% NuSerum	K1-3T3 + 5% NuSerum	RPMI + 20% FCS
Substratum	Plastic	Collagen gel	Plastic
Morphology	Flat; cobblestone	Flat; polygonal	Spindle-shaped
Growth	Contact-inhibited monolayers	Contact-inhibited monolayers	Hills and valleys at confluency
Dome Formation	No	Yes	No
Angiotensin-converting enzyme	Yes	No	No
Heparin sensitivity	No growth effect	No growth effect	Inhibits growth
Cilia	No	Yes	No
Contractile	No	No	Yes
Effect of D-valine	None	None	None
C3b receptors	ND	Yes	No
Myosin type	ND	platelet > smooth muscle; fibrillar	smooth muscle > platelet; diffuse
PAN sensitivity	ND	Yes	ND

ND, not done; PAN, puromycin aminonucleoside.
Mesangial and epithelial data from references 1, 6, and 7.

tions may be studied and compared. A summary of the morphologic, pharmacologic, biochemical, and growth characteristics of cloned rat glomerular cell types based on our studies[1,6,7] is presented in Table 2; this reflects our own experiences with these cells and is not intended to be a review of the literature. It is worth noting that the different cell types can be distinguished by several criteria, including nutritional and substrate requirements, morphologic features, differentiated properties (eg, contractility, dome formation), biochemical characteristics (ie, angiotensin converting enzyme), and heparin sensitivity.

Our data suggest that GEnCs and GECs may play a role in the pathogenesis of a diverse range of kidney diseases in which MC proliferation occurs, including some forms of glomerulonephritis,[23] snake bites,[24] homocysteinuria,[25] and hypoxia-induced erythrocytosis.[25] It is interesting to note that both anticoagulant and nonanticoagulant heparin species have been shown to ameliorate the injury found in the renal ablation model,[26-28] in the spontaneously hypertensive rat,[29] and in the habu snake venom model.[30] These lesions are characterized in part by MC hyperplasia. The study of glomerular cell-cell interactions and the growth-regulatory role of heparinlike species should lead to a better understanding of MC function, and may also provide a rational basis for therapeutic intervention in proliferative glomerular disease.

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