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Modulation of Mesangial Cell Proliferation by Endothelial Cells in Coculture

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The effects of direct cell contact between endothelial (ECs) and mesangial cells (MCs) on MCs proliferation were examined in a coculture system* in vitro.** ***Mitomycin C treated ECs (M-ECs) were plated on culture dishes and MCs were cocultured with these M-ECs. Cell number was measured at the end of days 1, 3, and 5. In the coculture system with direct contact, the growth of cocultured MCs was modulated as follows: 1) the growth of MCs was inhibited up to day 3, and 2) a high level of proliferation was observed between days 3 and 5. This biphasic pattern of growth could not be detected in coculture of fibroblasts with MCs. In coculture without direct contact, using intercup chambers, the kinetics in cell proliferation between cocultured MCs and MCs alone were essentially the same. Conditioned media derived from cocultures up to day 3 in a contact-dependent manner inhibited the ^{3}H-thymidine uptake of MCs. From these results, it would thus appear that MCs proliferation is regulated by intercellular contact with ECs. (Am J Pathol 1991, 139:949–957)

In the glomerulus, the endocapillary region is a closed space surrounded by the glomerular basement membrane comprised of a capillary lumen, endothelial cells (ECs), mesangial cells (MCs), and mesangial matrices. Communication circuits between cells or cells and matrices is important for maintenance of normal tissue physiology and for the initiation and persistence of pathophysiologic abnormalities such as glomerular inflammation which may lead to glomerulosclerosis.

Monocyte/macrophage-derived cytokines, representative extrinsic mediators, possess mesangial cell-growth–stimulating[1,2] and enhancing activity for extracellular matrix production by mesangial cells.[3] However, the cellular and molecular mechanisms governing the growth of mesangial cells through interactions of intrinsic endothelial cells are poorly understood.

There is accumulating evidence that ECs are recognized as important effector cells. For example, they play active roles in proliferation of intimal smooth muscle cells in blood vessels together with extrinsic cells of macrophages and platelets.[4,5]

Recently, using a cell culture technique, capillary endothelial cell growth was found to be inhibited by pericytes, and this inhibition was found to be dependent on physical contact or proximity between ECs and pericytes.[6]

In consideration of the aforementioned, an examination was conducted on the effects of ECs on MCs proliferation under a condition of cell-cell contact, using an *in vitro,* coculture system.

Materials and Methods

Cell Culture

Endothelial Cells (ECs)

ECs from human umbilical veins were prepared according to the method described previously.[7] The cells suspended in complete medium, i.e., MCDB 151 (Sigma Chemical Co., St. Louis, MO) containing 15% FCS, 50 μg/ml ECGS (Collaborative Research, Lexington, MA), 50 μg/ml heparin (Sigma Chemical Co., St. Louis, MO) were cultured in tissue flasks (Falcon #3013, Becton Dickinson Labware, Lincoln Park, NJ) coated with fibronectin. Third- or fifth-passage cells were used for subsequent experiments.

Identification as ECs was made on the basis of morphology under phase-contrast microscopy and indirect immunofluorescence, using rabbit anti-human factor VIII antigen serum (DAKO PATTS a/s, Glostrup, Denmark).

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Mesangial Cells (MCs)

Six- to eight-week-old male Wistar rats were used. MCs were cultured from isolated glomeruli as described previously.[8] Briefly, intact glomeruli were obtained by serial sieving of cortical minces. Tubular fragments were removed by allowing the glomeruli to sediment. The sediment was centrifuged at 900 g for 1 minute, and the supernatant was discarded. Trypsin (0.25%)-EDTA (0.01%) in Ca^{++}, Mg^{++} free phosphate buffered saline (PBS) was added to the pellet that was then incubated in a water bath at 37°C for 15 minutes. Trypsin-EDTA was subsequently neutralized by adding a medium supplemented with 20% FCS containing Dulbecco's modified Eagle medium (Nissui Pharmaceutical Co., Tokyo) (D-MEM); 10,000 isolated glomeruli were cultured in a plastic 25 cm^2 flask (Falcon #3013) containing 4 ml of D-MEM supplemented with 20% FCS, penicillin, streptomycin, L-glutamine. The tissue-culture flasks were incubated at 37°C in a humidified 5% CO_2 atmosphere. The cultures were re-fed with culture medium 4 to 6 days after plating and the medium was subsequently changed twice a week. Under these conditions, glomerular epithelial cell outgrowth was allowed to continue for 7 to 10 days. The epithelial cells were noted to senesce and become detached, accompanied by the appearance of MCs which spread out and away from the glomerular cores; 3 to 4 weeks after plating, the cultured cells used as mesangial cells became confluent and appeared as a homogeneous cell population, based on the following previously described[9,10] criteria: 1) morphologically elongated cell shape and multilayered growth, 2) positive immunofluorescence with antialpha smooth muscle actin of intracellular longitudinal fibrils and positive desmin staining, 3) positive immunofluorescence of the cell surface with monoclonal and polyclonal antibodies against Thy 1-1, and 4) absence of immunofluorescence with the antifactor VIII antibody or antikeratin antibody. Confluent MCs were washed, removed with 0.25% trypsin and 0.02% EDTA in PBS solution, and suspended in culture medium and plated onto culture flasks. Experiments were conducted on two- to five-passage MCs cultures.

Coculture Studies

Coculture with Direct Contact

To examine the effects of ECs on MCs in the coculture system, ECs were treated with mitomycin C(MMC) so that cell division would not occur,[11] and thus increase in total cell number should be attributed to MCs growth. MMC has selective inhibition of DNA replication[11] and MMC treated cells retain their function under adequate conditions.[12,13]

Subconfluent ECs were treated with MMC (0.5 μg/ml; Sigma, St. Louis, MO) for 2 hours at 37°C. The MMC-treated ECs (M-ECs) were rinsed with PBS containing 0.02% EDTA, removed by trypsinization (0.05% trypsin), and plated at a density of 5×10^4 cells per well into fibronectin coated 24-well tissue culture dishes (Falcon, #3047) in growth media. By the following day, a subconfluent M-ECs monolayer formed. After removing the media and washing with PBS, the MCs were plated in wells containing M-ECs at a density of 2×10^4 cells per well in D-MEM containing 15% FCS and 50 μg/ml ECGS. Control cells consisting of MCs and M-ECs were cultured alone under identical conditions.

The uptake of 1, 1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate labeled acetylated low density lipoprotein (DiI-Ac-LDL) (Funakoshi, Japan) of the M-ECs, indicating preservation one of EC function, was monitored during the experiment as described previously.[14] For this purpose, M-ECs and/or MCs were cultured on fibronectin-coated Lab-Tek glass slides. The cells were incubated with 10 μg/ml DiI-Ac-LDL at 37°C in media for 4 hours. The media was removed and cells were washed with PBS and fixed with 10% phosphate-buffered formalin for 5 minutes. The slides were mounted in 90% glycerol and examined with an immunofluorescent microscope.

The angiotensin-converting enzyme (ACE) activity of ECs and M-ECs was assayed with colorimetry using p-hydroxybenzoyl-glycyl-L-leucin as a substrate.[15] Prostacyclin (PG I_2) production by ECs and M-ECs was determined by assaying the amount of PG I_2 released into culture medium using a radioimmunoassay for the 6-keto derivative of prostaglandin F1 alpha, a stable catabolic product of PG I_2.

To examine the specificity of cell-cell interaction, mitomycin C treated (MMC 1.0μg/ml) fibroblasts (M-FBs) with MCs and mitomycin C treated (MMC 1.0μg/ml) mesangial cells (M-MCs) with MCs were co-cultured using the same direct contact method as described earlier. Fibroblasts (FBs) were derived from human forearm skin. The effectiveness of MMC in arresting M-FB and M-MC growth was confirmed by the finding that cell number was not increased during the 5 days of culture (data not shown).

Coculture Without Contact

In this system, M-ECs and MCs were cultured in the same well but physical contact was prevented by Intercup chambers (Sanko Junyaku, Japan). Briefly, Intercup chambers were floated on the wells of 24-well plates whose bottoms were 1 to 2 mm above the layer of M-ECs. MCs were then plated in the chambers. The cell concentration was the same in the coculture with direct contact.

The bottom of each Intercup chamber was a cellulose membrane coated with fibronectin, and had 0.45 μm pores to permit diffusion of the soluble factors.

Cell Proliferation Assay

Cell number was measured with a coulter counter to determine cell proliferation in the coculture system.

Direct Count of Cell Number in Coculture with Direct Contact

M-ECs and MCs in coculture were directly counted according to the following method. A cell mixture consisting of M-ECs and MCs obtained by trypsinization from a cocultured well was centrifuged, the sediment was smeared on a slide glass, fixed with ethanol for 5 minutes, and washed with PBS. This specimen was examined by indirect immunofluorescence using rabbit antihuman factor VIII antigen. The number of positive cells /100 cells was measured.

Conditioned Medium (CM)

Media (D-MEM containing 15% FCS and 50 μg/ml ECGS) were conditioned by coculture or control culture for 48 hours (from day 1 to day 3), removed and centrifuged at 2,000 rpm for 10 minutes, filtered through a 0.22 μm filter and stored at 4°C until use.

Mesangial Cell Proliferation Assay on Conditioned Medium

Confluent MCs were harvested as follows: the medium was aspirated and the cells were washed twice with PBS and treated with 0.25% trypsin and 0.02% EDTA solution. The MCs were then resuspended in 20% FCS D-MEM and adjusted to a concentration of 25,000 cells/ml; 200 μl of this cell suspension (i.e. 5,000 cells/well) were plated on a 96-well microculture plate (Falcon #3096) coated with fibronectin. After 24 hours culture in a 5% C02 humidified atmosphere at 37°C, the medium was replaced with 0.5% FCS D-MEM twice and incubated for another 48 hours. The whole culture media were replaced with conditioned media and incubated for another 48 hours. ^{3}H-thymidine (0.5 μCi/ml) was added to each culture during the last 18 hours. After labeling, the media were aspirated and 200 μl of trypsin-EDTA solution were added to each well. MCs were harvested and measured for ^{3}H-thymidine uptake.

Effect of Anti–TGF-beta Antibody on MC Growth-suppressive Activity

To examine whether TGF-beta is related to inhibitory activity of MC proliferation in conditioned medium from coculture with direct contact, a neutralizing assay was performed using anti–TGF-beta antibody as follows.

As described earlier, MCs were plated at a density of 5,000 cells/well into a fibronectin-coated 96-well plate in 20% FCS D-MEM. The following day, the medium was replaced with 0.5% FCS D-MEM twice and incubated for another 48 hours. After removing the media, 150 ul of conditioned medium obtained from coculture of M-ECs and MCs with direct contact, and 50 ul of diluted neutralizing rabbit anti-human TGF-beta 1 (King Brewing, Co., LTD, Japan) or normal rabbit serum (NRS) were added to each well and incubated for another 48 hours. The final concentration of anti-TGF-beta was from 2.5 μl/ml to 0.025 μl/ml because higher concentration of anti-TGF-beta markedly inhibited MC proliferation even in basal medium (15% FCS D-MEM and 50 μg/ml ECGS) in our preliminary experiment (data not shown). ^{3}H-thymidine (0.5 μCi/ml) was added to the culture during the last 18 hours and ^{3}H-thymidine uptake was measured.

Statistics

Calculations were made by the unpaired *t*-test.

Results

Coculture with Direct Contact

The effects of vascular ECs on MC proliferation in a coculture system with intercellular contact were examined.

Figure 1 shows cell counts of M-ECs alone, MCs alone, and total cell counts of cocultured MCs including M-ECs. The effectiveness of mitomycin C for arresting M-EC growth was confirmed by the finding that cell number was not increased during the period of examination in culture of M-ECs growing alone. Cultured M-ECs all were capable of taking up DiI-Ac-LDL throughout the experiment (Figure 2). The angiotensin-converting enzyme activity and prostacycline production were equivalent to control levels (untreated endothelium) during 5 days of culture (data not shown).

As noted previously, the increase in total cell number in the direct coculture system should be attributed to the increase in MCs only. Based on this assumption, cocultured MC number data (Figure 3) was calculated indirectly by subtracting the M-EC number (derived from M-ECs growing alone) from the total direct coculture cell

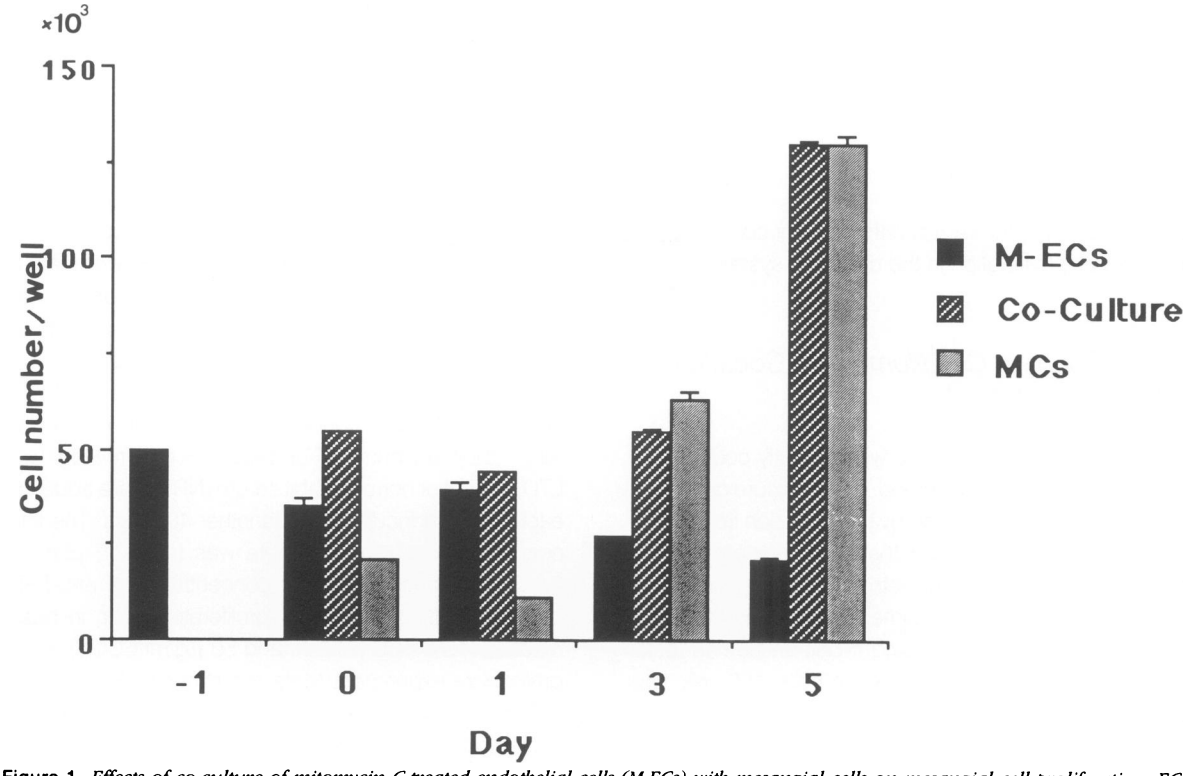


Figure 1. *Effects of co-culture of mitomycin C-treated endothelial cells (M-ECs) with mesangial cells on mesangial cell proliferation. ECs treated with mitomycin C (0.5 μg/ml) (M-ECs) were plated (5 × 10^4 cells/well) in 24-well dishes. The following day (day 0), MCs were plated (2 × 10^4 cells/well) on wells containing M-ECs and co-cultured. As controls, M-ECs or MCs were plated (M-ECs: 5 × 10^4 cells/well, MCs: 2 × 10^4 cells/well) and cultured alone. Results were mean values of six wells ± 1SD. (■): Cell number of M-ECs cultured alone, (▨): Total cell number of co-cultured cells consisting of M-ECs and MCs. (▩): Cell number of MCs cultured alone.*

number. The validity of the above assumption and indirect calculations was confirmed by direct count of cells in coculture as described in Materials and Methods. M-ECs in coculture all stained positively with factor VIII antigen and the population of cells negative for factor VIII increased as the indirectly calculated number of MCs in coculture increased.

A comparison of the number of cocultured MCs with that of MCs cultured alone is shown in Figure 3. The numbers of MCs cocultured with M-ECs showed no significant difference at days 1 and 5, but a 56% decrease at day 3, in contrast to the growth of MCs cultured alone ($P < 0.01$). This indicated that after day 3, the rate of increase in the number of cocultured MCs exceeded that of MCs cultured alone. The percent attachment after an overnight incubation of cocultured MCs was the same as that of MCs cultured alone (data not shown).

To determine whether these phenomena are specific for the interaction between ECs and MCs, MCs were cocultured with mitomycin C treated fibroblasts (M-FBs) or mitomycin C treated mesangial cells (M-MCs) in a contact dependent manner. In no case could a higher rate of increase in the number of cocultured MCs be detected. The proliferation rate of cocultured MCs was always less than that of MCs cultured alone (Figure 4).

Coculture Without Contact

The role of cell contact for mesangial cell proliferation was determined in a culture system using intercup chambers, in which no physical interaction could take place but which did allow for the exchange of humoral factors be-

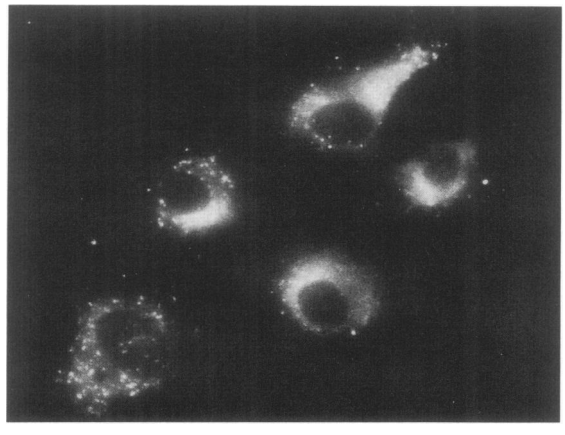


Figure 2. *Fluorescence micrograph of M-ECs labeled with 1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate Labeled Acetylated Low Density Lipoprotein (DiI-Ac-LDL). ECs cultured for 5 days after mitomycin C treatment, were incubated with 10 μg/ml DiI-Ac-LDL for 4 hours at 37°C. DiI-Ac-LDL was taken up into the cytoplasm of all M-ECs, ×1700.*

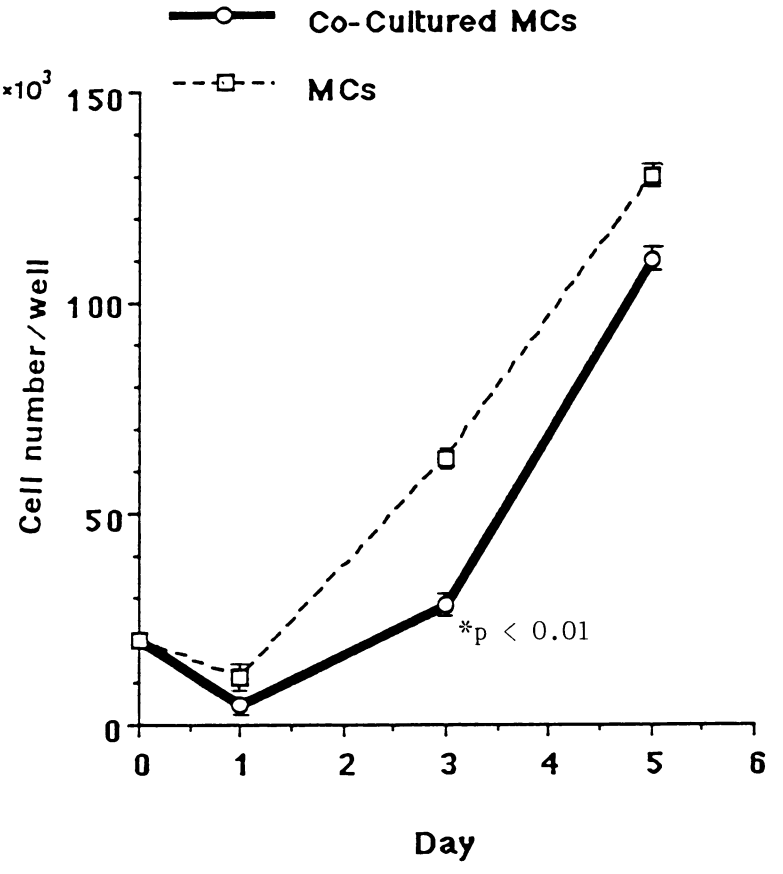


Figure 3. *Effects of co-culture with direct contact of M-ECs and MCs on MCs proliferation. The influence of cell contact on the cell proliferation of MCs was examined. The number of co-cultured MCs was expressed by subtracting the mean number of M-ECs from the mean of the total number of co-cultured cells shown in Figure 1. Results were mean values of six wells ± 1SD. (—○—): Cell number of co-cultured MCs, (—□—): Cell number of MCs cultured alone.*

tween M-ECs and MCs. As shown in Figure 5, the kinetics of cell number was the same as cocultured MCs and MCs cultured alone.

Effects of Conditioned Medium (CM)

Media conditioned for 48 hours (from day 1 to day 3) by individual cultures of M-ECs, MCs and cocultures of the two cells were examined for their effects on MC proliferation. Two experiments following the same schedule indicated the CM from the direct contact co-culture of M-ECs and MCs significantly inhibited the ^{3}H-thymidine uptake of MCs ($P < 0.05$) compared with M-ECs and MCs cultured alone. This inhibitory effect could not be detected using CM from the co-culture of M-ECs and MCs without physical contact (using intercup chambers) (Figure 6a). The CM from the direct contact coculture of M-FBs and MCs had no MC growth suppressing activity (Figure 6b). Our preliminary examination showed MC growth suppressing activity of CM from the coculture of M-ECs with direct contact in a dose response manner. Namely, ^{3}H-thymidine uptake of MCs cultured in control medium (i.e., at 0% CM) was 6385 ± 215 dpm; at 100% CM, it was 2038 ± 190 dpm (32% of control); at 75% CM, it was 4096 ± 242 dpm (64% of control); at 50% CM, it was 4903 ± 607 dpm (77% of control); and at 25% CM, it was 5771 ± 173 dpm (90% of control).

Effect of Anti–TGF-beta Antibody on MC Growth-suppressive Activity

As described earlier, the CM from the direct-contact coculture of M-ECs and MCs showed MC growth-suppressive activity. Rabbit antiserum against TGF-beta was used to examine whether TGF-beta is responsible for this growth-suppressive activity. In the presence of higher doses of neutralizing anti–TGF-beta (2.5 μl/ml and 0.25 μl/ml), the inhibitory effect was diminished in comparison with ^{3}H-thymidine uptake of MC in the absence of a-TGF-beta antibody (control) (Table 1), although the differences were not statistically significant.

Discussion

An examination was made of the effects of vascular endothelial cells on mesangial cell proliferation. Using a coculture system with intercellular contact, the following results were obtained regarding co-cultured MC growth:

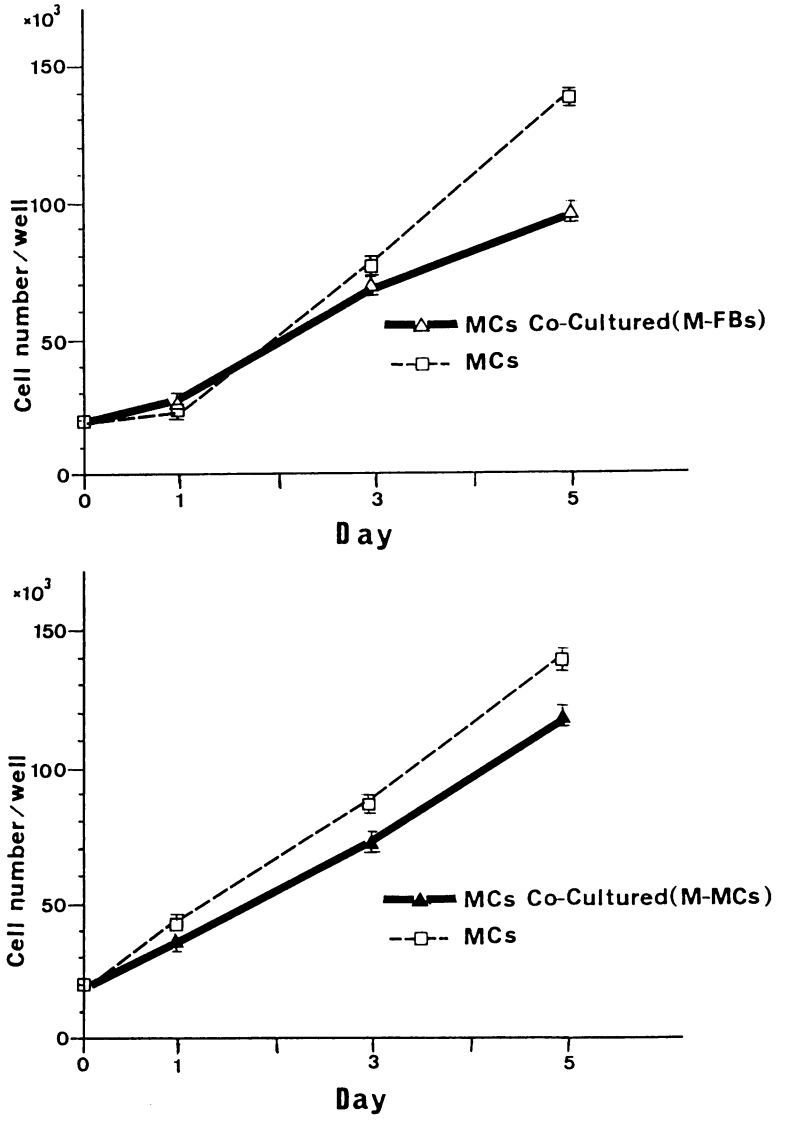


Figure 4. *Effects of co-culture of MCs with fibroblasts (FBs) and mesangial cells (MCs). FBs and MCs were treated with mitomycin C (M-FBs, M-MCs) as described in Materials and Methods, and plated in 24-well dishes (5 × 10^4 cells/well). The following day, MCs were plated (2 × 10^4 cells/well) in wells containing each type of mitomycin C treated cells. Results were mean values of 6 wells ± 1SD. The number of co-cultured cells was expressed as the difference between total cell number of co-cultured cells and number of M-FBs, or M-MCs. (—△—): Cell number of co-cultured MCs with M-FBs. (—▲—): Cell number of co-cultured MCs with M-MCs. (—□—): Cell number of MCs cultured alone.*

1) significant inhibition occurred up to day 3, and 2) there was a high level of proliferation between days 3 and 5. These results indicate that proliferation of cocultured MCs occurs in two phases; an arresting phase and a proliferating phase.

In the coculture system without direct cell contact, growth modulation of cocultured MCs was not observed. The kinetics in cell number was the same for cocultured MCs and MCs cultured alone. In the coculture of fibroblasts with MCs, and MCs with MCs, this biphasic pattern of modulation could not be found. The proliferation rate of cocultured MCs was virtually constant. The modulation of MCs proliferation noted in the coculture with direct contact would thus appear to depend on "contact" (or "close proximity") and could be detected particularly between ECs and MCs in vitro.

The cellular mechanisms of this modulation remains to be determined exactly. Since modulation depends on contact between ECs and MCs, the essential process appears to occur at an early stage when mutual contact between ECs and MCs is maintained most effectively. With the passage of time, MCs grow on top of each other and the number of MCs having no direct contact with ECs increases. In the early stage up to day 3, inhibition of MC growth was observed in cocultured MCs compared with the control. High-cell proliferation was noted after this. This biphasic pattern of cell growth may be explained in terms of cell-cycle regulation. In the arresting phase, MCs become synchronized in a cell-cycle phase preceeding the DNA synthesis phase. As the regulatory effect diminished or is antagonized, MCs simultaneously begin DNA synthesis. The cells enter the proliferative phase. In the direct coculture system, this was seen from days 3 to 5.

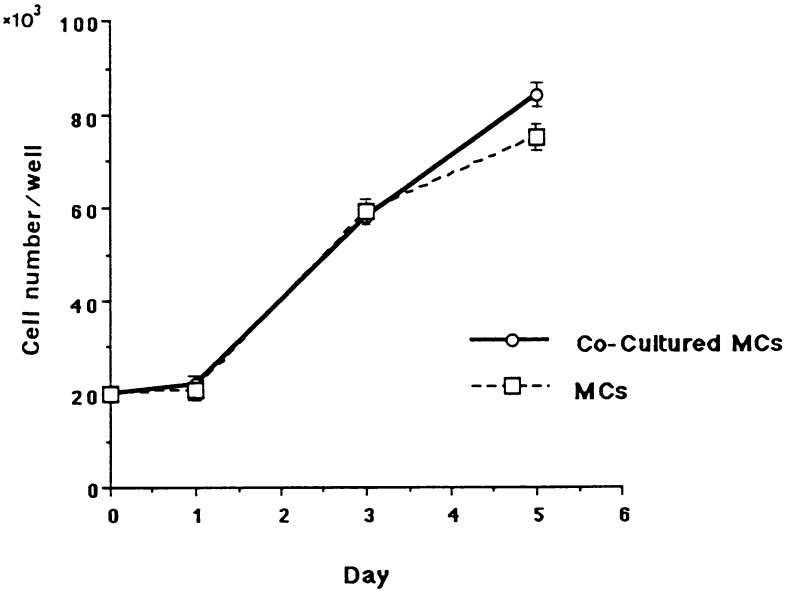
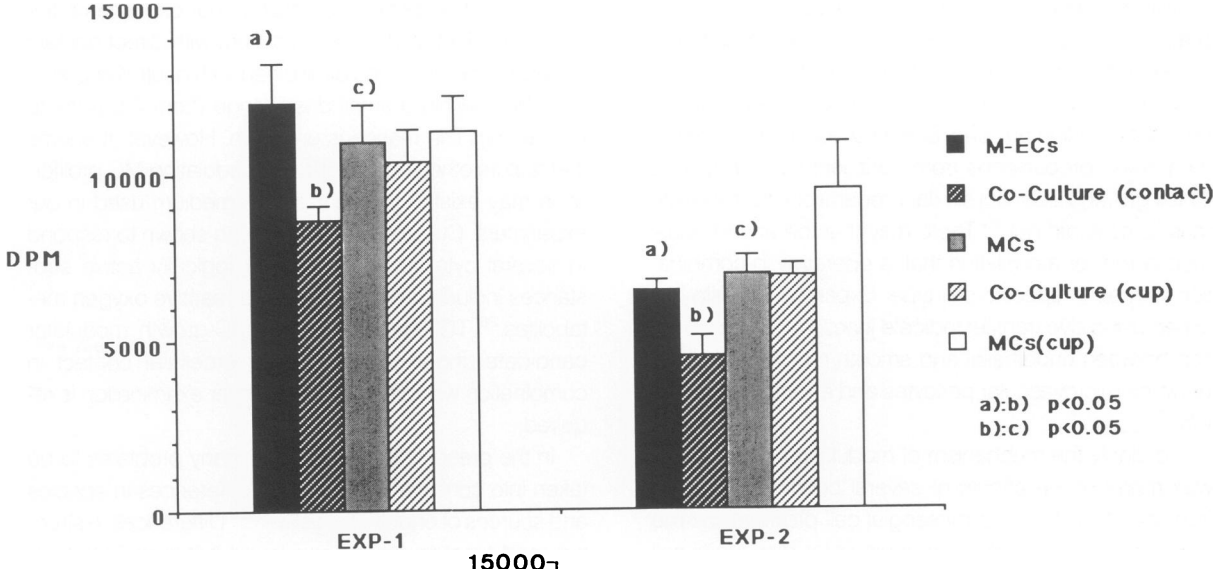


Figure 5. *Effects of co-culture without contact of M-ECs with MCs on MCs proliferation. M-ECs were plated in a 24-well dish (5 × 10^4 cells/well). The following day, MCs were plated (2 × 10^4 cells/well) in Intercup chambers, whose bottom was a membrane having 0.45 μm pores. The chambers were inserted into the walls containing M-ECs, followed by co-culturing two types of cells. Results were means of 6 wells ± 1SD. (—○—): Cell number of MCs co-cultured with M-ECs. (—□—): Cell number of MCs cultured alone in Intercup chamber.*



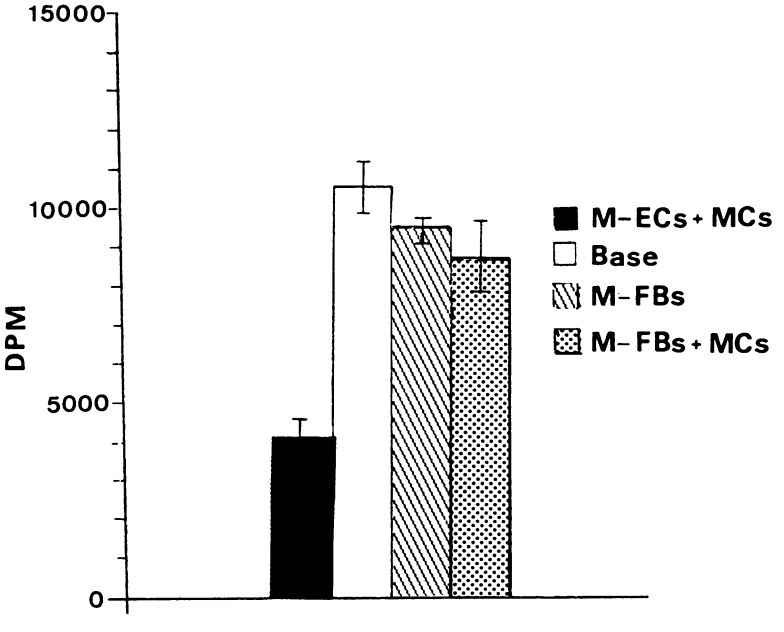


Figure 6. *Effects of conditioned media (CM) on mesangial cell proliferation. Media conditioned for 48 hours (from at day 1 to day 3) by co-cultures between M-ECs and MCs or control cultures were assayed for effects on ^{3}H-thymidine incorporation rate of MCs (Figure 6a). Results were mean values of triplicate wells ± 1SD. 1SD were less than 15% the mean. (■): CM obtained from M-ECs cultured alone. (▩): CM obtained from co-culture with direct contact between M-ECs and MCs. (■): CM obtained from MCs cultured alone. (▨): CM obtained from co-culture without direct contact between M-ECs and MCs using an Intercup chamber. (□): CM obtained from MCs cultured alone in the Intercup chamber. a),b): $P < 0.05$, b),c): $P < 0.05$. Effects of CM derived from co-culture between M-FBs and MCs with direct contact on ^{3}H-thymidine incorporation rate of MCs are shown in Figure 6b. Results were mean values of triplicate wells ± 1SD. 1SD were less than 10% the mean. (■): CM obtained from co-culture of M-ECs and MCs with direct contact. (□): Basal growth media. (▨): CM obtained from M-FBs cultured alone. (▩): CM obtained from co-culture of M-FBs and MCs with direct contact.*

Table 1. *Effects of Neutralizing Anti–TGF-β on Mesangial Cell Proliferation in Conditioned Medium from Coculture with Direct Contact*

	[^{3}H]-thymidine uptake of MCs*	
	Mean dpm ± SD‖	% control
Control: CM† + NRS‡ (2.5 μl/ml)	4696 ± 139	100%
1 CM + a–TGF-β (2.5 μl/ml)	5240 ± 795	112%
2 CM + a–TGF-β (0.25 μl/ml)	5119 ± 531	109%
3 CM + a–TGF-β (0.05 μl/ml)	4234 ± 773	90%
4 CM + a–TGF-β (0.025 μl/ml)	4316 ± 915	92%

* MCs were plated in 96 well plates (5 × 10^3 cells/well). After serum starvation, they were incubated with conditioned medium and various concentrations of neutralizing anti–TGF-beta for 48 hours. ^{3}H-thymidine was added during the last 18 hours of incubation (0.5 μCi/ml) and uptake was measured (see Materials and Methods).

† Conditioned medium derived from coculture of M-ECs and MCs with direct contact.

‡ Normal rabbit serum.

‖ Values were averaged from triplicate wells ± SD.

There are several reports on other cell systems in which cell–cell interactions induce modulation of cell growth. For example, the inhibition of cell growth with a plasma membrane preparation, similar to density-dependent growth inhibition, has been demonstrated in endothelial cells,[16] 3T3 cells,[17–19] hepatocytes,[20] and fibroblasts.[21] Heimark and Schwartz found that plasma membrane preparations from confluent ECs to actively inhibit growing ECs, but similar preparations from smooth muscle cells did not.[16] There may thus be an individual mechanism of modulation that is operative in combination with each specific cell type. Experimental data obtained using dye transfer indicate junctional communication between endothelial and smooth muscle cells,[22] or between microvascular pericytes and endothelial cells *in vitro*.[23]

To clarify the mechanism of modulation, examination was made of the effects of several conditioned media from day 1 to day 3 on mesangial cell proliferation. The results indicated a soluble modulator for mesangial cell proliferation dependent on physical contact (or close proximity) between pertinent cells and such a modulator may be associated, at least to some degree, with the initial phenomenon. Inhibitory factors for mesangial cell proliferation produced in the conditioned media of ECs such as heparinlike substances have been reported.[24] The humoral factor demonstrated in this study appears to be different from heparinlike substances because of the necessity for cell contact. An activated form of transforming-growth–factor beta (TGF-beta) was recently found to be produced by cocultures of endothelial cells and pericytes.[12] In that study, media conditioned by coculture of ECs and pericytes contained activated TGF-beta and mediated EC growth inhibition. Media conditioned by each of the cell types alone showed a latent form of TGF-beta. TGF-beta has been shown to be a bifunctional regulator of mouse mesangial cell proliferation.[25] In sparsely plated cells, TGF-beta diminished or ablated the proliferative response, but stimulated the proliferation of MCs cultured more densely. The proliferative response of culture MC to TGF-beta is complex. In our experiment, the inhibitory effect of CM from coculture with direct contact on MC proliferation was diminished with neutralizing anti-TGF–beta within a small dose range (from 2.5 μl/ml to 0.25 μl/ml). The reason is unknown. However, it seems that factors other than TGF-beta–modulating MC proliferation may exist in the conditioned medium used in our experiment. Cultured MCs have been shown to respond to several cytokines and other biologically active substances including eicosanoids and reactive oxygen metabolites.[26] TGF-beta remains a MC-growth modulator candidate under conditions of intercellular contact in combination with other factors. Further examination is required.

In the present study, there are many problems to be taken into consideration, such as differences in species and sources of endothelial cells.[27,28] Differences in structure and function have been found between ECs from large vessels and those from the microvasculature.[27] Ideally, to clarify tissue specific interactions between ECs and MCs, glomerular ECs should have been used. Since these cells have been difficult to obtain and maintain by other laboratories[24,29] and our own, human umbilical vein ECs were chosen in our study. Nonetheless this line of approach using coculture with or without cell contact may be expected to improve our understanding of the fundamental mechanisms regulating the growth of mesangial cells. We are pursuing the idea that the disturbance of the relationship between ECs and MCs is probably induced by cellular and humoral factor, eventually leading to glomerulosclerosis.

Acknowledgment

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References

1. Lovett DH, Ryan JL, Sterzel RB: Stimulation of rat mesangial cell proliferation by macrophage interleukin 1. J Immunol 1983, 131:2830–2836
2. Morioka T, Narita I, Shimizu F, Oite T: The production, by cultured human monocytes, of mesangial cell proliferation factor(s) differing from Interleukin 1 and Interleukin 6. Clin Exp Immunol 1990, 83:182–186
3. Narita I, Morioka T, Yoshida K, Shimuzu F, Arakawa M: Monocyte secretes factors that regulate glycosaminoglycan (GAG) synthesis by mesangial cells (MCs) *in vitro*. Kidney Int 1989, 35:357(abst.)
4. Ross R, Glomset J: The pathogenesis of atherosclerosis. N Engl J Med 1976, 295:369–420
5. Koo EWY, Gotlieb AI: Endothelial stimulation of intimal cell proliferation in a porcine aortic organ culture. Am J Pathol 1989, 134:497–503
6. Orlidge A, D'Amore PA: Inhibition of capillary endothelial cell growth by pericytes and smooth muscle cells. J Cell Biol 1987, 105:1455–1462
7. Jaffe EA, Nachman RL, Becker CG, Minick CR: Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. J Clin Invest 1973, 52:2745–2756
8. Morita T, Oite T, Kihara I, Yamamoto T, Hara M, Naka A, Ohno S: Culture of isolated glomeruli from normal and nephritic rabbits. I. Characterization of outgrowing cells. Acta Pathol Jpn 1980, 30:917–926
9. Sterzel RB, Lovett DH, Foelimer HG, Perfetto M, Biemesderfer D, Kashgarian M: Mesangial cell hillocks. Nodular Foci of exaggerated growth of cells and matrix in prolonged culture. Am J Pathol 1986, 125:130–140
10. Striker GE, Striker LJ: Glomerular cell culture. Lab Invest 1985, 2:122–131
11. Tomasz M, Chowdary D, Lipman R, Shimotakahara S, Veiro D, Walker V, Verdine GL: Reaction of DNA with chemically or enzymatically activated mitomycin C: Isolation and structure of the major covalent adduct. Proc Natl Acad Sci USA 1986, 83:6702–6706
12. Antonelli-Orlidge A, Saunders KB, Smith SR, D'Amore PA: An activated form of transforming growth factor B is produced by cocultures of endothelial cells and pericytes. Proc Natl Acad Sci USA 1989, 86:4544–454
13. Jozaki K, Marucha PT, Despins AW, Kreutzer DL: An *in vitro* model of cell migration: Evaluation of vascular endothelial cell migration. Anal Biochem 1990, 190:39–47
14. Voyta JC, Via DP, Butterfield CB, Zetter BR: Identification and isolation of endothelial cells based on their increased uptake of acetylated-low density lipoprotein. J Cell Biol 1984, 99:2034–2040
15. Kasahara Y, Ashihara Y: Colorimetry of angiotensin-1 converting enzyme activity in serum. Clin Chem 1981, 27:1922–1925
16. Heimark RL, Schwartz SM: The role of membrane-membrane interactions in the regulation of endothelial cell growth. J Cell Biol 1985, 100:1934–1940
17. Whittenberger B, Raben D, Lieberman MA, Glaser L: Inhibition of growth of 3T3 cells by extract of surface membranes. Proc Natl Acad Sci 1977, 74:2251–2255
18. Whittenberger B, Glaser L: Inhibition of DNA synthesis in culture of 3T3 cells by isolated surface membranes. Proc Natl Acad Sci USA 1978, 75:5457–5461
19. Vale RD, Peterson SW, Matiuck NV, Fox CF: Purified plasma membrane inhibit polypeptide growth factor-induced DNA synthesis in subconfluent 3T3 cells. J Cell Biol 1984, 98:1129–1132
20. Nakamura T, Yoshimoto K, Nakayama Y, Tomita Y, Ichihara A: Reciprocal modulation of growth and differentiated functions of mature rat hepatocytes in primary culture by cell-cell contact and cell membranes. Proc Natl Acad Sci USA 1983, 80:7229–7233
21. Wieser RJ, Oesch F: Contact inhibition of growth of human diploid fibroblasts by immobilized plasma membrane glycoproteins. J Cell Biol 1986, 103:361–367
22. Davies PF, Ganz P, Diehl PS: Reversible microcarrier-mediated junctional communication between endothelial and smooth muscle monolayers: An *in vitro* model of vascular cell interactions. Lab Invest 1985, 85:710–718
23. Larson DM, Carson MP, Haudenschild CC: Junctional transfer of small molecules in cultured bovine brain microvascular endothelial cells and pericytes. Microvasc Res 1987, 34:184–199
24. Castellot JJ, Hoover RL, Karnovsky MJ: Glomerular endothelial cells secrete a heparinlike inhibitor and a peptide stimulator of mesangial cell proliferation. Am J Pathol 1986, 125:493–500
25. MacKay K, Striker LJ, Stauffer JW, Doi T, Agodoa LY, Striker GE: Transforming growth factor-B; Murine glomerular receptors and responses of isolated glomerular cells. J Clin Invest 1989, 83:1160–1167
26. Lovett DH, Sterzel RB: Cell culture approaches to the analysis of glomerular inflammation. Kidney Int 1986, 30:246–254
27. Folkman J, Haudenschild CC, Zetter BR: Long-term culture of capillary endothelial cells. Proc Natl Acad Sci USA 1979, 76:5217–5221
28. Lau DCW, Wong KL, Tough SC: Regional differences in the replication rate of cultured rat microvascular endothelium from retroperitoneal and epididymal fat pads. Metabolism 1987, 36:631–636
29. MacKay K, Striker LJ, Elliot S, Pinkert CA, Brinster RL, Striker GE: Glomerular epithelial, mesangial, and endothelial cell lines from transgenic mice. Kidney Int 1988, 33:677–684