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Glomerular epithelial cell products stimulate mesangial cell proliferation in culture

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Glomerular epithelial cell products stimulate mesangial cell proliferation in culture. Glomerular epithelial cells (GEC) and mesangial cells (MC) are both involved in glomerular diseases. To elucidate potential interactions between these glomerular cell types, we examined whether products of GEC affect the proliferative activity of MC. We found that cultured rat GEC secrete soluble factors into the supernate (GEC-CM) that induce proliferation of quiescent rat MC. The mitogenic activity was trypsin sensitive and partially heat-labile. Biochemical analysis of GEC-CM by gel filtration HPLC, reverse phase HPLC, and isoelectric focusing revealed at least three mitogenic fractions as well as inhibitory activity present in GEC-CM. Competitive binding assays with ^{125}I-labeled PDGF did not show significant amounts of PDGF in GEC-CM. The biochemical features of the GEC-derived MC growth factors are distinct from IL-6, PDGF, bFGF, and endothelin, previously described GEC-derived MC growth factors. Additionally, significant contributions of known growth factors such as IL-1, IL-2, IL-3, IL-4, IL-5, TNFα, TGFβ, and GM-CSF are unlikely. The results indicate that GEC produce several biochemically-distinct MC growth regulators. While these epithelial cell-derived mitogens for MC require further characterization, they may play an important role in the regulation of MC replication, such as during embryogenesis and glomerular disease.

The glomerular mesangium containing mesangial cells (MC) and extracellular matrix is involved in many pathological processes of the kidney. Proliferation of normally quiescent MC is a common occurrence in several forms of glomerulonephritis, including IgA nephropathy, membranoproliferative glomerulonephritis, and lupus nephritis [1]. By the use of cell culture techniques, several factors regulating MC turnover *in vitro* could be identified [2], including various cytokines such as IL-1 [3], PDGF [4], basic FGF [5] and IL-6 [6]. *In vitro*, many of the growth factors are produced by MC and are thought to stimulate MC proliferation in an autocrine manner. There is increasing evidence [7–10] that these factors are present in various forms of glomerulonephritis, regulating MC proliferation *in vivo* as well.

The origin of such cytokines in glomerular disease is still unclear. They may be secreted *in vivo* by inflammatory cells or platelets, which are present in many pathological processes of the glomerulus [7]. However, in certain forms of glomerular injury no infiltrating cells are present, and the mitogenic agents are produced locally within the glomerulus. As outlined above, MC can produce growth factors with autocrine activity. Additionally, resident neighboring glomerular cells are capable of producing growth factors, thereby stimulating MC in a paracrine manner. Glomerular visceral epithelial cells (GEC) [11, 12] and glomerular endothelial cells [13] have been shown in cell culture experiments to produce potential MC growth factors. Direct stimulation and inhibition of MC growth by podocyte derived factors has been observed [11], however, the growth factors were not further characterized.

GEC (or podocytes) play an important role in the maintenance of normal glomerular architecture and permselectivity [14, 15]. It is also evident that GEC contribute to the initiation and propagation of glomerular diseases, such as in membranous nephropathy [16, 17]. Injury to GEC may lead to abnormal glomerular permeability and to structural alterations of the integrity of the glomerular basement membrane leading to proteinuria. Further epithelial damage may finally result in glomerulosclerosis, seen in Heymann nephritis or puromycin aminonucleoside-induced nephropathy, diseases thought to have an initial podocyte damage [14–17]. In idiopathic nephrotic syndrome seen in childhood, diffuse mesangial proliferation is found in combination with podocyte damage [16]. The mechanisms, however, which lead to mesangial dysfunction after podocyte damage are presently unclear. It seems likely that growth factors or cytokines, such as PDGF, are important mediators of glomerular injury [1, 2]. To elucidate potential interactions between glomerular cells, we investigated whether products of GEC can directly stimulate MC proliferation in culture.

METHODS

Animals

Male Sprague-Dawley rats, 150 to 200 g (The Charles River Breeding Laboratories, Wilmington, MA, USA) were used to isolate glomeruli.

Key words: glomerular epithelial cells, mesangial cells, growth factors, mitogens.

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Medium and reagents

To grow MCs, we used DMEM (Gibco, Grand Island, NY, USA) supplemented with 5 μg/ml bovine insulin (Sigma, St. Louis, MO, USA), 50 U/ml penicillin, 50 μg/ml streptomycin and 10% heat-inactivated (56°C, 30 min) fetal calf serum (FCS; Gibco). The GECs were grown in K1 medium containing DMEM-Ham's F10 (1:1; Gibco) with 2.5% NuSerum (Collaborative Research, Bedford, MA, USA), hormone supplements (insulin 5 μg/ml; PGE_1 25 ng/ml; T3 0.325 ng/ml; Na_2SeO_3 1.73 ng/ml; Transferrin 5 μg/ml; Hydrocortisone 18.12 ng/ml; Sigma) and antibiotics (50 U/ml penicillin, 50 μg/ml streptomycin; Sigma) added. Recombinant human PDGF and ^{125}I-PDGF (BB dimer) were purchased from BTI (Stoughton, MA, USA); recombinant human TGF-β1 was purchased from R&D Systems (Minneapolis, MN, USA). All other recombinant cytokines (human IL-1α and human IL-1β; human TNFα; human IL-6; human IL-2; murine IL-3; murine IL-4; murine IL-5; murine GM-CSF) were purchased from Genzyme Corp. (Boston, MA, USA).

Glomerular cell culture

Primary cultures of MC were obtained from outgrowths of collagenase-treated isolated rat renal glomeruli as described [3, 6, 18]. Briefly, intact glomeruli were prepared by serial sieving of cortex homogenates of blood-free kidneys. The glomerular preparations had less than 5% tubular cell contamination. They were incubated with bacterial collagenase (type IV; Sigma) for 30 minutes at 37°C, a process that results in shedding of glomerular epithelial cells. Washed glomerular remnants, consisting of MC and endothelial cells, were plated at a density of 200 to 300 remnants/cm^2 in 20% FCS containing medium and incubated at 37°C in a humidified 5% CO_2 atmosphere. Under these conditions, a homogeneous outgrowth of elongated or stellate cells appeared within seven days and reached confluency within two to three weeks. These cells are considered vascular smooth muscle-like MC using morphological and immunohistochemical criteria [3, 6, 18]. In addition, all cells stained strongly positive with a monoclonal Thy 1.1 antibody, a marker for rat MC in kidney sections and in cell culture [18, 19]. Endothelial cells and resident glomerular macrophages are not viable under the growth conditions employed. At confluence, no cells stained positive for factor VIII, Ia antigen and rat leukocyte common antigen. Fibroblast contamination was excluded by growing the MC in D-valine containing media. Passaged MCs were obtained by incubating a washed confluent monolayer with a solution of trypsin (0.5 mg/ml; Sigma) and EDTA (0.2 mg/ml) for five minutes at 37°C. At this time, MCs were freed by gentle agitation and 10% FCS medium was added. The subcultures of MCs revealed stable morphologic and functional features.

GEC were established from glomerular explants as previously described [20]. In brief, whole glomeruli from male Sprague Dawley rats were explanted onto culture dishes (Falcon, Becton Dickinson, Oxnard, CA, USA) that were coated with collagen (Vitrogen Collagen, Palo Alto, CA, USA). After seven to nine days, colonies of GEC were marked on the culture dish and were excised with the collagen. Cells were replated onto collagen-coated multiwell dishes, and wells growing pure colonies of GEC were then expanded. The cells were grown on collagen matrices in K1 medium. Passaged GEC were obtained by incubating a washed confluent monolayer with a solution of collagenase IV (0.2%; Worthington Biochemical, Freehold, NJ, USA) for 30 minutes at 37°C. At this time, GEC were spun (200 g) for three minutes, the supernatant discarded, and the cells incubated in 0.5 ml trypsin-EDTA (trypsin, 0.5 mg/ml; EDTA, 0.2 mg/ml; Sigma) for five minutes at 37°C. To break up cell clumps the suspension was vigorously pipetted with a Pasteur pipet. To stop the reaction K1 medium was added, the cells were spun, the supernatant discarded, and the cells in K1 medium resuspended. Passaged GEC from a continous cell line [20–22] were used for all experiments. The epithelial nature of these cells was established by their polygonal shape and cobblestone apperarance at confluence, cytotoxic susceptibility to low doses of aminonucleoside of puromycin, positive immunofluorescence staining for cytokeratin, and presence of junctional complexes by electron microscopy. Additionally these cells secrete collagen and laminin in a polarized fashion, express complement regulatory proteins, $\alpha_3\beta_1$ integrins, and antigens that are present on rat GEC and that contribute to subepithelial immune deposits *in vivo*. Thus these cells have several characteristics displayed by visceral GEC *in vivo* [20–22].

Preparation of conditioned medium

Subconfluent monolayer of GEC in 75 cm^2 flasks were washed twice with HBSS and 13 ml serum-free medium was added (without FCS, NuSerum, or hormones). Control medium had identical consistency with conditioned medium, except that it was not incubated with glomerular epithelial cells. Control medium was prepared by incubation of 13 ml serum-free medium on collagen coated flasks. It was always processed in parallel, and treated in the same way as the conditioned medium. After incubation the medium was collected and spun at 400 × g to remove cellular debris. The cell free supernatant was concentrated 10- to 20-fold by allowing it to equilibrate in dialysis tubing (Spectrapor No.1; molecular wt cutoff 6 to 8000; Spectrum Medical Ind., Los Angeles, CA, USA) surrounded by polyethylene glycol compound (Sigma) at room temperature. The retentate was dialyzed against low conductivity H_2O, filtered with a 0.22 μm filter (Millex GS; Millipore Corp., Bedford, MA, USA), aliquoted and stored at −20°C. To achieve further concentration for biochemical characterization of MC-CM, the aliquots were dried under vacuum centrifugation and resuspended in a smaller volume of low conductivity H_2O. For initial biochemical characterization GEC-CM and control medium were incubated with trypsin (Sigma; 50 μg/ml in RPMI) for three hours at 37°C. Subsequently the reaction was stopped by adding soybean trypsin inhibitor (Sigma; 1.25 mg/ml in RPMI) for one hour at 37°C. Control medium was incubated with soybean trypsin inhibitor and RPMI alone. In two experiments heparinase (Sigma; 7.25 U/ml) was added for three hours atr 37°C. Additionally, GEC-CM and control medium were incubated for 30 minutes at 60°C or boiled for 10 minutes at 100°C.

MC proliferation assay

MC proliferation was determined by measuring the incorporation of [^{3}H]thymidine as previously described [18]. Briefly, MC (4th to 10th passages) were plated at a density of 2 to 3000 cells/well in 96-well flat bottom tissue culture plates (Costar). MC were grown to subconfluency in 10% FCS containing medium and subsequently starved for four to six days in 200 μl 0.5% FCS containing medium in order to make MC quiescent. At this point, control medium or growth factors were added and [^{3}H]thymidine

incorporation was determined after 24, 48 and 72 hours. For the final 24 hours of incubation MC were pulsed with 1 μCi/well ^{3}H-thymidine (^{3}H-TdR; specific activity 1 Ci = 37 GBq, 6.7 mCi/mmol; NEN), diluted in PBS. The cells were washed twice with PBS without Ca^{2+} and Mg^{2+}, trypsinized for 15 minutes (0.5 mg/ml trypsin and 0.2 mg/ml EDTA) at 37°C and collected with an automated cell harvester (Cambridge Technologies Inc.). ^{3}H-TdR incorporation was measured in a Searle liquid scintillation counter. All values represent a mean determination (± SD) of at least quadruplicate samples.

MC counts were done as previously described [6]. In short, 4 × 10^4 MC in complete MC medium were added to each well of a 24-well flat bottom tissue culture plate (Costar). Adherence, washing, period of quiescence, and experimental conditions were identical to the steps described above. At the indicated time points the MCs were washed twice with PBS. After incubation at 37°C with 200 μl trypsin/EDTA per well for 15 minutes the trypsin reaction was stopped with 150 μl MC medium containing 20% FCS. Trypan blue (50 μl, 0.4%) was added to each well before aliquots of the single cell suspension were counted using a hemocytometer. All values represent a mean determination (± SD) of at least triplicate samples.

Reverse-phase high pressure liquid chromatography (HPLC)

Reverse-phase HPLC was performed with a 250 × 4.6 mm HiPore RP318 C18 column (Bio-Rad Laboratories, Rockville Center, NY, USA) using a Bio-Rad Gradient Processor HPLC system as previously described [6, 18]. Solvent A was 0.05% trifluoroacetic acid in water and solvent B was 0.05% trifluoroacetic acid in acetonitrile. All reagents were HPLC grade. Samples (0.5 ml) were filtered with a 0.45 μm fluoropolymer HPLC filter (Gelman Sciences, Ann Arbor, MI, USA), and injected into the C18 column. A linear gradient from 33 to 67% solvent B over 45 minutes was initiated at a flow rate of 1.0 ml/min. Fractions (1.0 ml) were collected in 12 × 75 polypropylene tubes, dried under vacuum centrifugation, and maintained at −20°C until assay when samples were resuspended in 0.5 ml medium. Optical density was continuously monitored at 220 nm.

Gel filtration HPLC

Samples (0.5 ml) were analyzed by injection into a Bio-Sil TSK 125 (Bio-Rad Laboratories) column using a Bio-Rad Gradient Processor HPLC system [6, 18]. The material was eluted with PBS (0.15 M NaCl, 10 mM phosphate, pH 7.4) at a flow rate of 1.0 ml/min and 0.4 ml fractions were collected in 12 × 75 mm polypropylene tubes. The fractions obtained following gel filtration-HPLC were tested for functional activity. Optical density was continuously monitored at 220 nm. The column was calibrated using chromatography molecular weight standards (Bio-Rad Laboratories).

Granulated-bed isoelectric focusing

Concentrated GEC-CM was obtained as described above, dialyzed extensively against deionized water, and further concentrated with a vacuum centrifuge. Ampholytes, pH 3.5 to 10 (LKB, Piscataway, NJ, USA) were added to 1.5 ml of dialyzed, concentrated GEC-CM for a final ampholyte concentration of 2%. The material was added to a 20 × 20 cm electrofocusing tray containing 50 ml of electrofocusing gel (Bio-Rad Laboratories) with 2% ampholytes [18]. The anode solution was 1 N H_3PO_4. The cathode solution was 1 N NaOH. IEF was carried out in a Bio-Phoresis Horizontal Electrophoresis Cell (Bio-Rad Laboratories) at a constant power of 14 Watts for 3.5 hours at 4°C [17]. Gel fractions were removed, 1.5 ml of H_2O was added, and the pH was measured with a combination pH electrode. Each sample was then extensively dialyzed against deionized water, filtered with a 0.45 μm fluoropolymer HPLC filter (Gelman Sciences), dried under vacuum centrifugation and maintained at −20°C until assay when samples were resuspended in 1.2 ml medium.

Competitive receptor binding assay with ^{125}I-PDGF

After washing with DMEM confluent MC in 24 well plates (Costar) were incubated twice (1 hr at 37°C) with binding medium (4 mg/ml BSA in DMEM) according to a previously published method for rat MCs [23]. The cells were co-incubated for two hours at 4°C with ^{125}I-labeled PDGF (recombinant hPDGF, BB-dimer; BTI Technologies; specific activity 50 μCi/μg, 10 ng/ml) and unlabeled PDGF, GEC-CM or control medium, respectively. For negative control ^{125}I-PDGF was incubated with binding medium alone. Nonspecific binding was determined by adding 1 μg/ml unlabeled rhPDGF and did not exceed 12%. After washing the cell bound ^{125}I-PDGF was determined and competetive binding units were calculated (1 unit = 1% binding inhibition). All experiments were done in triplicate and the mean ± SD of a representative experiment (N = 4) is shown.

RESULTS

All preparations of GEC-CM (10th to 20th GEC passages) induced the proliferation of quiescent rat MC (3rd to 20th passages). Control medium did not induce MC proliferation. First, we analyzed the time course of GEC-CM on MC proliferation. GEC-CM (20%, vol/vol, 4 experiments) inceased MC proliferation 2.4-fold (± 1.7) after 24 hours, 5.8-fold (± 1.8) after 48 hours, and 4.1-fold (± 2.5) after 72 hours, respectively. Concentrated (×12), dialyzed GEC-CM obtained after 48 hours of incubation (4 different preparations) increased ^{3}H-TdR incorporation by MC 2.9-fold (± 0.6) at 24 hours, 6.7-fold (± 2.5) at 48 hours, and 8.0-fold (± 3.6) at 72 hours of incubation at 1.25% (vol/vol). A representative experiment is shown in Figure 1. In comparison to arginine vasopressin (AVP), a potent growth factor for quiescent MC [24], GEC-CM had a similar growth-promoting activity at 48 hours and 72 hours. However, GEC-CM showed only weak mitogenic acitivity during the initial 24 hours of incubation. For most subsequent experiments, we analyzed ^{3}H-TdR incorporation of MC after 48 hours incubation. Cell count experiments confirmed these data, showing a steady rise in MC number over five days (Fig. 2). After adding 20% GEC-CM (vol/vol), we observed a 2.9-fold increase in MC number, 10% GEC-CM (vol/vol) led to an twofold increase (not shown). Control medium did not induce MC proliferation. As positive control, 5% FCS increased MC proliferation more than 14-fold after five days.

Next, we analyzed the time course of the appearance of mitogenic activity present in GEC-CM. As shown in Figure 3, there was a constant rise of mitogenic activity during the first 48 hours, suggesting active secretion into the medium by GECs. After 48 hours, we observed a decline of mitogenic activity, possibly due to inhibitory factors present in the conditioned medium since further dilutions of GEC-CM led to an increase in mitogenic activity. However, after prolonged incubation (> 48 hr) with serum-free medium, we observed many detached GEC.

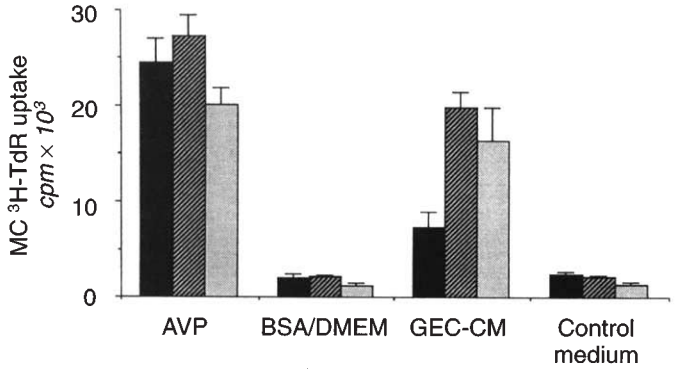

Fig. 1. Mesangial cell proliferation assay. Arginine vasopressin (10^{-6} M, AVP, positive control), 0.5% BSA in DMEM (negative control); GEC-CM (1.25%, vol/vol, 12-fold concentration, obtained after 48 hr incubation) and control medium (1.25%, vol/vol, 13-fold concentration, obtained after 48 hr incubation) were added to subconfluent quiescent (5 days 0.5% FCS) rat MC. MC proliferation was determined 24 hours (■), 48 hours (▨) and 72 hours (▩) after factor addition. Mean ± SD of a representative experiment (N = 4) is shown.

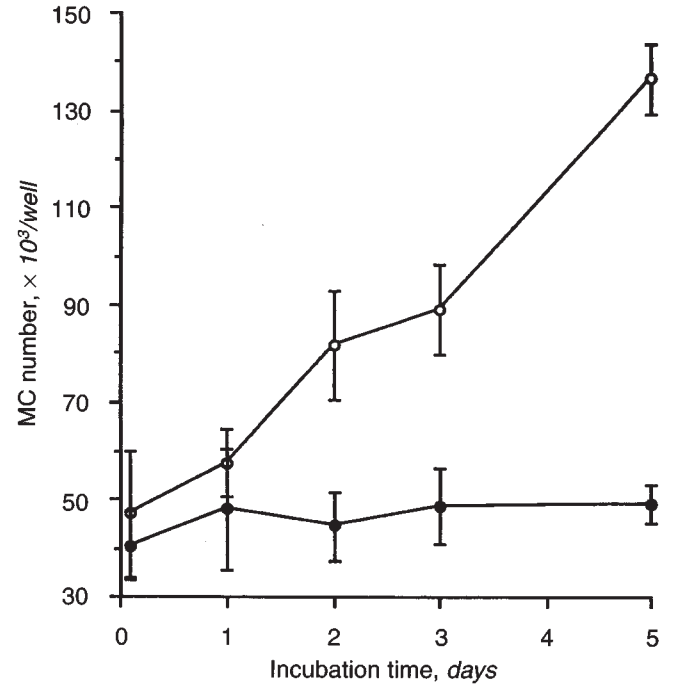

Fig. 2. Mesangial cell counts. GEC-CM (○) (20%, vol/vol, obtained after 48 hr incubation) and control medium (●) (20%, vol/vol, obtained after 48 hr incubation) was added to subconfluent quiescent (5 days 0.5% FCS) rat MC. Before and on days 1, 2, 3, and 5 after factor addition MC number was determined in triplicates (24-well plate). Mean ± SD of a representative experiment (N = 3) is shown.

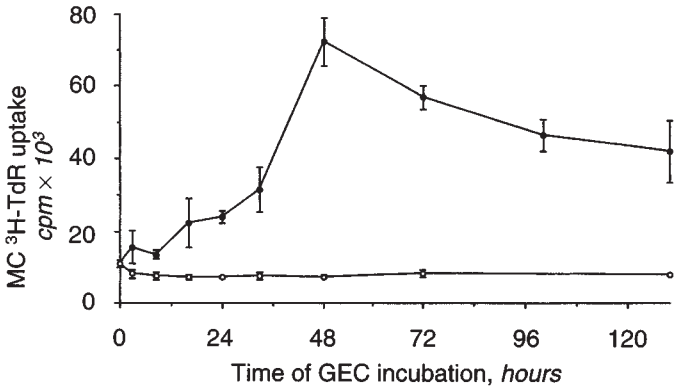

Fig. 3. Mesangial cell proliferation assay. GEC-CM (●) and control medium (○) were collected at the indicated times and added (5%, vol/vol) to subconfluent quiescent (5 days 0.5% FCS) rat MC. Proliferation (mean ± SD) was determined 72 hours after factor addition.

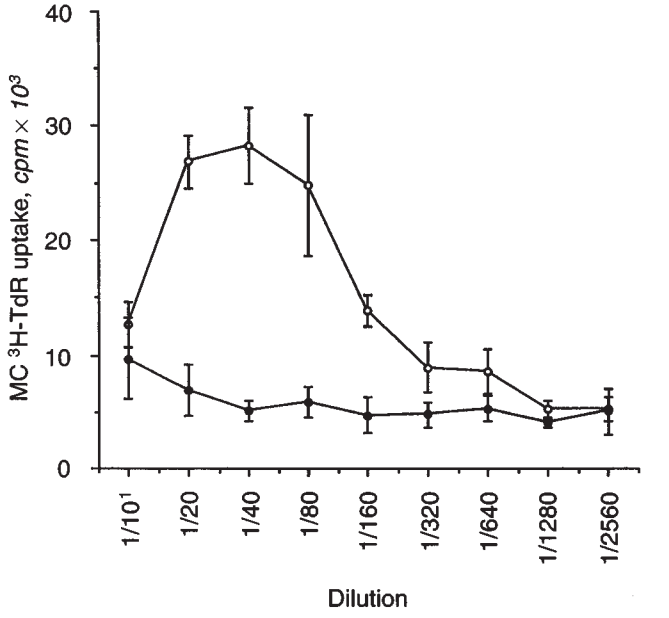

Fig. 4. Mesangial cell proliferation assay. Dose response analysis of concentrated (12-fold) dialyzed GEC-CM (○, obtained after 48 hr incubation) and concentrated (13-fold) dialyzed control medium (●, obtained after 48 hr incubation) on MC proliferation. The indicated dilutions were added to subconfluent, quiescent (5 days 0.5% FCS) rat MC. Proliferation (mean ± SD) was determined 48 hours after factor addition.

These cells clearly were viable (> 95% by Trypan blue exclusion) and GEC could be used for further passaging. In most subsequent experiments, we used GEC-CM harvested after 48 hours of incubation. Dose response analysis of unconcentrated GEC-CM demonstrated the same or sometimes higher mitogenic activity in diluted medium. This effect was even more pronounced in concentrated GEC-CM (Fig. 4). The initial dilutions led to an increase in MC proliferation, with subsequent dilutions showing a dose-dependent reduction of mitogenic activity. We found no toxic effect on MCs (> 95% viability by Trypan blue exclusion). These results indicated the presence of stimulatory and inhibitory components in GEC-CM.

Trypsin treatment (3 experiments) totally abolished the stimulatory effect of GEC-CM. Boiling (10 min at 100°C, 4 experiments) and heating (30 min at 60°C, 4 experiments) substantially reduced mitogenic activity by 55% and 46%, respectively. Initial biochemical characterization suggested partially heat-labile proteins responsible for mitogenic activity. After treatment with heparinase (2 experiments) ^{3}H-TdR uptake by MC was doubled, suggesting inhibitory heparin-like activity present in GEC-CM.

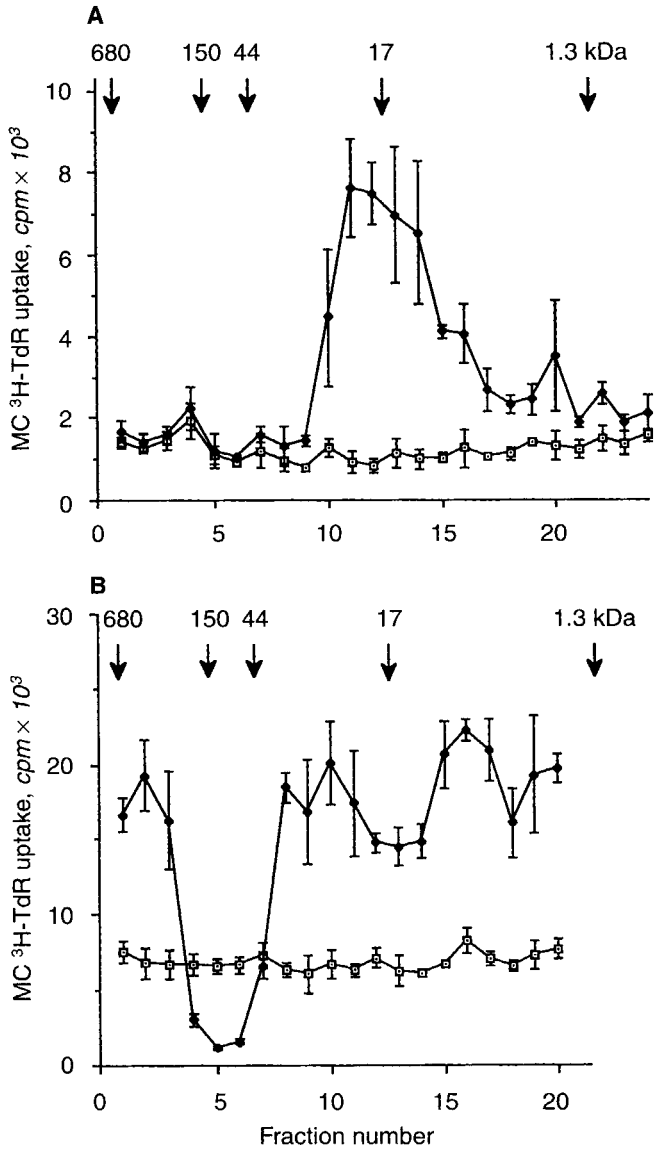

Fig. 5. Mesangial cell proliferation assay. Gel filtration HPLC analysis of GEC-CM (♦) and control medium (⊡) (*A*) and concentrated (28-fold) dialyzed GEC-CM (♦) and concentrated (22-fold) dialyzed control medium (⊡) (*B*). A total of 0.5 ml of GEC-CM and of control medium (obtained after 48 hr incubation) was separated on a TSK 125 gel filtration HPLC column. Individual fractions (50 μl, 20% vol/vol) were assayed for their ability to affect MC proliferation (mean ± SD) after 48 hours. The column was calibrated using chromatography molecular weight standards (Bio-Rad Laboratories).

Using gel filtration HPLC we further characterized the mitogenic activity. In unconcentrated GEC-CM we found a single peak of activity between 15 and 20 kDa, which was maximal around 17 kDa. A representative experiment is shown in Figure 5A. Concentrated GEC-CM repeatedly showed at least three mitogenic fractions with molecular weight species > 400 kDa, broad activity between 10 and 40 kDa, and between 7.5 to 1.3 kDa (Fig. 5B). Inhibitory fractions were found in the range of 150 to 45 kDa. Control medium did not alter MC proliferation.

Analysis of concentrated GEC-CM using reverse phase HPLC (Fig. 6) revealed three distinct mitogenic fractions, whereas the control medium was negative. One fraction did not bind to the C18 column, another major peak of activity eluted between 42 and 44% acetonitrile and a third minor peak eluted between 49 and 51% acetonitrile.

Isoelectric focusing of GEC-CM (Fig. 7) showed at least two distinct pI forms of the mitogenic activity of concentrated GEC-CM. Major activity was found at pI of 3.2, and 4.2 to 4.9. Minor activity was detectable between pI of 5.1 to 5.8 and very weak activity between pI of 6.1 to 6.9. A second experiment confirmed these data. Control medium was negative as was unconcentrated GEC-CM.

Additionally, we looked for the presence of PDGF, a potent mitogen for MC [12]. Using a competitive receptor binding assay for PDGF on mesangial cells we could not detect significant amounts of PDGF in GEC-CM (Table 1) in four experiments. Even concentrated GEC-CM (12-fold) did not contain PDGF as detected by this assay.

Analysis of potential effects of different recombinant cytokines (Fig. 8) in the MC proliferation assay excluded major contributions of these peptides to the mitogenic activity present in GEC-CM since they did not induce mesangial cell proliferation at 24 hours, 48 hours and 72 hours. Recombinant human IL-1α (100 to 0.1 U/ml), human IL-1β (100 to 1 U/ml), human IL-2 (20 to 2 U/ml), murine IL-3 (500 to 5 U/ml), murine IL-4 (1000 to 1 U/ml), murine IL-5 (500 to 5 U/ml), human TNFα (500 to 1 ng/ml), murine GM-CSF (500 to 5 ng/ml), and human TGF-β1 (100 to 0.2 ng/ml) did not induce proliferation of quiescent MC. In three experiments, high concentrations of human IL-2 (200 U/ml) seemed to inhibit MC proliferation, whereas human IL-6 (100 to 10 U/ml) exhibited stimulatory activity (2-fold increase of ^{3}H-TdR incorporation), as previously demonstrated [6].

DISCUSSION

While renal injury in various forms of GN is known to involve MC and GEC the mechanisms by which the stimuli induce these changes are only beginning to be understood. The regulation of MC turnover in the glomerulus may play an important role in a number of physiologic and pathologic processes, including differentiation, aging and various forms of glomerular disease. Cultured MC and GEC are a useful tool in studying cell physiology. GEC in culture secrete basement membrane components as well as their degrading enzymes [14, 25]. Recent studies have shown the production of endothelin [26], PDGF [12], and bFGF [27] by cultured GEC, factors which are known to be potent stimuli for MC proliferation [2]. Castellot et al [11] showed that heparin-like proteins produced by GEC are effective inhibitors of MC proliferation *in vitro*. In addition to this inhibitory activity, the authors found growth promoting activity in the supernatant of GECs, however did not further characterize this activity.

The MC growth factors are produced and released into the supernatant under serum-free conditions, extending previous observations by other investigators [11, 12, 27]. Proliferation of quiescent MC was detectable after 24 hours of incubation, however, proliferation was maximal between 48 hours and 72 hours of incubation, suggesting possible direct and/or indirect (via the release of mitogens by stimulated MC) stimulation of MC

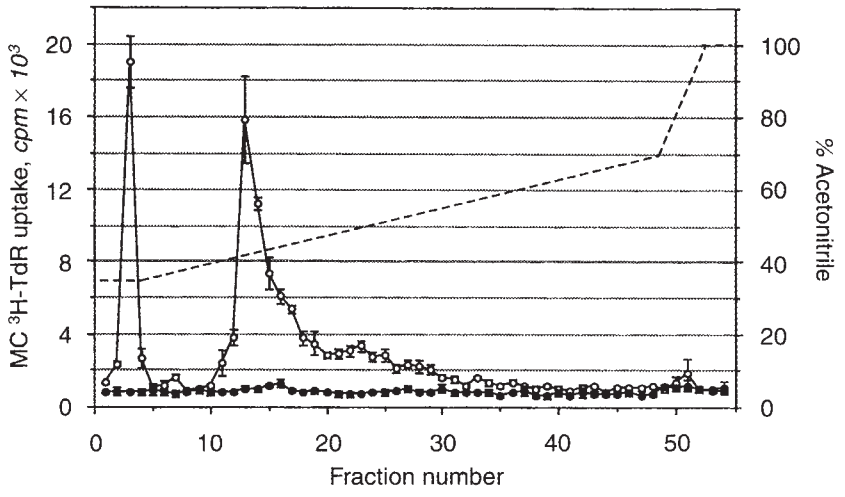

Fig. 6. Mesangial cell proliferation assay. Reverse phase HPLC analysis of GEC-CM (○) and control medium (●) collected after 48 hours. Concentrated (28-fold), dialyzed GEC-CM and concentrated (22-fold), dialyzed control medium were separated on a C-18 reverse-phase column. A gradient of acetonitrile (35 to 70%; dashed line) and H_2O with TFA was used to elute the column. Individual fractions (1 ml) were recovered from the column, dried under vacuum centrifugation, and resuspended in DMEM. Individual fractions (50 μl, 20% vol/vol) were assayed for their ability to affect MC proliferation (mean ± SD) after 48 hours.

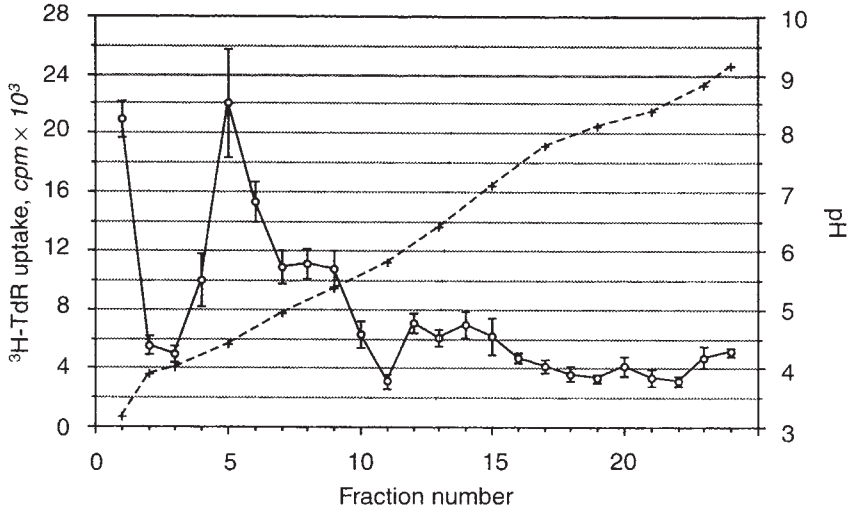

Fig. 7. Mesangial cell proliferation assay. Isoelectric focusing analysis of GEC-CM. Concentrated (26-fold) GEC-CM (○) was dialyzed extensively against low conductivity H_2O and separated by horizontal isoelectric focusing. A pH range from 3.2 to 9.2 (dashed line) was assayed to detect the presence of isoelectric forms at the extremes of physiologic pH. Individual fractions (50 μl, 20%, vol/vol; open circles) were assayed for their ability to induce MC proliferation (mean ± SD). DMEM (50 μl, 20% vol/vol) was added for negative control: 5954 ± 316 cpm.

proliferation. In order to chacterize the mitogenic activity, we performed biochemical characterizations, which showed the presence of several biochemically distinct mitogenic fractions in GEC-CM.

PDGF is one of the most important MC mitogens [2, 4]. PDGF, a basic heat-stable protein with an apparent molecular wt of 30 kDa, is released by many different cell types, including MC. Floege, Johnson and Alpers [12] observed weak expression of PDGF mRNA transcripts in GEC maintained in serum containing medium. After stimulation with IL-1, an increase of PDGF mRNA was found in GEC. First, we looked for the presence of PDGF protein in GEC-CM. Since medium supplemented with serum contains small amounts of PDGF we used GEC-CM obtained under serum-free conditions. Results of the radioreceptor binding assay excluded significant amounts (> 6 ng/ml) of PDGF in our preparations of GEC-CM. The molecular weight of the mitogenic activity found in unconcentrated GEC-CM was approximately 17 kDa, which further excluded PDGF (30 kDa) as the main mitogen. Finally, we could not detect basic proteins that had mitogenic activity. Taken together these results exclude major contributions of PDGF to the mitogenic activity present in GEC-CM.

Table 1. Competitive receptor binding assay with ^{125}I-PDGF

	Inhibition units (mean ± SD)
PDGF	
60 ng/ml	35.8 ± 4.4
24 ng/ml	20.3 ± 3.1
12 ng/ml	5.8 ± 1.4
6 ng/ml	0.5 ± 2.5
Control	0.0 ± 4.1
GEC-CM	0.1 ± 2.3
Control medium	0.3 ± 2.4
GEC-CM (11.5-fold conc.)	2.8 ± 5.8
Control medium (14.9-fold conc.)	2.3 ± 5.5

Confluent rat MC were co-incubated with unlabeled PDGF, GEC-CM, control medium, and ^{125}I-labeled PDGF (10 ng/ml) for 2 hr at 4°C. For negative control, ^{125}I-PDGF was incubated with binding medium alone. After determining cell bound ^{125}I-PDGF, competitive binding units were calculated (1 unit = 1% inhibition). The mean ± SD of triplicate determinations of a representative experiment ($N = 3$) is shown.

Takeuchi et al [27] observed the production of bFGF by GEC. Basic FGF is a cationic protein (pI 9.6) with potent mitogenic activity for cultured MC [28]. Due to the lack of typical signal

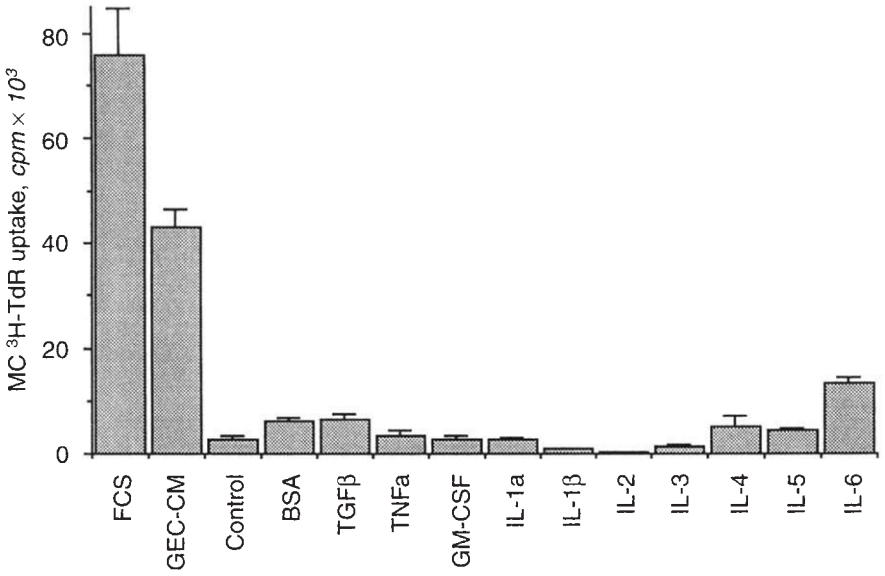

Fig. 8. Mesangial cell proliferation assay. FCS (10%, vol/vol), GEC-CM (5%, vol/vol; obtained after 48 hr incubation), control medium (5%, vol/vol; obtained after 48 hr incubation), 0.5% BSA/RPMI and different recombinant cytokines (human TGFβ, 20 ng/ml; human TNFα, 100 ng/ml; murine GM-CSF, 500 ng/ml; human IL-1α, 100 U/ml; human IL-1β, 100 U/ml; human IL-2, 200 U/ml; murine IL-3, 500 U/ml; murine IL-4, 1000 U/ml; murine IL-5, 500 U/ml; human IL-6, 100 U/ml) dissolved in 0.5% BSA/RPMI were added to subconfluent quiescent (5 days 0.5% FCS) rat MC. Seventy-two hours after addition of these factors MC proliferation (mean ± SD) was determined.

sequences, which are characteristic for secreted proteins, bFGF is probably released predominantly during cell injury [27, 28]. Once released, bFGF rapidly binds to matrix components, mainly heparan sulfate proteoglycan [27]. Recently, Francki et al [28] found small amounts of a 16 kDa isoform of bFGF in the supernatant of stimulated MC; however, most activity was present in cellular MC fractions. Even in the presence of serum-containing medium Takeuchi et al [27] detected only small amounts of bFGF mRNA in GECs using RT-PCR. These observations make the presence of significant amounts of bFGF in the supernatant of unstimulated GEC unlikely. Moreover, our data using isoelectric focusing of GEC-CM did not show any contribution of cationic proteins to the mitogenic activity. Therefore, we conclude that bFGF is not responsible for the GEC-derived mitogenic activity.

Endothelin peptides are important MC growth factors [2]. Recently, Kasinath et al [26] observed that GEC under serum free conditions secrete endothelin-1 into the supernatant. Endothelin-1 has a molecular weight of approximately 2.5 kDa, which is much smaller than the mitogenic activity we observed in GEC-CM. Additionally, the dialysis tubing used during the preparative process has a molecular weight cut-off between 6 to 8 kDa, which excludes contributions of smaller peptides, such as endothelin and epidermal growth factor (molecular wt 6 kDa) in concentrated GEC-CM. The presence of smaller stimulatory molecules in gel filtration-HPLC of concentrated GEC-CM therefore suggests the presence of degraded higher molecular weight mitogens resposible for mitogenic activity below 6 kDa, which was underlined by the presence of a large smear in the protein readout despite previous dialysis.

IL-1 has been shown to be an autocrine growth factor for MC [3], mainly in combination with other cytokines or serum components. In our experiments IL-1 did not elicit a growth response on its own, which is in keeping with other reports [2, 28, 29] and makes contributions of IL-1 to the observed GEC-derived mitogenic activity unlikely. Other cytokines such as IL-2, IL-3, IL-4, IL-5, TNFα, GM-CSF [18] and TGFβ did not induce MC proliferation in our test system.

Confirming our previous data [6], high concentrations of IL-6 are mitogenic for cultured rat MC. Recently, Moutabarrik et al [30] and Osawa et al [31] reported up-regulated IL-6 secretion by GECs after cytokine stimulation. Both groups found only minor constitutive secretion of IL-6 by GEC. In order to provoke a relatively modest growth response of cultured MC, high concentrations of IL-6 are necessary. In contrast, our results demonstrate that unconcentrated GEC-CM elicits a strong mitogenic response. Additionally, IL-6 has a different molecular weight-profile on gel filtration HPLC [6] and a different profile on reverse phase HPLC [6] as compared to the mitogenic activity in GEC-CM. Thus, our results do not provide any evidence for a significant role of IL-6 in the growth response of cultured MCs elicited by GEC-CM. Taken together, these results virtually exclude contributions of several known MC growth factors to the mitogenic activity present in GEC-CM. It is therefore likely that we have described an additional and distinct GEC-derived MC growth factor.

In addition to growth-promoting activity, GEC produce inhibitory molecules. This extends previous observations by Castellot et al [11]. They attributed the inhibitory activity found in GEC-CM to heparin-like species because heparinase treatment abolished the antimitogenic effect on MC proliferation. Confirming their finding, our data show that heparinase treatment leads to a substantial increase in GEC-derived mitogenic activity. The presence of growth-inhibitory molecules was further suggested by our dilution experiments and by gel filtration HPLC, showing inhibitory fractions between 150 and 45 kDa. The fact that mitogenic activity of GEC-CM did not further increase may suggest that the molecule was partially degraded during the preparative step or copurified with an inhibitor. Because MCs were clearly viable after incubation with GEC-CM, true growth inhibition rather than toxic effects on MC growth can be assumed. So far, growth-inhibitory activity for MCs has been demonstrated only for few cytokines [2]. TGFβ in higher concentrations decreased serum or cytokine-induced MC proliferation [31]. In addition, antiproliferative activity has been attributed to atrial natriuretic factor, interferon γ, prostanoids and nitric oxide [2]. However, all these antimitogenic substances have considerably lower molecular weights excluding contributions to the inhibitory activity observed

by us. Several reports described the inhibitory effects of heparin and heparin-like species on MC proliferation [2, 32, 33]. Whether the large molecular weight fractions of GEC-CM contain heparin-like species such as heparan sulfate (such as in heparan sulfate proteoglycans) was not investigated in this study. Production of such components by GEC with a similar molecular weight has been shown previously [33]. Their potential contribution to the inhibitory activity of GEC-CM requires further analysis.

The GEC-derived mitogens and growth inhibitors may play a role under different physiological and pathophysiological conditions. Glomerular cells in two-dimensional culture appear to reflect an activated cell phenotype, as seen in glomerular disease or in morphogenesis [2, 29]. One could speculate that GEC-derived growth regulators may provide proliferative and inhibitory signals for quiescent MCs *in vivo*. In some forms of human glomerulonephritis (for example, childhood idiopathic nephrotic syndrome with diffuse mesangial cell proliferation or in development of focal glomerulosclerosis) initial podocyte injury is thought to lead to MC proliferation [14, 17]. In such circumstances, GEC-derived cytokines could be the first step in the progression of glomerular disease. It is also conceivable that GEC-derived cytokines are growth factors or chemoattractants for other cell types that enter the urinary space of the glomerulus, such as mononuclear cells seen in crescents and parietal epithelial cells. Additionally, the GEC-derived factors may provide a growth regulatory signal for tubular cells under physiological and pathophysiological conditions. Due to the process of glomerular ultrafiltration outward flow across the glomerular capillary wall should oppose access and action of these cytokines to the mesangial space. However, as pointed out by Kasinath et al [26], it is evident that GEC are directly adjacent to MC in the perimesangial areas, separated only by the glomerular basement membrane of the capillary tuft. It is conceivable that molecules can diffuse across the glomerular basement membrane from GEC toward MC in glomerular disease with occlusion of capillaries and glomerular basement membrane derangement. Under such conditions GEC-derived proteins might diffuse into the mesangium and affect MC behavior. Additionally, such GEC-derived regulatory molecules may be involved in nephron development [34, 35].

In conclusion, the results of this study have characterized new GEC-derived mitogenic activity. Significant contributions of known MC growth factors produced by epithelial cells are unlikely. Further identification of the major individual mitogenic fractions is a worthwhile goal, since GEC-derived growth regulators may play a role in the interactions between GECs and MCs in glomerular diseases, and during development.

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