Transplanted Corneal Stromal Cells in Vitreous Reproduce Extracellular Matrix of Healing Corneal Stroma

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Purpose. To characterize the extracellular matrix (ECM) formed by corneal stromal cells after injection into the vitreous. This will provide a basis for future studies on the function of corneal ECM macromolecules.

Methods. Cell line from rabbit dermal fibroblasts (RAB9) and primary cultures of rabbit corneal stroma fibroblasts (NRCF) were grown to confluence. For each cell type, approximately $1 \times 10^6$ cells suspended in basal medium were injected into the vitreous of normal rabbits and observed periodically with a slit lamp. After 1, 2, and 4 weeks, eyes were processed for transmission electron microscopy (TEM), immunohistochemistry, immunocytochemistry, and in situ hybridization.

Results. All cells showed gradual growth within the vitreous along the needle track. Occurrence of retinal detachment and inflammation was variable. Transmission electron microscopy of NRCF confirmed the deposition of ECM reminiscent of the organization of normal fetal corneal stroma. Similar matrices were produced by RAB9. NRCF deposited collagen fibrils similar in diameter to those seen in normal developing and healing corneal stroma. RAB9 produced collagen fibrils with larger diameters. NRCF-transplanted cells synthesized proteoglycans and collagen immunologically identical to decorin proteins and type VI collagen, indicating that the expression of specific ECM is maintained after transplantation. In addition, in situ hybridization showed that type XII collagen mRNA is synthesized by transplanted NRCF similar to healing corneas.


The cornea contains extracellular matrices (ECM) with distinct structures, macromolecular compositions, and functions. The stroma forms the bulk of the cornea and is unique among connective tissues because it is transparent though it retains the requisite mechanical properties to ensure the integrity of the eyeball. The small- and uniform-diameter collagen fibrils in the stroma form lamellae, which run parallel to the corneal surface. The interfibrillar matrix contains nonfibrillar collagen, proteoglycans (PGs), and other macromolecules. Interactions between PGs and other macromolecules in the extracellular space generally are assumed to contribute to the functional properties of the tissue.

Previous studies in our laboratory have shown marked morphologic and molecular similarities between normal developing and healing adult cornea. Differences in these processes have indicated why healing corneas fail to produce a transparent tissue, but the precise function of individual macromolecules in corneal morphogenesis still evade us. What are the specific functions of the ECM macromolecules? Recent attempts to determine the factors controlling fibrillogenesis have implicated proteoglycans or the ratio of collagens. These in vitro experiments fail to address the relevancy of the temporal and spatial restrictions of an in vivo environment. There is clearly a need for a system to test the function of...
within the vitreous support the contention that this system may be useful to study the function of macromolecules during morphogenesis of connective tissues in vivo.

MATERIALS AND METHODS

Transplantation of Fibroblast Cells Into Rabbit Vitreous

Cell line rabbit dermal fibroblasts (RAB9) obtained from American Type Culture Collection (Rockville, MD), rabbit corneal stroma fibroblasts (NRCF), and rabbit scleral fibroblasts in their third passage after outgrowth from tissue fragments were grown to confluence in Dulbecco's modified Eagle medium containing penicillin (100 U/ml) and streptomycin (100 μg/ml), supplemented with 10% fetal bovine serum (all from Gibco, Gaithersburg, MD). Approximately 1 \times 10^6 cells suspended in Dulbecco's modified Eagle medium were injected into the vitreous of normal rabbits using a published technique,\textsuperscript{9} with some modifications. Briefly, we carefully injected 50 or 100 μl of cell suspension into the vitreous at a site 4 mm to 5 mm posterior to the corneal limbus through a 30- or 25-gauge needle with an acute angle toward the midvitreous cavity. Animals were handled and cared for according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All cells showed gradual growth within the vitreous without any clinically visible inflammatory response or other complications. As controls, basal medium without cells or tissue-cultured cells derived from scleral tissue was injected.

Transmission Electron Microscopy

At 1, 2, and 4 weeks after injection, the rabbits were killed with an overdose of sodium pentobarbital. Eyes were enucleated and fixed in a fresh mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) overnight at 4°C, and the vitreous was dissected carefully to isolate the new tissue. Tissues were rinsed in 0.1 M cacodylate buffer, postfixed in 1% OsO4 for 1 hour at 4°C, dehydrated in a graded ethanol series, and embedded in Poly/Bed 812 (Polysciences, Warrington, PA) to obtain ultrathin sections on grids. Grids were stained in 4% uranyl acetate in 50% methanol for 25 minutes, followed by Reynolds' lead citrate for 5 minutes, and examined with a Philips 410 transmission electron microscope (Eindhoven, The Netherlands). Transmission electron microscopy of tissues produced by transplanted cells were enlarged to \times47,500 magnification, and for each tissue sample, the diameter of 850 collagen fibrils...
FIGURE 2. Transmission electron microscopy of NRCF (a,b) and RAB9 (c,d), 4 weeks after transplantation. (a) Fibroblasts organized in a parallel array with abundant ECM. (b) At higher magnification, collagen fibrils are uniform in diameter and generally are organized into bundles. Groups of microfibrils occasionally were seen. (c) Fibroblasts organized in a parallel array and extensive ECM. (d) At higher magnification, collagen fibrils are large and variable in diameter. F = fibroblast; ECM = extracellular matrices; Co = collagen fibrils; MF = microfibrils.

from several fields was measured with an ocular micrometer.

**Immunofluorescence Microscopy**

Cells were analyzed for decorin synthesis in vitreous transplants. NRCF cells were transplanted into the rabbit vitreous and allowed to grow for 1, 2, and 4 weeks. Tissues obtained from vitreous transplants were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.2. Transplants were embedded in Tissue-Tek OCT compound (Miles, Elkhart, IN), cryostat-sectioned at 6-μm thickness, and mounted on gelatin-coated slides. After three PBS washes containing 1% bovine serum albumin (BSA), tissue sections were incubated with monospecific polyclonal antibodies to corneal decorin (aDSPG/CP), as previously described. The secondary antibody was fluorescein isothiocyanate-conjugated rabbit anti-sheep immunoglobulin G (IgG) antibody (Cappel, Malvern, PA). Nonimmune sheep serum was substituted for primary antibody as controls. Binding of antibodies was detected with a Zeiss Axiophoto microscope (Oberkochen, Germany).

**Immunocytochemistry**

Cryostat sections, 10 to 12 μm thick, of NRCF vitreous transplants 1, 2, and 4 weeks after injection were mounted on gelatin-coated microscope slides. After three washes in PBS-BSA, tissues were incubated with aDSPG/CP (1:100) overnight at 4°C. The samples were washed in PBS-BSA for 2 hours, and tissues were incubated overnight at 4°C in 5 nm colloidal gold-conjugated rabbit anti-sheep IgG (1:3) (E-Y Laboratories, San Mateo, CA), followed by three rinses in PBS-BSA. Tissue samples were then processed for TEM as described above. Some sections of vitreous transplants were used for double-labeling experiments. After incubation with aDSPG/CP and 5-nm gold-conjugated rabbit anti-sheep IgG, sections were incubated overnight at 4°C with monoclonal mouse-anti-type VI collagen antibody (1:500) (Gibco), followed by an extensive rinse in PBS-BSA. Localization of anti-type VI collagen was shown by incubation overnight at 4°C with 15 nm gold-conjugated rabbit anti-mouse IgG (1:3) (E-Y Laboratories). As controls, nonimmune sheep and mouse serum were substituted for primary antibodies.
In Situ Hybridization of Type XII Collagen

NRCF cells were transplanted into the rabbit vitreous and allowed to grow for 2 weeks. After sacrifice of the rabbit, the eyes were enucleated and fixed in 4% paraformaldehyde in PBS. Tissues from NRCF vitreous transplants were processed for paraffin embedding, sectioned at 5-mm thickness, and mounted on microscope slides. Antisense and sense oligonucleotide probes of type XII collagen were prepared as previously described. In situ hybridization was performed as previously described.

Cell-Specific Distribution of Collagen Fibril Diameters

Each cell type produced a distinctive distribution of collagen fibril diameters (Fig. 4). NRCF deposited collagen fibrils of diameters ranging from 21 to 47 nm, similar to that seen in normal developing and in healing corneal stroma. RAB9 produced collagen fibrils with diameters ranging from 23 to 59 nm. All samples contained fine collagen fibrils with diameters of 9 to 19 nm. They were identified as vitreal collagen because the diameters of collagen fibrils in the vitreous tissue injected with basal medium were from 9 to 19 nm (not shown). Scleral cells deposited collagen fibrils of diameters from 21 to 47 nm, similar to that seen in normal developing sclera, and slightly larger than NRCF collagen (Fig. 5).
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FIGURE 4. Collagen fibril diameter in tissue produced by NRCF and RAB9 cell types, 4 weeks after transplantation. Each cell type produced a distinctive distribution of collagen fibril diameters. % = percent of total number of fibrils measured. Diameter = diameter of collagen fibril cross-sections.

Needle Track Extracellular Matrix Synthesis and Structure Mimic Healing Cornea

Immunohistochemical analysis showed that αDSPG/CP, specific for decorin core protein, stained ECM produced by NRCF-transplanted cells (Fig. 6). The staining pattern was filamentous and associated with collagen bundles.

Cryostat sections of the vitreous transplants treated with αDSPG/CP and anti-type VI collagen for double-labeling immunocytochemical analysis contained sparse bundles of collagen fibrils and abundant immunogold particles between the fibrils (Fig. 7). The immunogold particles were associated primarily with the interfibrillar filamentous material and showed that both antibodies were co-localized in the tissues. Controls failed to show immunogold particles.

Type XII collagen mRNA, abundant in corneal stroma only during development and healing, also is seen in NRCF vitreal-transplanted cells labeled with antisense probe ASXII1 specific for type XII collagen mRNA (Fig. 8).

DISCUSSION

The three-dimensional structure of the vitreous, the restriction of cells to the needle track, and an environment containing many of the growth factors and other cytokines needed for matrix production constitute ideal conditions for duplicating many of the processes found in the formation of ECM in vivo. As previously shown, our results indicate that transplanted cells recapitulate the formation of orthogonal ECM reminiscent of the corneal stroma. Our new findings add...
FIGURE 7. Double-labeling immunocytochemistry of DSPG and type VI collagen in extracellular matrix produced by NRCF 2 weeks after transplantation. (a) Binding of antibodies to the core protein of DSPG and type VI collagen was detected by 5-nm and 15-nm gold-conjugated secondary antibodies, respectively. Small and large gold particles are associated with interfibrillar filamentous structures. (b) Control. Arrowheads point to gold particles. Bar = 100 nm.

FIGURE 8. In situ hybridization of type XII collagen mRNA in sections of rabbit vitreous with transplanted NRCF, 2 weeks after transplantation. (a) Specific probe ASXII1 binding is associated primarily with the fibroblasts. (b) No specific labeling is seen with the SXII1 sense probe. Arrowheads point to labeled fibroblasts. Bar = 15 μm.
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healing, as evidenced by orthogonal bundles of collagen fibrils with small and variable fibril diameters. The rate of ECM deposition, however, is decreased relative to that in healing cornea. The reason for this is unknown.

Intraocular transplantation techniques have been used in developing experimental models such as proliferative vitreoretinopathy (PVR). Experimental PVR models have shown that cells of various origin injected into the vitreous produced fibrillar ECM; however, the investigators did not specifically analyze the ECM components or their organization. Our study showed that the vitreous provided some of the physical and humoral factors for cells from various sources to retain their original characteristics. Furthermore, the survival of a human cell line HS27 in the rabbit vitreous for 4 weeks (unpublished observation) supports the contention that the vitreous cavity is an immunologically privileged site for allografts. This indicates that cells from different animal species can be transplanted into the rabbit vitreous, survive for several weeks, and synthesize ECM. Therefore, the vitreous cavity is a good culture chamber, and this in vivo model may be useful to study the morphogenesis of connective tissues from various tissue sources.

We must consider the possibility of contamination or replacement by cells from other sources in the eye. There is a possibility that some cells might be introduced by perforating through the sclera and the neural retina. Some cytokines, such as growth factors and interleukins, are elevated in the vitreous humor of patients with PVR, which is a response to injury to the internal limiting membrane of the retina. In addition, intravitreal membranous tissues in PVR have included retinal pigment epithelial cells, glial cells, and inflammatory cells. These cells may be called on by the cytokines in the vitreous. Although retinal cells may contribute to ECM formation, their matrix is physically separate from the matrix produced in the needle track. We have shown that collagen fibrils produced by scleral cells are ultrastructurally distinguishable from those produced by NRPF. In addition, injection of basal medium into the vitreous did not elicit cell migration from the injection site. Finally, adenovirus, AdCMV/βA.nlacz, transfected NRPF cells transplanted into the vitreous express reporter gene activity (β-galactosidase) within the needle track 2 weeks after injection (unpublished observations). We, therefore, believe the matrix produced in the needle track is from exogenous cells transplanted into the vitreous.

We conclude that transplantation of corneal stromal cells from monolayer cultures synthesizes a three-dimensional ECM highly reminiscent of the healing corneal stroma, which satisfies our first criterion for an in vivo ECM morphogenetic system. Moreover, preliminary studies show that alterations in protein synthesis in vitro are maintained in vivo after vitreal transplantation of transfected NRCF cells. We hypothesize that the alteration in the synthesis of ECM proteins in cell culture by viral constructs can be maintained when these cells are transferred to the vitreous. Analysis of the altered tissue synthesized in the vitreous will provide a clue to the function of specific ECM proteins. Future studies will test this hypothesis.

**Key Words**
collagen, corneal fibroblasts, extracellular matrix, proteoglycan, transplantation, vitreous

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