Trigeminal Ganglion Neurons Affect Corneal Epithelial Phenotype
Influence on Type VII Collagen Expression In Vitro

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Purpose. To examine whether trigeminal ganglion (TG) neurons in tissue culture influence expression of Type VII collagen (a major component of the anchoring fibrils involved in the attachment of the epithelium to the underlying stroma) by cultured corneal epithelial cells.

Methods. A two-chambered coculture system was used. Fetal rabbit TG neurons were cultured into a central chamber on collagen- or laminin-coated tissue culture dishes. After good neurite outgrowth (average 7 days), rabbit corneal epithelial explants were placed into the outer chamber. Once neurite-epithelial cell interaction occurred, the cultures were immunostained for Type VII collagen. Direct coculture of TG neurons onto confluent passaged rabbit corneal epithelium also was studied.

Results. Neurites in contact with the epithelial cells in the outer chamber formed branching complexes, but staining for Type VII collagen was negative. In cocultures of TG neurons onto confluent passaged rabbit corneal epithelium, there was extensive neurite branching on and around the epithelial cells within a week. Scattered epithelial cells, many in clusters, were found to express Type VII collagen, as determined by immunofluorescence staining.

Conclusions. Based on the finding from this study that TG neurons influence production of Type VII collagen by rabbit corneal epithelium in vitro, it is likely that TG neurons influence corneal epithelial phenotypic characteristics that are critical to the maintenance of healthy epithelium in vivo. Invest Ophthalmol Vis Sci. 1993;34:137-144.
This study was designed to investigate the effect of TG neurons on Type VII collagen expression by rabbit corneal epithelial cells, which do not normally express Type VII collagen in vitro. The influence of the interaction between neurite outgrowth and epithelium and that of TG neurons seeded on confluent passaged epithelium on type VII collagen production was investigated.

METHODS

The isolation and culture of TG neurons with corneal epithelial cells were accomplished using modifications of the procedures described by Forbes et al.10 and Chan et al.11 All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Preparation of Two-Chambered Tissue Culture Dishes

Tissue culture dishes (35 mm) were coated with 1 ml of Type I collagen (Vitrogen 100; Collagen Corp., Palo Alto, CA) or laminin (Collaborative Research, Inc., Lexington, MA). Parallel linear scratches were made using a pin rake, as described by Ziegler and Herman.12 A sterile 8 mm glass cloning cylinder, coated on one end with sterilized high-vacuum silicone grease, was dipped in 0.6% methyl-cellulose in HDMEM (Dulbecco’s modified Eagle’s medium with 10% horse serum and 10% fetal bovine serum), and then was filled with DMEM 10/10 (DMEM with 10% horse serum and 10% fetal bovine serum), and 2 ml of cornea medium with nerve growth factor (1:1 DMEM and Ham’s F-12, 5% fetal bovine serum, 0.25 IU/ml insulin, 1 µg/ml hydrocortisone, 50 µg/ml gentamicin, 25 mmol/l HEPES buffer, 0.125 µg/ml 7S nerve growth factor; Sigma Chemical Co., St. Louis, MO) was added to the outer chamber. The plates were incubated for several hours, then checked for leaks. Only plates with intact seals were used.

Isolation of Rabbit Trigeminal Ganglia

Day 27 timed-pregnant New Zealand white rabbits were anesthetized with a 2 ml intramuscular injection of 1:10 xylazine (100 mg/ml)-ketamine (100 mg/ml) solution. Once an overdose injection of pentobarbital (390 mg/ml) was administered through an ear vein, the fetuses were immediately delivered and killed with intracardiac pentobarbital. An average of 10 fetuses were obtained per pregnant rabbit. The trigeminal ganglia were isolated, placed into Hanks’ balanced salt solution (BSS), and processed by the methods described below.

Initial Method of Trigeminal Ganglia Processing

Under a dissecting microscope, the trigeminal ganglia were gently teased apart with jewelers forceps, transferred into 5 ml of Hanks’ BSS with 0.25% trypsin, then incubated for 15 min at 37°C in 5% CO2:95% air. This was followed by centrifugation at 1,500 rpm for 10 min, aspiration of the supernatant, washing with DMEM 10/10, and recentrifugation at 1,500 rpm for 10 min. The pellet then was resuspended in DMEM 10/10 with 1 µg/ml 7S nerve growth factor (250 µl per pair of TGs), triturated into cell suspension, and plated into the central well of the prepared tissue culture dishes (200–250 µl per dish). The dishes were incubated at 37°C in 5% CO2:95% air. On the first day, the medium in the central well was changed to HDMEM with 10 mmol/l cytosine arabinoside to minimize nonneuronal cell growth.13 Subsequently, every 24–48 hr, HDMEM without cytosine arabinoside was replaced in the central well, as was the cornea medium with nerve growth factor in the outside well. Once neurite outgrowth into the outer chamber occurred, limbal epithelial explants were plated in the outer chamber.13 The cultures were immunostained after 2–3 days of direct neurite-epithelial cell interaction.

Trigeminal Ganglia Processing for Direct Coculture Onto Passaged Epithelium

Confluent passaged epithelium (passage 2 or 3) was used to study the effect of direct co-culture on Type VII collagen expression. Cloning cylinders coated on one end with silicone grease were placed centrally on 35 mm dishes of confluent passaged epithelium in the same manner as described above for the collagen- or laminin-coated dishes, either with or without making scratches with a pin rake. Dishes with intact seals were used. Initial investigations used trigeminal ganglia isolated and plated as described above.

The procedure described by Chan and Haschke11 was used to obtain TG cultures essentially free of nonneuronal cells. Trigeminal ganglia obtained from day 27 fetal rabbits were pooled in Hanks’ BSS and gently teased apart using jewelers forceps. Successive passage through 18 G, 20 G, and 23 G needles dissociated the neurons into a cell suspension. This suspension then was passed through a 13 mm Swinney filter (Fisher Scientific, Pittsburgh, PA) with a “sandwich” filter of #60/#200/#60 stainless steel mesh, followed by centrifugation at 1200 rpm for 2 min. The pellet was resuspended in DMEM 10/10 (0.25 ml per pair of TGs) with 1 µg/ml of nerve growth factor. Aliquots of 200–250 µl were plated into the central chamber of each dish. In control dishes without neurons, the medium in the central wells also was replaced with 250 µl of DMEM 10/10 with 1 µg/ml of nerve growth factor.
Cornea medium with 0.125 μg/ml nerve growth factor was placed in the outer chamber of all the dishes. After 1 day, the medium in the central well was changed to cornea medium with nerve growth factor as well. The medium then was changed every 24–48 hr. Cultures were immunostained on day 7.

**Indirect Immunofluorescence Staining Procedure**

Cultures were washed in phosphate buffered saline solution (PBS), fixed in cold methanol at 4°C for 10 min, and immunoreacted with specific antibodies using an indirect fluorescein isothiocyanate (FITC)-conjugated or rhodamine-conjugated antibody technique, as described previously. In brief, fixed cultures were reacted with 10% heat-inactivated rabbit serum in PBS for 45 min, then reacted with a monoclonal antibody for 30 min. The first antibody was either anti-neurofilament antibody, a gift from Carl Lageaur (The University of Pittsburgh School of Medicine); anti-Type VII collagen monoclonal antibody designated 9C3, a gift from Jonathan Jones (Northwestern University School of Medicine); anti-keratin (PKK1) antibody from Labsystems Inc. (Morton Grove, IL); or an anti-keratan sulfate antibody (for negative control). The cultures then were reacted with FITC-conjugated rabbit anti-mouse IgG (Organon Teknika Corp., Durham, NC) at 1:200 dilution in 10% heat-inactivated rabbit serum or with rhodamine-conjugated rabbit anti-mouse IgG (Organon Teknika) at 1:20 dilution in 10% heat-inactivated rabbit serum for 40 min. The stained sections were mounted in Aqua-Mount (Lerner Labs, New Haven, CT) and examined and photographed with a photomicroscope (Vanox; Olympus Optical Co. Ltd., Tokyo, Japan) with fluorescence attachments and epifluorescence objectives.

**RESULTS**

Four pregnant rabbits were used to obtain fetal rabbit trigeminal ganglia to study the effect of neurite outgrowth on Type VII collagen expression by primary cultures of rabbit corneal epithelium. When neurons were plated into the central well of collagen- or laminin-coated dishes, neurite extensions into the outer chamber were seen, extending along and between the tracts made with the pin rake. Neurites on the collagen surface were qualitatively thicker in appearance and appeared more firmly attached than those on the laminin-coated dishes. After good neurite outgrowth had occurred, limbal epithelial explants were placed into the outer chamber along the linear scratches, approximately 5 mm from the cloning ring. Epithelial outgrowth from the explant was evident within 24–48 hr. Neurite-epithelial cell interactions occurred within 2–5 days after the explants were plated(Figs. 1 and 2). After 2–3 days of neurite-epithelial cell interaction, immunofluorescence staining for Type VII collagen was performed. As positive controls, cryostat sections of rabbit corneas, treated identically, were included for immunostaining with the 9C3 antibody. These controls (Fig. 3) verified that 9C3 reacts specifically with the corneal epithelial anchoring fibril zone that contains type VII collagen, and indicated that the epitope recognized by this antibody is not shared by any other antigens in the cornea. None of the cultures reacted with this antibody, indicating the absence of Type VII collagen expression (Fig. 4).

Four rabbits were used for the experiments of direct coculture of fresh TG cell isolates onto passaged...
FIGURE 3. Immunofluorescence staining of a rabbit corneal section with anti-Type VII collagen antibody (9C3). Note a linear staining in the basement membrane zone and a punctate staining (arrow) extending into Bowman’s membrane. (Original magnification X60; bar = 5 μm.)

confluent epithelium. After 7 days of coculture, immunofluorescence staining with neurofilament antibody revealed extensive neurofilaments on and around the epithelial cells in the central well (Fig. 5A), with no neurofilament immunostaining in the outer chamber in the dishes without scratches. Indirect immunofluorescence staining with anti-type VII collagen antibody revealed clusters of positive epithelial cells within the central well (Fig. 5B). Neurites extended to the outside wells in the dishes with scratches (Fig. 5C). The epithelial cells in the outside well of these cocultures (Fig. 5D), in the outside well of the dishes without scratches (Fig. 5E), and in the central well without neurons (not shown) were negative to Type VII antibody staining. Initially, these results were obtained with the neurons isolated according to Forbes et al.10 These results were verified with the second method of Chan and Haschke,11 which provided cultures essentially free of nonneuronal cells. Figure 6 shows epithelial cultures seeded with TGs (prepared according to Chan et al) immunostained with antikeratin antibody as a marker of epithelium, anti-neurofilament antibody as a marker for ganglion cells and their neurite extensions, or anti-Type VII collagen antibody. Most of the cells in the monolayer reacted with antikeratin antibody (Fig. 6A). TGs and neurite extensions on and around the epithelial cells, in clusters, were evident from immunostaining with anti-neurofilament antibody (Fig. 6B). Clusters of epithelial cells in the central well, cocultured with neurons (Fig. 6C), reacted with anti-Type VII collagen antibody. The epithelial cells in the outer chamber, without interaction with neurons or neurite extensions, did not react with this antibody (Fig. 6D).

DISCUSSION

The etiology of corneal pathology in neurotrophic keratitis is unclear. Some investigators, including ourselves, believe that the TG has a neurotrophic influence on the cornea, in addition to its better-known sensory role. In our previous studies, we observed that rabbit corneal epithelial cells in cultures, with or without fibroblasts, did not express Type VII collagen (unpublished observations). The current study demonstrates that the coculture of TG neurons with rabbit
FIGURE 5. Immunofluorescence staining of cocultures of corneal epithelial cells and trigeminal ganglion neurons. Trigeminal ganglia were isolated and grown in culture according to Forbes et al.\textsuperscript{19} Fixed and permeabilized cell cultures were reacted with (A) anti-neurofilament antibody and (B and D and E) anti-Type VII collagen antibody. Note extensive network of neurites, extending from trigeminal ganglion neurons in the center well immunostained with anti-neurofilament antibody. In an identical culture, discrete groups of epithelial cells in the central well show reactivity with anti-Type VII collagen antibody (B). A phase contrast micrograph (C) shows that the neurites (arrows) extended under the cloning ring (removed) and reached the epithelial cells (CE) in the outer well. These cells did not react with anti-type VII collagen antibody, as shown in the fluorescence micrograph in D. Epithelial cells in the outer wells of unscratched dishes, which did not have contact with the neuronal cells, exhibited no reactivity with anti-Type VII antibody (E). (Original magnifications ×60; bar = 5 µm.)
corneal epithelium induces expression of Type VII collagen in corneal epithelial cells. Thus, the TG may have a trophic influence on the phenotypic characteristics of corneal epithelium.

Metabolic and structural changes in the corneal epithelium of experimental models in which corneal innervation has been interrupted support this view. Sigelman and Friedenwald found a decrease in the rate of mitoses of the corneal epithelium in rats with trigeminal denervation, and Mishima found similar results in rabbits. Alper studied the effect of the destruction of TG and the ophthalmic division of the trigeminal nerve on the cornea in monkeys. He demonstrated with light microscopy that the corneal epithelium was thinner on the denervated side, whereas the stroma remained unchanged. Clinically, ulceration and keratitis developed in the denervated corneas of those monkeys whose tarsorrhaphies broke down, but not in those with an intact tarsorrhaphy or with normal innervation.

Beuerman et al investigated the effects of corneal denervation by light, scanning, and transmission electron microscopic analyses of rabbit corneas after thermocoagulation of the TG. A disorganized appearance
of the corneal epithelial cells, with a decrease in hemidesmosomes and loss of cytoplasmic tonofilaments after corneal denervation, was observed. Also noted was a loss of surface microvilli and abnormal desquamation of the epithelium. In contrast, Cintron et al\(^6\) reported there was no difference between normally innervated and denervated corneas regarding glycogen content, thickness of the tissue, number of basal cell nuclei, or DNA content, when a cyclocryosurgical method was used to denervate the rabbit corneas. Schimmelpfennig and Beuerman\(^7\) subsequently reported that, with their method of thermocoagulation the TG, the corneal epithelium maintained normal thickness, but demonstrated disorganization of the basal and wing cell layers. They also had reported increased epithelial permeability and delayed wound healing in denervated corneas.\(^9\) More recently, Gilbard and Rossi\(^1\) demonstrated changes in the corneal epithelium of rabbit eyes denervated by thermocoagulation of the TG. These changes included a decrease in glycogen content, loss of surface microvilli, intracellular swelling, and increased epithelial cell desquamation. On slit-lamp examination, filament-like lesions and diffuse rose Bengal staining were seen. One difference between the investigations that found changes in the cornea and those that did not was the method of denervation of the cornea, thermocoagulation versus cyclocryosurgery. Whether this is the reason for the observed difference is unknown. Regardless, most of the studies in the literature do implicate some regulatory or trophic influence of the trigeminal nerves on the corneal epithelium.

In vitro experiments to investigate the interaction of TG neurons and corneal epithelium by Chan et al have emphasized the neuronotrophic effect of the epithelium on neurons in culture.\(^11,17-20\) To our knowledge, the effect of TG neurons on the production of various proteins by corneal epithelial cells in vitro has not been reported previously.

We chose to investigate Type VII collagen because of the role it is believed to have in helping maintain the firm attachment of corneal epithelium to underlying stroma via anchoring fibrils in vivo.\(^8,9\) Because chronic epithelial defects occur clinically with neurotrophic keratitis,\(^21\) it was hypothesized that there may be an absence or abnormality in the adhesion proteins in the basement membrane zone of sensory-denervated corneas. The coculture system used in the present experiment permitted the investigation of any neural effect on Type VII collagen production by rabbit corneal epithelial cells, which do not normally express Type VII collagen in vitro under the culture conditions used in this study.

As noted in the results, direct coculture of neurons onto a monolayer of passaged epithelium resulted in Type VII collagen expression, whereas areas not in contact with neurons (ie, epithelium in the outside well of the tissue culture dish) and cultures free of neurons were negative. The mechanism by which the neurons induced the expression of Type VII collagen is unknown. In vivo, epithelial cells are not in direct contact with TG neurons, but communicate with them through the neurites. That we obtained positive results only with direct coculture may suggest that direct neuronal-epithelial contact was necessary for the stimulation of type VII collagen production. Our findings, however, do not exclude the possibility that the interaction between neurites and the epithelial cells was responsible for the stimulation of type VII collagen production in cocultures. This hypothesis also was based on the observation that there was a similarity in the pattern of neurite network around distinct clusters of epithelial cells and in the pattern of immunostaining for type VII collagen, which was in isolated clusters of the epithelial cells in these cocultures.

Because there was no stimulation of Type VII collagen in the epithelial cells that interacted with the neurites in the outer well, we speculate that a factor or factors from the TG neurons responsible for influencing synthesis of Type VII collagen was short lived and was diminished by the time the neurites made contact with the epithelial cells in the outer well. On the other hand, in the cocultures, the neurites established contact with the adjacent epithelial cells in a much shorter time.

Our preliminary studies with conditioned media from TG cultures have failed to promote Type VII synthesis by corneal epithelium, but further investigation is needed to confirm whether or not there is a diffusible factor released by TG neurons and to elucidate the mechanism of neural influence on the corneal epithelium.

In summary, our study used a two-chambered coculture system to demonstrate that trigeminal ganglion neurons have an influence on the expression of Type VII collagen by rabbit corneal epithelial cells in vitro. Although passaged rabbit corneal epithelial cells in culture alone or in a separate chamber from TG neurons were negative to indirect immunostaining for Type VII collagen, those cells in direct coculture with neurons were positive. These results suggest that the trigeminal nerves may play a role in the adhesion of the epithelium to its underlying stroma; the loss of this effect may be one factor in the development of neurotrophic keratitis. Additional studies are needed to further characterize the nature of the neuronal-epithelial cell interaction and to identify any factors that may be involved.

**Key Words**
corneal epithelium, neurons, tissue culture, trigeminal ganglions, type VII collagen.
References


