Formation of Capillary-Like Tubes by Vascular Endothelial Cells Cocultivated With Keratocytes

Kiyoo Nakayasu,* Nobuhiro Hayashi,* Shigekuni Okisaka,* and Noboru Sato†

This report describes an in vitro system that could be useful for investigating corneal neovascularization. When isolated bovine capillary endothelial cells (CEC) were cocultivated with rabbit corneal keratocytes, capillary-like cords extended actively from the endothelial cells in the multilayers of the keratocytes. The developing cords continued to elongate, branch out, and anastomose with each other, forming a capillary-like network by the 12th day of coculturing. Electron microscopic observation revealed that the cords were tubular structures composed of three to seven endothelial cells in the multilayers of keratocytes, and that a basal lamina-like matrix was situated at the abluminal face of the endothelial cells. The growth of cellular cords from the cloned CEC also occurred when CEC were seeded onto a cell-free extracellular matrix that had been synthesized by keratocytes. Keratocyte-conditioned medium alone did not stimulate proliferation of CEC. These observations clearly indicate that the formation of capillary-like structures by CEC depended upon the presence of an extracellular matrix produced by keratocytes, rather than upon the keratocytes themselves or any other keratocyte byproduct. This simple in vitro experimental system is proposed as a useful tool for studying corneal neovascularization. Invest Ophthlamol Vis Sci 33:3050–3057, 1992

The absence of blood vessels in the normal cornea contributes to its transparency. However, under certain pathologic conditions, capillaries extend into corneal tissue from the limbal vascular plexus, compromising corneal transparency. Pathologic neovascularization of the cornea has been studied by many investigators, and several contributing factors have been proposed. Still, the contribution of corneal cellular components or the extracellular matrix to the regulation of neovascularization is unclear and requires further study.

The formation of capillary-like tubular structures has been reported in cultures of endothelial cells from various kinds of blood vessels, eg, bovine capillaries,1-2 bovine aortas,3 human umbilical veins,4 and rat capillaries.5,6 However, lack of a simple in vitro experimental system significantly hampers further elucidation of corneal neovascularization mechanisms. The purpose of this report is to explore the possibility that a combination of capillary endothelial cells (CEC) and factors produced by corneal keratocytes could be a simple and useful tool for studying corneal neovascularization.

Materials and Methods

All investigations described in this report conformed to the ARVO Resolution on the Use of Animals in Research.

Preparation of Keratocytes and Capillary Endothelial Cells

Seven adult New Zealand albino rabbits, weighing 2–3 kg each, were killed by an intravenous overdose of pentobarbital sodium solution (Abbott Labs., North Chicago, IL). The eyes were propsected and the corneas were separated from the globes by a circumferential incision 1 mm within the limbus. In each case, the epithelium and Descemet’s membrane with corneal endothelium attached were removed with a razor blade and forceps. The remaining stroma, containing keratocytes, was cut into 2 mm cubes and the tissue pieces were placed in 35 mm culture plates (Sumitomo Co., Osaka, Japan) containing Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Grand Island, NY) and 10% fetal bovine serum (FBS; Gibco) supplemented with penicillin/streptomycin, 100 U/ml (Flow Labs., Irvine, Scotland). Keratocytes migrated from the explants and multiplied to give confluent cultures in about 2 wk.

For the secondary culture of keratocytes, the cells were harvested with 0.25% trypsin-0.05% EDTA (Difco, Detroit, MI) in modified Hank’s balanced salt solution without sodium bicarbonate, calcium ions, and magnesium ions. The cells were subcultured at a

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1:4 split ratio and maintained in DMEM plus 10% FBS at 37°C in a humidified atmosphere of 5% CO₂/95% air for about 3 wk. The culture medium was changed every 3 days.

Capillary endothelial cells (CEC) were prepared from bovine adrenal cortex by the method of Folkman et al. and used at the 10th through 14th passage. The cells from the capillary (Fig. 1) were identified as endothelial cells by morphologic features and by positive immunoperoxidase staining for factor VIII-related antigen using a Stravigen biotin-streptavidin kit (Biogenex Labs., San Ramon, CA). CEC also were cultivated in DMEM supplemented with 10% FBS.

Preparation of Dishes Coated With an Extracellular Matrix of Keratocytes

Secondary cultured keratocytes, which had been maintained in the medium for about 2 wk, were cultured for an additional 3 wk. The cell layer then was dissolved by exposure to 0.5% triton X-100 in Dulbecco’s phosphate buffered saline (DPBS) for 2 min at room temperature, leaving the underlying extracellular matrix. Remaining nuclei and cytoskeletons were removed by a 2-3 min exposure to 0.025N NH₄OH, followed by four washes with DPBS.

Preparation of Dishes Coated With Type I Collagen Gel Matrix

Seven volumes of 0.3% type I collagen in HCl solution (pH 3.0, cellmatrix type I-A; Nitta Gelatin Co., Osaka, Japan) were rapidly mixed with 2 volumes of 5× DMEM and 1 volume of 0.08 N NaOH and HEPES (47.7 mg/ml) in a sterile flask kept in ice to prevent immediate gelatin. The cold mixture then was dispensed into 24-well plastic culture dishes (Falcon Plastics, Oxnard, CA) and allowed to gel for 15 min at 37°C.

Coculturing

Three × 10⁴ of the cultured bovine CEC at passage 10-14 were seeded onto keratocytes that had been secondarily cultured in 35 mm dishes for about 3 wk. The combination was cultured an additional 12 days in an atmosphere of 5% CO₂/95% air. The same number of CEC also was cultured for 12 days in the dishes coated with the extracellular matrix of keratocytes and in dishes with type I collagen gel matrix. The cultured CEC were observed daily under a phase contrast microscope (Nikon, Tokyo, Japan). DMEM plus 10% FBS was used as the medium for these cultures. The medium was changed every 3 days.

Preparation of Samples for Electron Microscopy

After the cocultured cells were washed three times with a 0.1 mol phosphate buffer (pH 7.4), they were prefixed with a mixture of 1% glutaraldehyde and 4% formaldehyde in the phosphate buffer for 3 hr and rinsed three times with the same buffer. They were postfixed with 1% osmium tetroxide in the phosphate buffer. Fixed materials were dehydrated in a graded ethanol series and embedded in epoxy resin. After trimming, ultrathin sections were double-stained with uranyl acetate and lead citrate and examined on a JEM 100C electron microscope (Nihon Densi, Tokyo, Japan).

Staining for Factor VIII-Related Antigen

CEC cocultured with keratocytes were stained on Lab-Tek chamber slides for tissue culture (Nunc Inc., Naperville, IL). Cells on the slides were fixed with 3% formaldehyde for 5 min at 4°C, washed in DPBS, and incubated with 0.1% pepsin (2900 U/mg; Sigma, St. Louis, MO) in 0.01 N HCl for 10 min at 37°C. The fixed cells then were stained with factor VIII-related monoclonal antigen using a Stravigen biotin-streptavidin kit (Biogenex). After rinsing in DPBS, the slides were mounted with 30% polyvinylpyrrolidone solution (Sigma) and photographed through a photomicroscope (Wild Leitz, Germany).

Preparation of the Medium Conditioned to Rabbit Keratocytes

Media (2 ml/35 mm dish) conditioned by keratocytes that had migrated from the explants of rabbit corneal stroma were collected on the 16th and 18th days of culturing. Cell debris was separated from the conditioned medium by centrifugation at 2000 × g for 5 min. The population of keratocytes in dishes...
Measurement of Cell Number and DNA Synthesis of Bovine CEC

The effect of medium conditioned to rabbit corneal keratocytes on the proliferation of bovine CEC was examined. Bovine CEC at passage 14 were plated in 35 mm culture dishes (Sumitomo) at densities of $4 \times 10^4$ cells/dish. After 24 hr of cultivation in DMEM supplemented with 10% FBS, the medium was replaced with 2 ml of fresh medium with or without 20% of the keratocyte-conditioned medium. At day 5, the medium was removed and the culture dishes were rinsed twice with PBS (pH 7.4). The cells were harvested by trypsinization with 650 μl of 0.25% trypsin-0.02% EDTA Hank’s solution, and the number of cells was counted in 50 μl of cell suspension by a hemocytometer plate. The remaining cells, in 600 μl of trypsin-EDTA Hank’s solution, were divided into two 96-well black microtiter fluoroplates (Flow Labs). DNA fluorometric assay was performed by the method of Richards et al.8 To each well, 100 μl of serum-free DMEM containing 10μg/ml of Hoechst 33342 (Polysciences, Warrington, PA) was added. The cells were incubated for 45 min at 38°C, and the number of fluorescent units per well was measured by a Titertek Fluoroscan (Flow Labs.) equipped with a 365 nm filter for the excitation beam and a 450 nm filter for the emitted light.

Results

Culture of Bovine CEC

When bovine CEC were seeded onto rabbit corneal keratocytes that already had been cultured for about 3 wk and were spindle in shape, the CEC attached to the confluent layer or layers of keratocytes within 1 hr (Fig. 2A). Within 4 days, the seeded CEC had disappeared and long cellular cords extending over or into the underlying keratocyte cell layer were observed (Fig. 2B). The developing cords sometimes crossed over others and continued to elongate, branch out, and anastomose with each other, forming a capillary-like network by the 12th day (Fig. 2C). In contrast, CEC that had been cultured alone on nontreated culture plates grew to confluence and formed a typical monolayer without indication of cellular cords during this experiment (Fig. 3). Furthermore, CEC cultured on type I collagen gel became slightly elongated in shape but did not form cellular cords (Fig. 4). On the other hand, the development of capillary networks was observed in a culture dish coating an extracellular matrix produced by keratocytes (Fig. 5).

Electron Microscopic Observation of Cellular Cords

Viewed through the electron microscope, the cellular cords on the 12th day of coculturing CEC and keratocytes had formed a tubular structure in the multilayered keratocytes. The lumina usually were
composed of three to seven cells joined by intercellular junctions. Although Weibel-Palade bodies were not detected, the existence of micropinocytotic vesicles, tight junction-like structures, a basal lamina-like matrix, and associations of cells encircling lumina provided criteria sufficient to characterize the cord constituents as endothelial cells. A basal lamina-like matrix was observed at the abluminal face of the endothelial cells. On the other hand, the multilayered cells surrounding the capillary cord were spindle shaped and contained a well-developed granular endoplasmic reticulum and Golgi complexes (Fig. 6).

Detection of a Factor VIII-Related Antigen

To clarify the nature of cord-forming cells in this culture, a factor VIII-related antigen was stained using a Streptigen biotin-streptavidin system. Positive staining was weakly detected along cord-constituent cells on day 12 of culture, whereas keratocytes beneath the cellular cords were not stained at all (Fig. 7).

Effects of Keratocyte Conditioned Medium on Proliferation of CEC

Keratocyte-conditioned medium by itself failed to significantly affect proliferation of CEC. The number of CEC increased to 115% of the control value, and fluorometric DNA assay, using Hoechst 33342, showed a 5% increase compared to that of cells treated with unconditioned medium (Fig. 8). However, the differences were not statistically significant.

Discussion

Capillary-like cellular cords developed in vitro when cloned capillary endothelial cells, even after repeated passages in culture, were cocultivated with corneal keratocytes (Fig. 2). Electron microscopic examination and immunohistochemical staining for a factor VIII-related antigen revealed that the cells forming capillary-like cords were characteristic of vascular endothelial cells and had the correct cellular polarization (Fig. 6). Moreover, it is obvious that cultured CEC seeded on keratocytes invaded the multilayers of corneal keratocytes and proliferated, differentiated, and organized capillary-like cellular cords. However, when CEC were plated onto nontreated plastic culture dishes without keratocytes, they proliferated to form a confluent monolayer but did not organize into capillary cords (Fig. 3).

These findings suggest that corneal keratocytes play an important role in cord formation by CEC in vitro. What that role might be is unclear, but two possibili-
ties have been suggested: (1) keratocytes may synthesize and secrete some factors stimulating the proliferation, migration, and differentiation of CEC; and (2) the extracellular matrix synthesized by keratocytes may determine the endothelial phenotype and promote its growth and development. The first possibility is supported by earlier studies that demonstrated the stimulatory effects of medium conditioned to skin fibroblasts or to corneal epithelium on vascular endothelial cells, although direct corneal keratocyte participation in the growth of vascular endothelial cells has not been found in previous studies. More recent studies, by Baudouin et al. and Soubrane et al., demonstrated that neither acidic nor basic fibroblast growth factors were distributed in corneal keratocytes. Noji and colleagues also reported that mRNA expressions of acidic and basic fibroblast growth factors were not detected in rat keratocytes by in situ hybridization. Finally, in the present study, corneal keratocyte-conditioned medium did not significantly stimulate proliferation and DNA synthesis of bovine CEC (Fig. 8). Thus, there seems to be little possibility that keratocytes synthesize and secrete some special angiogenic factors, including fibroblast growth factors.

Nevertheless, results in the present study indicated that capillary-like cords could develop when CEC are seeded onto a keratocyte-free extracellular matrix that had been synthesized by keratocytes (Fig. 5). In other words, it is clear that an important factor for the differentiation of endothelial cells into capillary-like cords is the presence of the extracellular matrix synthesized and secreted by keratocytes, rather than the presence of keratocytes themselves. Previous studies have indicated that an extracellular matrix—e.g., collagenous substances— influenced not only the proliferation and migration of vascular endothelial cells but
also their capability to differentiate into capillary-like tubular structures. In the present report, we found that CEC seeded onto type I collagen gel became elongated but did not differentiate into capillary-like cords (Fig. 4). On the other hand, Montesano and colleagues showed that CEC embedded in a three-dimensional collagen matrix organized rapidly into a network of capillary-like tubes and that the cells had correct cellular polarization. Other investigators using various kinds of vascular endothelial cells, also succeeded in getting organized capillary-like tubes in a three-dimensional collagen matrix. Based on these observations, it appears that a three-dimensional extracellular matrix may play a cardinal role in differentiating vascular endothelial cells into capillary-like tubes and in determining the correct cellular polarization. In an environment wherein the extracellular matrix encircles the entire surface of the endothelial cells, the cells may be induced to form capillary-like tubes.

Specific angiogenic factors also could be necessary for the growth of vascular endothelial cells. Because the existence of the keratocyte itself is not necessary for the formation of capillary-like tubes, CEC themselves may produce some angiogenic factors that modulate capillary-like tube formation in an autocrine fashion. Indeed, it is generally accepted that CEC of bovine adrenal glands and other tissues produce and release basic fibroblast growth factor and express the basic fibroblast growth factor gene. Of course, the FBS in our endothelial cell culture medium also may be a source of angiogenic factors.

Regarding the pathogenesis of corneal neovascularization in vivo, in 1949 Cogan observed that the events in new vessel formation in cornea were initiated by corneal swelling that resulted in tissue looseness of the surrounding stroma. Therefore, he hypothesized that the corneal swelling was important in the induction of angiogenesis. Since then, several more hypotheses on corneal neovascularization have been proposed (summarized by Klintworth). These include the presence of anti-angiogenic substances and their destruction in cornea, production of angiogenic factors in cornea, manifestation of inflammation and hypoxia. In the present in vitro experiment, we were able to show: (1) that even after several passages, cultured CEC could grow and differentiate into capillary-like tubes in the multilayered keratocytes; and (2) that this organization seemed to be promoted by the extracellular matrix produced by keratocytes. Barring the remote possibility that noncellular components of inflammation exist in bovine serum, our culture system is believed to be free of factors that could induce an inflammatory reaction. That, and the fact that the CEC and keratocytes were cocultured in an atmosphere of 95% air, lead us to conclude that the cultured CEC were able to differentiate into capillary-like tubes in the absence of hypoxia or an inflammatory reaction. More likely, the CEC is enveloped in an environment formed by an extracellular matrix that is synthesized by keratocytes and that is one of the cardinal factors for the differentiation. However, it is conceivable that in vivo, several factors, including hypoxia or inflammation, may be involved in the induction and development of corneal neovascularization.

Regarding methodology in the present experiment, one point merits discussion. The CEC used in this experiment were not derived from limbal vessels of the conjunctiva; they were derived from bovine adrenal gland. Folkman et al pointed out that among CEC, each characteristic depends upon the individual
tissue in which the capillary is located in situ. Although we would have liked to have used endothelial cells derived from capillaries of the limbal conjunctiva in this experiment, neither we nor other investigators have been able to successfully isolate such cells from conjunctival capillaries. Attempts to establish a culture method for cells from rabbit conjunctival vessels are under way.

Most studies of corneal angiogenesis have used in vivo experimental systems. However, because of the complexity of the mechanisms, clearly elucidating the pathogenesis of corneal neovascularization may be too difficult when only in vivo systems or clinical observations are invoked. Therefore, several in vitro models have been proposed for studying angiogenesis in various tissues or under various conditions.

Corneal tissue normally is avascular, and its avascularity is one of the most intriguing questions in ocular embryology. The role of pericytes in the genesis of new vessels also is unclear.32 Regarding corneal tissue, two competing hypotheses have been put forth.33 The first proposes that pericytes of newly invading vessels originate from the endothelial cells of preexisting limbal vessels, whereas the other suggests that the pericytes develop from keratocytes in corneal stroma. In our experimental set-up, capillary-like cords were induced readily, and the cords, once formed, usually were retained for more than 3 wk. Therefore, we suggest that our simple in vitro experimental model could be used to test the competing hypotheses. Our model also could serve as a useful tool in studying corneal neovascularization mechanisms and in exploring factors that stimulate or inhibit corneal angiogenesis. More explicitly, this model might be used to shed light on unanswered questions, such as the exact interaction between the extracellular matrices and vascular growth regulators synthesized by keratocytes and vascular endothelial differentiation, the role of keratocytes in the avascularity of normal cornea, and the origin of neovascular pericytes in cornea, to name but a few.

Key words: angiogenesis, capillary endothelial cell, cell differentiation, extracellular matrix, keratocyte,

References

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