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LABORATORY INVESTIGATIONS

Combined Effect of Extracellular Matrices and Growth Factors on Bovine Corneal Endothelial Cells in Culture

Takuya Mitsumoto,*,† Tomohisa Nishimura,*
Shuji Toda,† Satoshi Okinami,* Shinji Oono* and Hajime Sugihara†

*Departments of *Ophthalmology, and*
†Department of Pathology, Saga Medical School, Saga, Japan

Purpose: First, to confirm that corneal endothelial cells in the confluent state have the capability to form cellular covering. Second, to establish a method to study the combined effect of extracellular matrices (ECMs) and growth factors on the biological response in corneal endothelial cells in culture.

Methods: Bovine corneal endothelial cells were cultured inside a cylinder set on a plastic dish. They formed a confluent cell nest on the dish coated with type I or type IV collagen, laminin, or fibronectin. After the removal of the cylinder, hepatocyte growth factor (HGF), epidermal growth factor, transforming growth factor-α or transforming growth factor-β_1 was added to the cultures. Each confluent cell nest enlarged outward, and its increased area size was measured. Cellular response in the nest, including cellular proliferation, was analyzed.

Results: The size of the increased area of the culture on type IV collagen plus HGF was the largest of all the combinations of ECMs and growth factors. The responses of component cells in the increased area consisted of cellular hypertrophy, proliferation, migration and giant cell formation. The treatment with type IV collagen plus HGF clearly promoted all the above responses.

Conclusions: The biological response of corneal endothelial cells was regulated by ECMs and growth factors. **Jpn J Ophthalmol 2001;45:115–124**

Key Words: Corneal endothelial cell, culture, extracellular matrix, growth factor.

Introduction

Corneal endothelial cells exist on Descemet's membrane, meaning that endothelial cells are in close contact with the components of the extracellular matrix (ECM), such as type IV collagen, laminin, etc.[1,2] The ECM plays an important role in the regulation of the biological response of cells, including adhesion, differentiation, proliferation and migration of many cell types,[3–5] including corneal endothelial cells.[6]

Furthermore, corneal endothelial cells in vivo are exposed to the aqueous humor. Growth factors, such as epidermal growth factor (EGF), hepatocyte growth factor (HGF), transforming growth factor-α (TGF-α) and -β_1 (TGF-β_1), have been detected in the aqueous humor or the corneal tissue.[7–10] The proliferation of corneal endothelial cells was promoted by EGF, HGF, TGF-α, and others.[10,11]

In other words, the biological responses of corneal endothelial cells, including wound healing, are affected by the cellular environment of the ECM and growth factors. To examine the combined effect of ECM and growth factors, our laboratory established an in vitro model. Corneal endothelial cells were cultured inside a cylinder on a plastic dish. They formed a confluent cell nest on the dish. After removal of the cylinder, the confluent cell nest expanded outward, and the size of the area was measured. This model, named "the outward growth method," enabled us to administer simultaneously an optional ECM and growth factor to the corneal endothelial cells.

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Correspondence and reprint requests to: Takuya MITSUMOTO, MD, Department of Ophthalmology, Saga Medical School, Nabeshima 5-1-1, Saga-Shi, Saga-Ken 849-8501, Japan

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Materials and Methods

Isolation of Bovine Corneal Endothelial Cells

Eyes from steers aged 3–4 years were enucleated in an abattoir immediately after slaughter and placed in tissue culture medium (Eagle's Minimum Essential Medium [MEM]; Nissui Pharmaceutical, Yuki, Ibaragi) supplemented with 40 mg/mL gentamicin and 5 mg/mL amphotericin B. The cornea was removed from the eyeball at the surgical limbus under sterile conditions. The full-thickness cornea was treated with 1.0×10^3 unit/mL protease solution (Dispase I, Goudoh-Shusei, Tokyo) at 37°C for 30 minutes.[12] Descemet's membrane, with the endothelium, was gently removed from the cornea, and placed into a 60-mm culture dish in MEM containing 10% fetal bovine serum (FBS) as a standard medium. Cultures were kept in a 5% CO_2 humidified atmosphere at 37°C. The standard medium was replaced at 3-day intervals. For several days, endothelial cells grew out from the explant. When the cells had covered 90% of the dish surface, they were rinsed twice with MEM, then rinsed in a mixture of 0.1% trypsin and 0.025% EDTA, and dispersed in a mixture of the same two substances for 5 minutes. In this way, endothelial cells from the primary culture were obtained and the following experiments were performed with these cells.

Conventional Monolayer Culture of Isolated Endothelial Cells

The freshly isolated adult bovine corneal endothelial cells formed a monolayer sheet in the culture. The culture cells ranged in size between 15 and 20 μm in diameter and were hexagonal in shape. These cells closely resembled the endothelial cells in the cornea in vivo. They were rinsed twice with MEM, then rinsed in a mixture of 0.1% trypsin and 0.025% EDTA, and dispersed in a mixture of the same two substances for 5 minutes. Then, 1.0×10^5 primary corneal endothelial cells were seeded and cultured on a 35-mm culture dish in the standard medium. Cultures were kept in a 5% CO_2 humidified atmosphere at 37°C. We defined the culture as a conventional monolayer culture in the present study. In this conventional monolayer culture, the bromodeoxy-uridine (BrdU) labeling index of endothelial cells was 83.7 ± 4.7% (mean ± SD) for 6 hours on culture day 2. Contaminating cells, such as fibroblasts or corneal epithelial cells, constituted less than 0.1% of all culture cells throughout this study.

Preparation of ECM-coated Culture Dish

The ECM-coated culture dishes were prepared using the following standard method, as described previously.[13] Briefly, type I or type IV collagen (Nitta Gelatin, Osaka) was diluted ×30 with 1 mM HCl to a final concentration of 100 μg/mL. Fibronectin or laminin (Nitta Gelatin) was diluted ×40 with distilled water to a final concentration of 12.5 μg/mL. One milliliter of each of these matrix solutions was placed on a culture dish 35 mm in diameter with 2-mm grids (inner size 1,925 μm and line width 75 μm) (Nalge Nunc International, Roskilde, Denmark) and kept at 25°C. After 1 hour, the solution was removed, and the dish was dried overnight at 25°C. A plastic uncoated dish with grids was used as the reference substratum in this study.

"The Outward Growth Method"

A sterilized cylinder 6 mm in diameter was set on each plastic dish with grids, or on the ECM-coated dish (Figure 1A). Inside the cylinder, 1.0×10^5 corneal endothelial cells were seeded and cultured (Figure 1B). When the cylinder was removed on culture day 5, a confluent cell nest was left on the culture dish (Figure 1C). We called this "the original nest" (Figure 1D), and continued to culture it. The cellular covering was observed with phase contrast microscopy. On each day after removal of the cylinder, the size of the nest was measured by the number of grids on the plate covered by the cells (Figures 1D and E). We defined "the increased area size" as the size of the expanding area outside the original nest.

Stimulation with Growth Factors

After the removal of the cylinder, 10 ng/mL of HGF (R&D Systems, Minneapolis, MN, USA), TGF-β_1 (R&D Systems), TGF-α (Becton Dickinson Labware, Bedford, MA, USA) or EGF (Upstate Biotechnology, Lake Placid, NY, USA) was added to the medium. The medium was replaced at 1-day intervals. Growth factors were also administered each day.

Examination of Cell Proliferation

Cell proliferation was examined by the BrdU labeling index. On day 6 after removal of the cylinder, BrdU (2 μL, 30 mg/mL) was added to the 2 mL of culture medium for 24 hours. Cells were fixed with acetic acid ethanol and immunostained with monoclonal anti-BrdU antibody by procedures for the BrdU kit (Cell Proliferation Kit, Amersham, Arling-

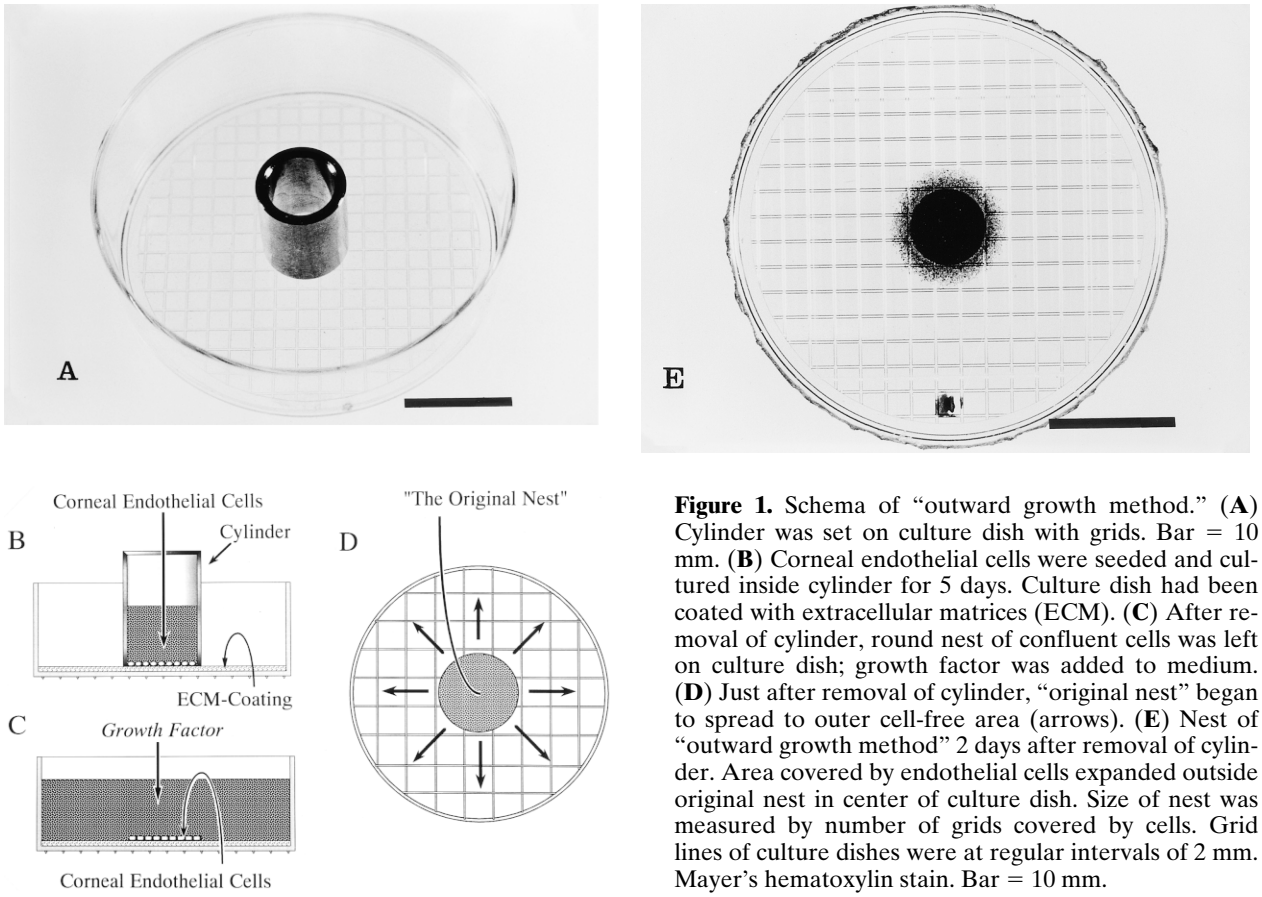

Figure 1. Schema of "outward growth method." (**A**) Cylinder was set on culture dish with grids. Bar = 10 mm. (**B**) Corneal endothelial cells were seeded and cultured inside cylinder for 5 days. Culture dish had been coated with extracellular matrices (ECM). (**C**) After removal of cylinder, round nest of confluent cells was left on culture dish; growth factor was added to medium. (**D**) Just after removal of cylinder, "original nest" began to spread to outer cell-free area (arrows). (**E**) Nest of "outward growth method" 2 days after removal of cylinder. Area covered by endothelial cells expanded outside original nest in center of culture dish. Size of nest was measured by number of grids covered by cells. Grid lines of culture dishes were at regular intervals of 2 mm. Mayer's hematoxylin stain. Bar = 10 mm.

ton Heights, IL, USA).[14] The BrdU antigen was visualized with 3,3′-diaminobenzidine tetrahydrochloride, and counterstaining of the nuclei was performed with Mayer's hematoxylin. To estimate the labeling index, a total of 1.0×10^3 cells in the peripheral part of the nest were counted, and the percentage of BrdU-positive nuclei was calculated.

Preparatory Experiments

Prior to investigating the combined effect of ECMs and growth factors, we carried out the following three experiments using the outward growth method. (1) To clarify the cellular response in the early stage of this outward growth, some cultures were fixed on day 3 after removal of the cylinder. In addition, BrdU was added to the culture medium for 24 hours on day 2 after removal of the cylinder. (2) To elucidate the effects of ECMs, endothelial cells were cultured on type I collagen-coated dishes, or type IV collagen-, laminin- or fibronectin-coated dishes using the outward growth method. (3) Under the conditions of the outward method, we administered 10 ng/mL of HGF or TGF-α to confirm the efficacy of each growth factor on the corneal endothelial cells in the plastic dishes.

Inhibition of Proliferation

To clarify the contribution of proliferation in the expanding area outside the original nest, we administered 4 μg/mL of aphidicolin (Wako Pure Chemical, Osaka) which inhibited proliferation of the cells.[5]

Results

Outward Growth Method

Within 5 days, endothelial cells grew to be confluent in a monolayer inside the cylinder. When the cylinder was removed, a round nest of confluent cells was left on the culture dish. We called this "the original nest." In the original nest, individual cells were a hexagonal shape, similar to endothelial cells in vivo. Thus, we regarded this shape as a morphological form of differentiated endothelial cells in vitro. Inside the cylinder, no significant difference in shape or density of the cells was observed among the type I

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collagen-coated, type IV collagen-, laminin-, or fibronectin-coated or plastic dishes. The reason for no significant difference in the cells inside the cylinder under ECM conditions was probably that endothelial cells seeded inside the cylinder were sufficient to attach and become confluent without further proliferation inside the cylinder.

The following results with type IV collagen coating represented typical results in the ECM substrata using the outward growth method. Within 12 hours after removal of the cylinder, individual cells at the leading edge of the original nest began to extend footprocesses from the cytoplasm (Figure 2). Other cells, which were several rows behind the edge, gradually enlarged their cytoplasm. Then, those cells demonstrated cellular hypertrophy, proliferation and migration in the peripheral part of the nest. Consequently, the nest spread to the outer cell-free area.

On day 3 after removal of the cylinder, the original peripheral part of the nest began to expand into two zones, which were morphologically distinct: an outer zone and an inner zone. In the outer zone, endothelial cells extensively proliferated. This zone was named "the hyperplastic zone" (Figure 3). In the inner zone, most endothelial cells were obviously hypertrophic. This zone was named "the hypertrophic zone." In the hypertrophic zone, cells were uniform in size and shape, and ranged in size from 20–30 μm in diameter (Figure 4). Three days after the last morphological examination, the inner area of the hyperplastic zone appeared to change into a hypertrophic zone, and cells inside the hypertrophic zone changed morphologically into a hexagonal shape, similar to endothelial cells in vivo. Outside the hyperplastic zone, many endothelial cells extended footprocesses and moved toward the cell-free area. They were apart from the edge of the nest (Figure 5); so we regarded their shapes as morphological forms of migrating cells.[15] At the edge of the nest, multinuclear cells with large cytoplasm occasionally appeared (Figure 6). These cells were more than five times larger than cells at the central part of the nest and

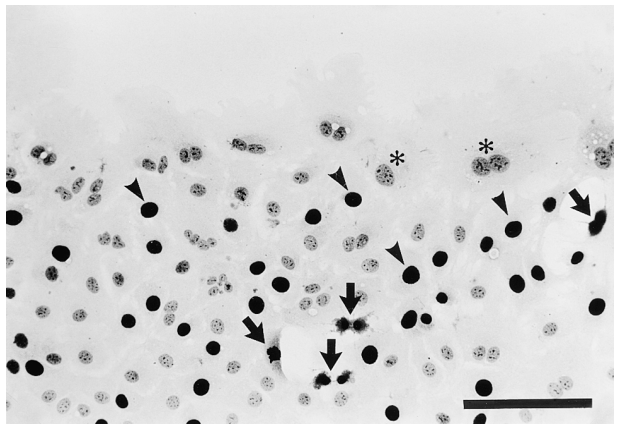

Figure 3. Hyperplastic zone 3 days after removal of cylinder. Peripheral part of nest is on upper side in this figure. In hyperplastic zone, endothelial cells extensively proliferated. These cells were immunostained with anti-bromodeoxy-uridine (BrdU) antibody. Mitotic cells (arrows) and BrdU-positive cells (arrowheads). BrdU-negative cells (*) were counterstained with Mayer's hematoxylin. Type IV collagen coating, no growth factor. Phase contrast figure. Bar = 100 μm.

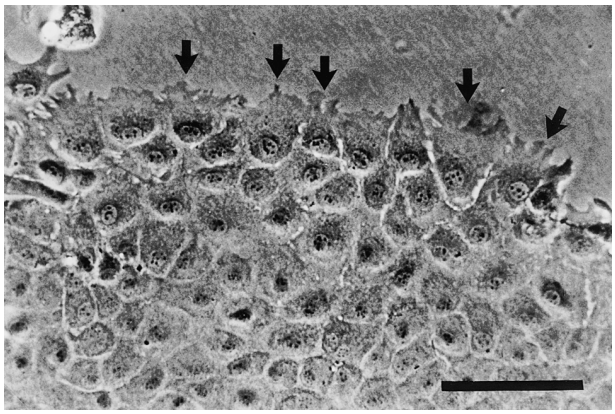

Figure 2. Peripheral part of nest 12 hours after removal of cylinder. Individual cells at leading edge of original nest began to extend foot processes from cytoplasm (arrows). Type IV collagen coating, no growth factor. Phase contrast figure. Bar = 100 μm.

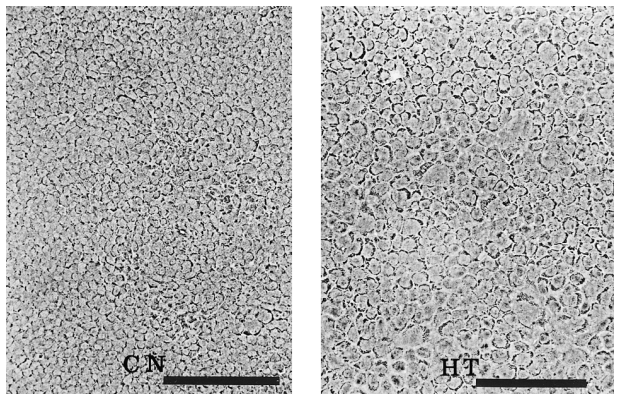

Figure 4. Left: Central part of nest (CN). Right: Hypertrophic zone (HT) 3 days after removal of cylinder. In the CN, where it corresponds to "original nest," cells are hexagonal in shape. In the hypertrophic zone (HT), cells are uniform in size and shape, and 20–30 μm in diameter. Type IV collagen coating, no growth factor. Phase contrast figure. Bar = 200 μm.

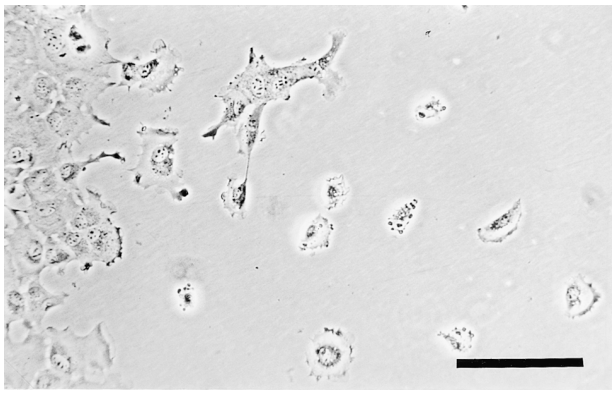

Figure 5. Six days after removal of cylinder, migrating cells originating from corneal endothelial cells were apart from edge of nest. These cells extended foot processes and moved toward cell-free area. Type IV collagen coating, no growth factor. Phase contrast figure. Bar = 100 μm.

were rarely more than 200 μm in diameter. We called these unusual cells "giant cells." They had at least two nuclei or one large nucleus, and sometimes they had more than 10 nuclei. The giant cells did not seem to revert to typical endothelial cells, which were a hexagonal shape throughout this study. In the central part of the nest and the hypertrophic zone, these giant cells were not observed.

On day 7 after removal of the cylinder, the hyperplastic zone and the hypertrophic zone were about 5% and 40% of the entire nest area, respectively (Figure 7).

As a result, the cellular response of the covering consisted of cellular hypertrophy, proliferation, migration, and giant cell formation. These phenomena appeared in the peripheral part of the nest. In contrast, cells in the central part of the nest maintained their shape and cell density throughout this culture.

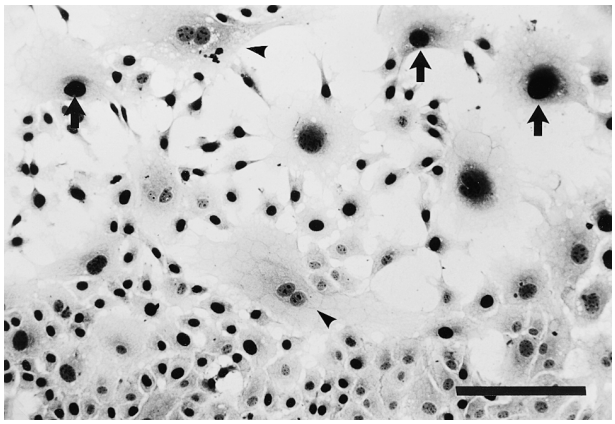

Figure 6. Giant cells originating from corneal endothelial cells were immunostained with anti-bromodeoxy-uridine (BrdU) antibody (arrows). Note BrdU-positive giant cells (arrowheads). Type IV collagen coating, no growth factor. Micrograph. Bar = 100 μm.

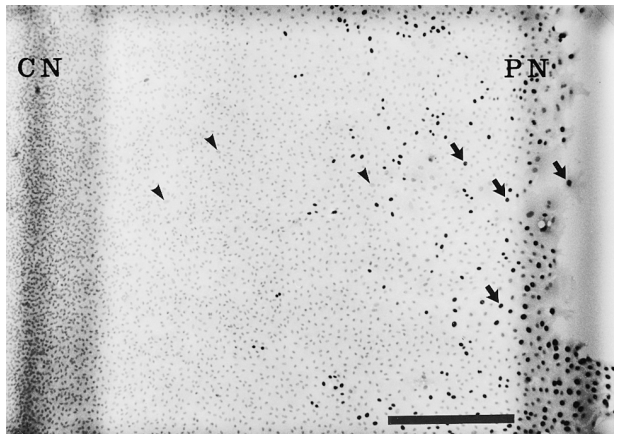

Figure 7. Examination of proliferative ability of endothelial cells by "outward growth method" on day 7 after removal of cylinder. In this figure, central part of nest (CN) is on left side, and peripheral part of nest (PN) is on right. Nuclei of endothelial cells were positively immunostained with anti-bromodeoxy-uridine (BrdU) antibody (arrows), and counterstained with Mayer's hematoxylin (arrowheads). BrdU-positive cells gradually increased toward edge of nest. Dark shadows are grids of dish. Type IV collagen coating, no growth factor. Micrograph. Bar = 500 μm.

Effects of ECMs

Experiments with ECMs were performed several times. On day 7 after removal of the cylinder, the increased area size with the type IV collagen-coating was the largest, 92.4 ± 7.3 mm^2 (mean ± SD); followed by the type I collagen-coating, 80.3 ± 12.3 mm^2; fibronectin-coating, 51.7 ± 5.3 mm^2; laminin-coating, 44.1 ± 5.8 mm^2; and the plastic dish, 33.1 ± 2.1 mm^2. These results are represented graphically in Figure 9. The experiments showed strikingly similar results. It was difficult, however, to get the average for the data among the experiments, because each experiment was independent from the others. On the BrdU labeling index, the fibronectin-coating was the highest, 79.3 ± 2.7% (mean ± SD); followed by laminin, 72.7 ± 11.9%; the plastic dish, 70.9 ± 4.5%; type I collagen, 64.6 ± 5.3%; and type IV collagen, 60.9 ± 5.3%. The number of migrating cells greatly increased under type I collagen-coating conditions. The number of giant cells slightly increased under type IV collagen-coating conditions (Table 1, Figures 8A–E).

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Table 1. Effect of Extracellular Matrices (ECMs)

ECM	BrdU Labeling Index (%)*	Number of Cells†		Increased Area Size (mm^2)‡
		Migrating Cells	Giant Cells	
Type I collagen	64.6 ± 5.3	62.6 (46–82)	5.6 (3–7)	80.3 ± 12.3
Type IV collagen	60.9 ± 5.3	5.0 (0–16)	19.5 (10–29)	92.4 ± 7.3
Fibronectin	79.3 ± 2.7	18.9 (1–47)	6.5 (4–10)	51.7 ± 5.3
Laminin	72.7 ± 11.9	26.3 (12–64)	5.2 (1–11)	44.1 ± 5.8
(−)	70.9 ± 4.5	26.3 (10–41)	8.2 (0–13)	33.1 ± 2.1

Effect of ECMs on day 7 after removal of cylinder.
*BrdU: Bromodeoxyuridine values are mean percentage ± SD.
†Values are mean number with minimum–maximum cells per grid in parentheses.
‡Values are size of entire nest minus size of original nest (mean increased area size ± SD).
(−) Plastic dish (control).

Effects of Growth Factors

Experiments with growth factors were performed several times. On day 7 after the removal of the cylinder, the increased area size with the HGF treatment was the largest, 78.4 ± 9.8 mm^2 (mean ± SD); followed by TGF-α treatment, 76.1 ± 9.1 mm^2; and without any growth factor, 33.1 ± 2.1 mm^2. These results are represented graphically in Figure 10. On the BrdU labeling index, HGF was the highest, 92.5 ± 2.3% (mean ± SD); followed by TGF-α, 82.2 ± 5.2%; and without any growth factor, 70.9 ± 4.5%. HGF and TGF-α obviously promoted the proliferation of corneal endothelial cells. The number of migrating cells and giant cells increased with the HGF treatment (Table 2, Figures 8A, F, and G).

Combined Effect of ECMs Plus Growth Factors

Because type IV collagen best promoted the enlargement of the nest in the prior experiments with ECMs, type IV collagen was mainly used to represent ECM in the following experiments with ECM plus growth factors. In addition, investigations of the combined effect with type I collagen, fibronectin and laminin were also performed in the same way (data not shown).

Experiments with ECMs plus growth factors were performed several times. On day 7 after the removal of the cylinder, among all combinations of ECMs and growth factors in this study, the increased area size with the type IV collagen coating plus HGF treatment was the largest, 221.2 ± 17.3 mm^2 (mean ± SD); followed by the type IV collagen plus TGF-α, 192.4 ± 9.4 mm^2; the type IV collagen plus EGF, 189.4 ± 24.0 mm^2; the type IV collagen without any growth factor, 92.4 ± 7.3 mm^2; and the type IV collagen plus TGF-β_1 treatment, 29.6 ± 1.3 mm^2. On the BrdU labeling index, the type IV collagen coating plus HGF treatment was the highest, 83.2 ± 2.3% (mean ± SD); followed by the type IV collagen plus TGF-α, 73.5 ± 8.2%; the type IV collagen plus EGF, 68.4 ± 3.6%; the type IV collagen without any growth factor, 60.9 ± 5.3%; and the type IV collagen plus TGF-β_1 treatment, 4.0 ± 2.4% (Table 3, Figures 8C, H, I, J, and K). These results are represented graphically in Figure 11.

The number of migrating cells increased greatly, and giant cells obviously increased with type IV collagen plus HGF. The labeling index was the highest with type IV collagen plus HGF. In the nest of type IV collagen plus HGF, the hypertrophic zone obviously expanded. Because the BrdU-positive cells were observed everywhere in the nest treated with TGF-α except for the central area of the nest, we could not distinguish whether they were in the hypertrophic zone or the hyperplastic zone.

On the other hand, the nest treated with TGF-β_1 had very narrow hypertrophic and hyperplastic zones. Cells treated with TGF-β_1, interestingly, changed into a spindle shape and appeared to overlap one another in the central area. The BrdU labeling index of the spindle cells was obviously higher (19.8%) than all the other conditions.

Treatment with Inhibitor of Proliferation

The aphidicolin-treated cells scarcely proliferated; the BrdU labeling indices in the entire nest were less than 1% for 24 hours. The size of the expanding area was reduced by half with the aphidicolin treatment; that is, cellular proliferation was estimated to represent about 50% of the increase in the size of the nest.

Discussion

Our model is based on the anatomical environment of the corneal endothelium in the in vivo cornea. That is, (1) corneal endothelial cells exist on Descemet's membrane in vivo, in close contact with

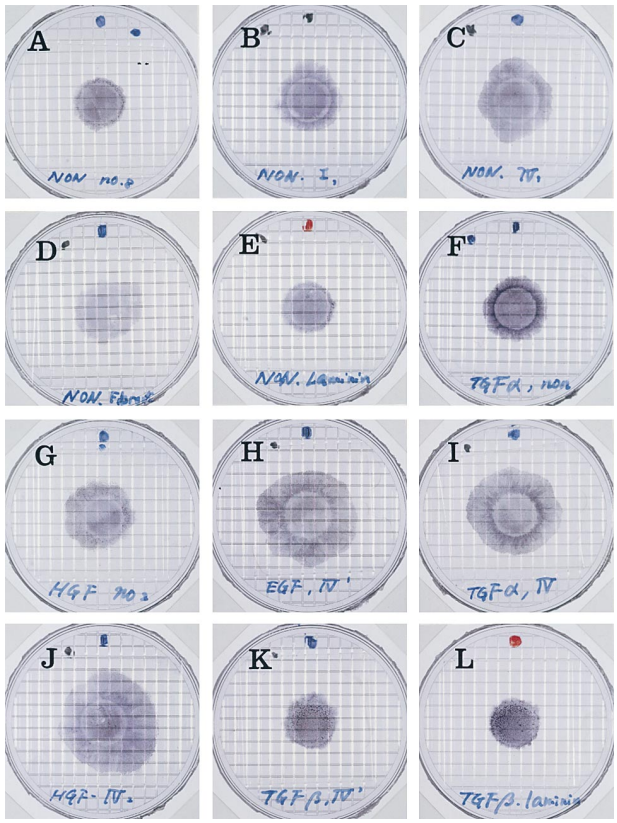

Figure 8. Typical effect of extracellular matrices and growth factors in our experiments 7 days after removal of cylinder. (**A**) Plastic dish. (**B**) Type I collagen coating. (**C**) Type IV collagen coating. (**D**) Fibronectin coating. (**E**) Laminin coating. (**F**) Transforming growth factor (TGF)-α treatment. (**G**) Hepatocyte growth factor (HGF) treatment. (**H**) Type IV collagen coating plus epidermal growth factor (EGF) treatment. (**I**) Type IV collagen coating plus TGF-α treatment. (**J**) Type IV collagen coating plus HGF treatment. (**K**) Type IV collagen coating plus TGF-β_1 treatment. (**L**) Laminin coating plus TGF-β_1 treatment. Size of increased area with type IV collagen coating plus HGF treatment (**J**) is largest. Size of increased area with laminin coating plus TGF-β_1 treatment (**L**) is smallest. (**A**)–(**L**), Mayer's hematoxylin stain. Macrograph. Original magnification ×0.5.

the ECM. In our model, endothelial cells exist on the ECM, as in vivo. (2) Furthermore, corneal endothelial cells in vivo are exposed to the aqueous humor. Growth factors, such as EGF, HGF, TGF-α and TGF-β_1, have been detected in the aqueous humor or the corneal tissue. In our model, HGF, EGF, TGF-α or TGF-β_1 was added to a standard culture medium. (3) The biological responses of corneal endothelial cells, including wound healing, are affected by the cellular environment of the ECM and growth factors. Our model enabled us to examine the effects of simultaneous administration of ECMs and growth

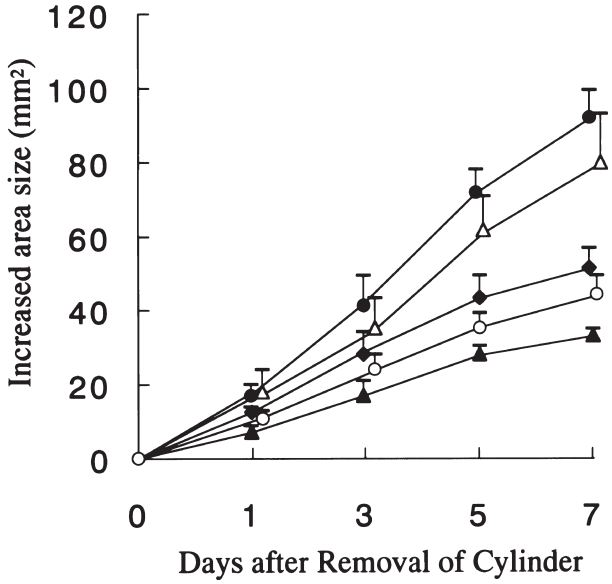

Figure 9. Effects of extracellular matrices: ●: Type IV collagen coating, △: type I collagen coating, ◆: fibronectin coating, ○: laminin coating, ▲: plastic dish. We defined size of increased area as size of expanding area outside original nest. Size of nest after cell migration minus size of original nest equaled size of increased area.

factors. Using this method, we demonstrated that the cellular covering in vitro is involved in cellular hypertrophy, proliferation, migration, and giant cell formation. The size of the expanding area was re-

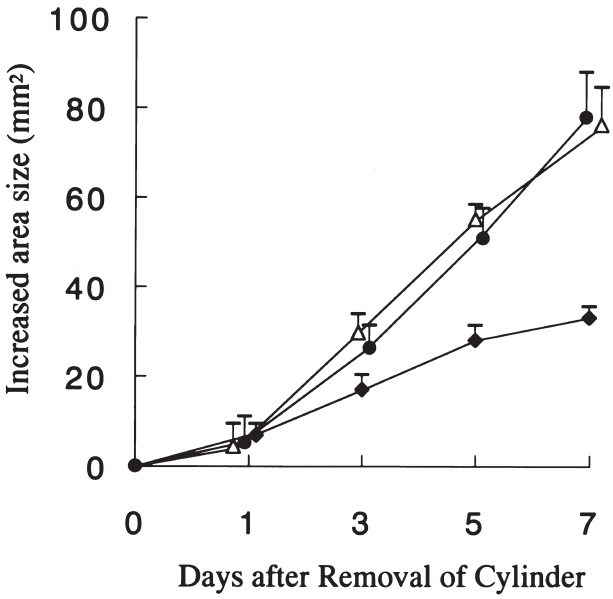

Figure 10. Effects of growth factors. ●: Hepatocyte growth factor treatment, △: transforming growth factor-α treatment, ◆: no growth factor. Definition of increased area size is same as in Figure 9.

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Table 2. Effect of Growth Factors*

Growth Factor†	BrdU Labeling Index (%)‡	Number of Cells§		Increased Area Size (mm^2)‖
		Migrating Cells	Giant Cells	
TGF-α	82.2 ± 5.2	16.1 (7–29)	4.6 (2–9)	76.1 ± 9.1
HGF	92.5 ± 2.3	43.8 (15–86)	12.0 (5–21)	78.4 ± 9.8
(−)	70.9 ± 4.5	26.3 (10–41)	8.2 (0–13)	33.1 ± 2.1

*Combined effect of growth factors on day 7 after removal of cylinder.
†TFG-α: Transforming growth factor-α, HGF, hepatocyte growth factor, (−): no growth factor (control).
‡BrdU: Bromodeoxyuridine. Values are mean percentage ± SD.
§Values are mean number with minimum–maximum cells per grid in parentheses.
‖Values are size of entire nest minus size of original nest (mean increased area size ± SD).

duced by half in the mitotically inhibited cultures. Our results showed that the simultaneous administration of ECMs and growth factors greatly affected the cellular covering.

Endothelial wound healing with "the wound closure model" has already been investigated in detail.[16–27] Proliferation, migration, and elongation of the cell shape were described. Joyce and Meklir[18] described the movement of migrating cells into the cell-free area as "spreading." Fukami et al[16] observed migration, elongation, coalescence and mitosis during the healing process. The cellular hypertrophy in our study was probably the same phenomenon as the elongation in their reports. The giant cell was also reported as the multinuclear cell in other studies.[21,25,26] It was considered that the multinuclear cell resulted from incomplete mitosis[28,29] amitotic division,[30–34] or cell fusion.[32,35–37] It seems more plausible that the nucleus repeatedly divided without cytokinesis. Interestingly, the giant cells always appeared in the peripheral part of the nest in the present study. As compared with wound closure models, our method does not require the mechanical denudation of the endothelial cells. Therefore, with our model it is possible to retain the ECM-coating on the dish. At the removal of the cylinder, the ECM-coating outside cylinder has never covered by endothelial cells. Our results were unaffected by any residual ECM resulting from denuded endothelial cells with the mechanical denudation model.

In this study, we gave great attention to the increased area size, because this encompassed the other parameters, such as proliferation and migration. Consequently, the simultaneous administration of type IV collagen plus HGF greatly affected the size of the increased area. Wilson et al detected messenger RNA coding for HGF and HGF receptor in human corneal endothelial cells.[10] The presence of HGF has been detected in the aqueous humor.[9] In this study, the number of migrating corneal endothelial cells increased with HGF treatment. On the other hand, type IV collagen has been identified in Descemet's membrane.[1,2] Type IV collagen promoted proliferation and migration in the present study: The size of the increased area with the type IV collagen coating was larger than that with HGF alone. In this study, the increased area size with type IV collagen coating plus HGF treatment was the largest. However, the BrdU labeling index of type IV collagen plus HGF was lower than that with HGF

Table 3. Combined Effect of Extracellular Matrices (ECMs) Plus Growth Factors*

ECM	Growth Factor†	BrdU Labeling Index (%)‡	Number of Cells§		Increased Area Size (mm^2)‖
			Migrating Cells	Giant Cells	
Type IV collagen	EGF	68.4 ± 3.6	5.8 (3–8)	12.6 (11–17)	189.4 ± 24.0
Type IV collagen	TGF-α	73.5 ± 8.2	7.8 (3–18)	11.5 (0–47)	192.4 ± 9.4
Type IV collagen	HGF	83.2 ± 2.3	73.4 (5–126)	21.0 (15–36)	221.2 ± 17.3
Type IV collagen	TGF-β1	4.0 ± 2.4	22.8 (5–42)	6.2 (3–11)	29.6 ± 1.3
Type IV collagen	(−)	60.9 ± 5.3	5.0 (0–16)	19.5 (10–29)	92.4 ± 7.3

*Combined effect of ECMs plus growth factors on day 7 after removal of cylinder.
†EGF: Epidermal growth factor, TGF-α: transforming growth factor-α, HGF: hepatocyte growth factor, (−): no growth factor (control).
‡BrdU: Bromodeoxyuridine. Values are mean percentage ± SD.
§Values are mean number with minimum–maximum cells per grid in parentheses.
‖Values are size of entire nest minus size of original nest (mean increased area size ± SD).

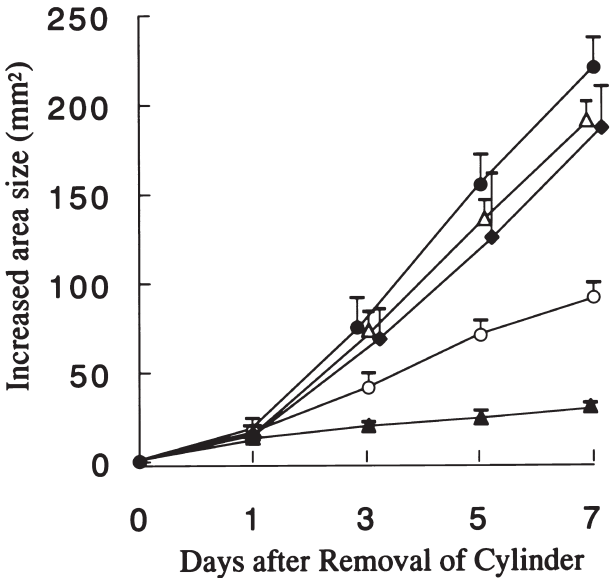

Figure 11. Combined effect of extracellular matrices and growth factor. ●: Type IV collagen coating plus hepatocyte growth factor treatment, △: type IV collagen coating plus transforming growth factor (TGF)-α treatment, ◆: type IV collagen coating plus epidermal growth factor treatment, ○: type IV collagen coating without any growth factor, ▲: type IV collagen coating plus TGF-β_1 treatment. Definition of increased area size is same as in Figure 9.

alone. These results suggest that cellular hypertrophy had priority over proliferation in the process of covering the corneal endothelial cells. The increased area size with only type IV collagen coating plus HGF was greater than the sum of the increased area sizes with type IV collagen and HGF separately. This result indicated how crucial combined effect of ECM and growth factors.

Conceivably, the subendothelial ECM preserved the growth factor molecules produced by endothelial cells, keratocytes, and so on, and maintained a high concentration of growth factors around the endothelial cells. The growth factors, which were trapped and condensed in the ECM, greatly affected endothelial wound healing through the so-called "matricrine" mechanism. Vlodavsky et al[38] suggested that corneal endothelial cells stored growth factors capable of autocrine growth promotion in two ways: by sequestering growth factor within the cell and by incorporating it into the underlying ECM.

In conclusion, we created an in vitro model of the cellular covering of the corneal endothelium. Using this method, we demonstrated that the cellular covering in vitro is involved in cellular hypertrophy, proliferation, migration, and giant cell formation. In particular, proliferation was the most effective response in the cellular covering process in vitro. This process was promoted by simultaneous treatment with ECMs plus growth factors. Type IV collagen plus HGF was particularly effective in this study. Our results indicated the crucial combined effect of ECM and growth factor in the cellular covering of the corneal endothelium. It is generally considered that human endothelial cells do not divide after birth. So, the wound healing processes for animal and man are probably different. The authors recognize the limitations of this study in clarifying the corneal wound healing process. However, we believe our method is useful for studying the cellular covering mechanism of the corneal endothelium.

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