In Vitro Evaluation of Fibroplasia in a Porous Polymer

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We evaluated a polybutylene/polypropylene blend in a blown microfiber configuration in vitro for potential use as the peripheral area of a keratoprosthetic device. Material properties such as ultimate tensile strength and ultimate elongation were measured. Stromal fibroblasts were seeded onto the material in vitro, and cell proliferation between uncoated and Type I collagen coated discs did not differ significantly. Fibroblasts could be seen migrating along the fibers and also traversing the fibers. The synthesis of connective proteins was examined. Laminin, fibronectin, and Type I collagen were detected by day 8. The experiments demonstrate that stromal fibroblasts can adhere onto the fibers, proliferate, and synthesize connective tissue proteins. Experiments are now being conducted to further evaluate the material in vivo. Invest Ophthalmol Vis Sci 31:1321–1326, 1990

In the past, a major cause of failure of various keratoprostheses has been the absence of healing between the periphery of the artificial device and the rim of host cornea. As a result, tissue necrosis, wound leakage, epithelial downgrowth, and intraocular infection often occurred.1–4 Therefore, the long-term success of a keratoprosthesis requires that the device heal securely to the surrounding cornea. To achieve this goal, the periphery of the device must be constructed of a porous material into which stromal fibroblasts can penetrate, proliferate, and synthesize connective tissue proteins. When this happens, "healing" will occur, creating a natural anchorage between the synthetic material and the host tissue.

The ideal keratoprosthesis will consist of two components: 1) an optically transparent center and anterior surface that will support the adherence and proliferation of epithelium5 and 2) a peripheral skirt that will support fibroplasia.

As part of our effort to construct a clinically useful keratoprosthesis, we have developed a melt-blown fibrous construction (MBFC) that permits stromal fibroblast ingrowth in vitro and in vivo,6 and have demonstrated the proliferation of fibroblasts and the synthesis of connective tissue proteins in vitro. Our results suggest that this material may serve as the peripheral skirt of a keratoprosthesis.

Materials and Methods

Characteristics of the MBFC

A fibrous melt-blown web prepared from a blend of 80% polybutylene (Shell 8510, Houston, TX) and 20% polypropylene (Exxon 3145, Baytown, TX) was obtained from the 3M Company (St. Paul, MN). The web has a basis weight of 28.7 g/m², a thickness of 0.29 mm, and a void volume of 87%. The values of the initial modulus (the initial slope of stress versus strain, which is a measurement of material stiffness) are 42.9 and 21.2 kg/cm² (patent pending). The fiber diameter of the blown microfiber ranges from 2 to 12 μm (Fig. 1). The material is cut into 6-mm-diameter discs for use in the experiments.

Preparation of Collagen Coated Discs

Solubilized collagen was prepared from lyophilized rat tail tendon collagen by solubilizing the protein in 3% acetic acid at 4°C. This was followed by exhaustive dialysis against 0.05 M Tris/0.15 M NaCl buffer (pH 7.4) at 4°C. The collagen preparations were shown to be essentially pure collagen by both amino acid analysis and by polyacrylamide gel electrophoresis. Other highly pure solutions of Type I collagen were obtained from American Biomaterials Research (Plainsboro, NJ).

To prepare the coated discs, discs were placed on 2.2-μm filters to which Type I collagen (2% solution)
was added. The discs were bathed in the solution for 1 hr at room temperature, after which the collagen solution was pulled through the discs with a house vacuum. Discs that were not treated with collagen were incubated in control medium.

Experiments were conducted to evaluate collagen adsorption. These discs were washed extensively with Puck's Saline G, and the amount of adsorbed protein was determined by hydrolyzing the discs in 6 N HCl. Uncoated discs were hydrolyzed as controls. The total collagen adsorbed onto the discs was then determined with a Beckman (Palo Alto, CA) 119 CL amino acid analyzer. These experiments were repeated three times.

**Cell Culture**

All investigations conformed to the ARVO Resolution on the Use of Animals in Research. New Zealand rabbits were sacrificed with 5 ml sodium pentobarbital (325 mg) administered intravenously. The corneas were excised and fibroblasts were cultured. Briefly, Descemet's membrane and the endothelium were removed with forceps, and the remaining cornea (epithelium and stroma) was incubated in modified Dulbecco's Eagle's medium (Gibco, Grand Island, NY) and 1.2 U/ml Dispase II (Boehringer Mannheim, Indianapolis IN) for 1 hr. Sheets of epithelium were removed from the basement membrane.7,8
remaining stroma (devoid of epithelial cells) was minced and incubated for 1 hr at 37°C in serum-free medium containing 125 μg/ml porcine pancreatic elastase (Type III; Sigma, St. Louis, MO) and 500 μg/ml bacterial collagenase Type 1A (Sigma, St. Louis, MO).

After 1 hr, the digested tissue was centrifuged at 800 g for 10 min, washed with medium containing 10% fetal bovine serum, and centrifuged. The pellet was resuspended in Dulbecco’s modified Eagle’s medium supplemented with 1% nonessential amino acids, 1% penicillin-streptomycin solution, 3.7 g/l NaHCO3, 0.5% insulin-transferrin-selenium (ITS; Collaborative Research, Bedford, MA) and 10% fetal bovine serum. Fibroblasts were seeded at 2 X 10⁵ cells/ml into Corning T-25 tissue culture flasks. After 1 week, the cells were subcultured and seeded onto uncoated or Type I collagen coated discs.

Cell Growth and Total DNA Determination

Cell number was monitored every 2 days. To determine cell proliferation, each disc was washed in Puck’s Saline with a shear rate of 60 cm-sec⁻¹, placed into an Acuvette II (Hialeah, FL) counter vial containing 0.5 ml of 0.05% trypsin and 0.02% ethylenediaminetetraacetic acid (trypsin-EDTA), and incubated with shaking at 37°C for 20 min. After enzymatic removal of the cells, Isoton II (Hialeah, FL) was added to the vials, and the cells and diluent were mixed vigorously. Three discs were counted for each time point, and the mean and the standard error was determined. These experiments were repeated three times.

The DNA content per two discs was determined with a 33258 Hoechst Fluorochrome, which demonstrates an increased fluorescence when the dye is complexed with DNA.

Microscopy

Scanning electron microscopy (SEM) was used to demonstrate the morphology of the MBFC. The discs were CO₂-critical-point dried, mounted, coated with gold palladium for 4 min at 22 mA in a minicoater (Ful lam), and examined on an AMR scanning electron microscope. Four discs were evaluated.

Collagen Synthesis

The synthesis of collagen was measured on uncoated and coated discs. Briefly, stromal fibroblasts cultured on the discs were pulsed with 25 μCi/ml ³H-proline on day 7. Prior to pulsing, the cells were washed with Puck’s Saline G and subsequently starved for 1 hr in saline. The cells were pulsed for 18 hr in proline-free and serum-free medium containing 20 μg/ml ascorbate. After 18 hr, the cell layer was removed, homogenized, dialyzed, and lyophilized. The medium was centrifuged to remove particulate matter and then dialyzed and lyophilized. The samples were resuspended in 6 N HCl, hydrolyzed in vacuo at 110°C for 20 hr, evaporated to dryness under nitrogen, and resuspended in 0.01 N HCl. Radiolabeled proline and hydroxyproline were analyzed with a Beckman 119 CL amino acid analyzer. The percentage of hydroxyproline as a function of total radioactive proline incorporated was calculated. Six discs were used for each experiment and the experiments were repeated three times. Mean and standard deviations were calculated.

Immunodot Blot Analysis

Immunodot blot analyses were carried out to determine which proteins were deposited within the matrix of the blown microfiber, and the quantity of each protein per disc was calculated. Six discs were pooled for each measurement, and the experiments were repeated three times. Antibodies to α-actinin, vinculin, laminin, and fibronectin were used. The antibodies were obtained from Collaborative Research. Cells were seeded onto uncoated and coated blown microfibers. After days 1 and 8, the cells were harvested, and the medium was removed. The discs containing the cells were homogenized, dialyzed, filtered, and lyophilized. Briefly, the lyophilized material was resuspended in distilled water and blotted onto nitrocellulose membrane (Schleicher and Schuell, Keene, NH) equilibrated with Tris/Tween buffer. The blots were blocked with 3% milk proteins in Tris/Tween for 20 min. The membranes were then incubated overnight at 37°C in a Tris/Tween solution containing 1% milk proteins and one of the following antibodies: anti-α-actinin (0.5 mg/ml), antivinculin (0.5 mg/ml), anti-fibronectin (2.5 μg/ml) and anti-laminin (2.5 μg/ml). As a control, the antibodies were run against bovine serum albumin (BSA; Sigma). The blots were washed extensively and in-
cubated for 1 hr with $^{125}$I-protein A on a shaker. After extensive washing, the blots were dried and exposed to Kodak (Rochester, NY) XAR-5 film with an intensifying screen for 16 hr at $-75^\circ$C. Analysis of immunodot blots was carried out with a Schimadzu (Schimadzu, CA) Chromato Scanner.

**Results**

**Cell Growth and Morphology**

Stromal fibroblasts in first passage were plated onto both uncoated and collagen coated discs, and cell growth was monitored. The amount of collagen adsorbed onto each collagen-coated disc was 4.2 μg. Two hr after seeding, 62% of the cells were adherent. Cell number increased with time on both the uncoated and coated discs. On the uncoated discs, cell doubling was not detected until 48 hr after seeding. A second doubling occurred at 7 days (Fig. 2). On the collagen-coated discs, a cell doubling occurred 24 hr after cell seeding. After 24 hr, there was no detectable increase in cell growth. In additional experiments on coated discs, total DNA was determined and correlated to cell number. Twenty-four hr after cell seeding, the cell number was $6.4 \times 10^3$ cells/ml and the total DNA was 16.8 μg/disc. On day 8 the cell number was $1.7 \times 10^4$ cells/ml and the total DNA was 35 μg.

To evaluate the morphology and penetration of the fibroblasts within the microfiber, cells were seeded onto discs and cultured. After 8 days the fibroblasts were fixed and processed for SEM. Stromal fibroblasts were detected migrating along the fibers. The cells possessed a typical fibroblast morphology and demonstrated filopodial extensions. Fibroblasts also were seen crossing fibers of different sizes, and in these cases the fibroblasts were not as elongated$^9$ (Fig. 3). A similar distribution of fibroblasts was detected on the unseeded surface (data not shown).

**Protein Synthesis**

Collagen synthesis (as determined by hydroxyproline content) was measured on day 8 on uncoated and
collagen-coated discs. (On day 0, less than 1% of the counts were in the disc.) The total proline (cell layer and medium) was greater for those cells seeded onto collagen-coated discs than those seeded onto the uncoated discs. After 8 days, the amount of proline incorporated into the cell layer was greater than that secreted into the medium (data not shown). Collagen synthesis also was greater on collagen-coated discs than on uncoated discs or on tissue culture plastic (Table 1). Histochemical evaluations of discs demonstrate that collagen is laid down throughout the MBFC (data not shown).

The appearance of α-actinin and vinculin was evaluated, since these proteins are critical for cellular adherence. α-actinin was detected on day 1 at a concentration of 1 ng/disc with immunodot blot and densitometric analysis. It also was detected after 8 days of incubation at a concentration of 2.0 ng/disc on uncoated and 0.4 ng/disc on coated discs. Vinculin was not detected at either time point with this technique. To date, we have not performed immunoelectron microscopy to determine the localization of the proteins.

The synthesis of laminin and fibronectin also was evaluated to determine whether these extracellular matrix proteins were being deposited within the interstices of the fibrous network in vitro. On day 8, fibronectin was present at a concentration of 1.4 ng/disc on both uncoated and coated discs. Laminin was present at higher concentrations for both conditions (0.2 μg/uncoated and 0.02 μg/coated).

### Table 1. Synthesis of collagen by stromal fibroblasts on blown microfiber discs

<table>
<thead>
<tr>
<th></th>
<th>Cell layer</th>
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<th>Medium</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>Total proline (cpm)</td>
<td>Collagen (%)</td>
<td>Total proline (cpm)</td>
<td>Collagen* (%)</td>
</tr>
<tr>
<td>Coated</td>
<td>62,576 ± 2389</td>
<td>4.3 ± 3</td>
<td>26,267 ± 6219</td>
<td>3.97 ± 0.65</td>
</tr>
<tr>
<td>Uncoated</td>
<td>39,899 ± 4103</td>
<td>0.39 ± 0.02</td>
<td>34,794 ± 17</td>
<td>3.5 ± 0.18</td>
</tr>
</tbody>
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Each value is the average of three separate experiments.

* Percent collagen = \( \frac{2(\text{cpmOHpro})}{5.4(\text{cpm pro-cpmOHpro}) + 2(\text{cpmOHpro})} \) × 100

Discussion

The purpose of these studies was to develop a biopolymer into which fibroblasts penetrate and produce “stromal healing.” By this we imply that the penetration and proliferation of stromal fibroblasts within the MBFC and their subsequent synthesis of connective tissue proteins can secure the material in the cornea without permanent dependence on sutures. Previous keratoprosthetic devices were constructed largely from impermeable, rigid plastic that supported neither surface epithelium nor stromal fibroplasia. These devices cannot be implanted with standard surgical techniques and have a poor history of retention. The devices that did remain in place elicited a high incidence of erosion, necrosis, and loss of peripheral corneal tissue adjacent to the keratoprosthesis, providing a fistulous track for the leakage of aqueous humor. This resulted in the loss of the anterior chamber, formation of anterior synechiae, secondary glaucoma, epithelialization of the anterior chamber, and chronic intraocular inflammation.

Other materials have been studied in vivo directly in the cornea, but we believe that this is the first time an in vitro procedure has been established for evaluating materials potentially useful in the construction of a keratoprosthesis. Previous in vivo experiments in skin, muscle, and arterial wall have shown the importance of pore size on cellular ingrowth. In 1980, investigators demonstrated that a carbon alloplastic material could be implanted in the cornea. However, this material had a high extrusion rate unless it was covered with conjunctival or corneal tissue. In 1987, another team of investigators demonstrated that a hydrogel with carbon fibers attached to its periphery could be implanted into cat eyes. Although fibroblasts did migrate along the carbon fibers, it is unlikely that there was much collagen deposition along the fibrils.

This report is the first demonstration that cultured rabbit stromal fibroblasts can penetrate a blown microfiber, migrate along the fibers, proliferate, and synthesize connective tissue proteins. The polypropylene/polybutylene 80:20 blown microfiber was chosen after evaluations of many fibrous constructions in vitro and subsequent evaluations of the promising materials in vivo (data not shown).

To examine the usefulness of each material we developed criteria for predicting its success. The criteria required that the cells penetrate the material, proliferate, exhibit a morphology typical of fibroblasts, and
blown microfiber configuration will support fibro-
synthesis of extracellular matrix proteins. The material chosen is comprised of fibers
that range in size from 2 to 12 μm. The fibers permit cellular migration, and the porosity of the material
also allows for the diffusion of nutrients and metabolites. The mechanical properties of the MBFC dem-
strate that the material has sufficient strength and flexibility to withstand surgical manipulation and
the long-term repeated stresses to which it would be ex-
posed in the eye.

Uncoated and collagen-coated blown microfibers
both were evaluated to determine the effect of the
substrate on cell proliferation. The cells used in these
experiments were always in first passage so that the
cellular response to a given surface could be evalu-
ated with a high degree of repeatability. Coating of
the MBFC was accomplished by pulling a collagen
solution through the disc with a house vacuum. The
amount of collagen adsorbed on the surface of indi-
vidual fibers was highly reproducible. This protein
seems to provide a substrate that enhances collagen
synthesis and early cell growth on the coated discs. In
contrast, cell proliferation was initially delayed on the
uncoated material. However, once the cells had be-
come established within the interstices of the un-
coated blown microfiber, cell growth was superior to
that of the coated material. We attribute this to the
synthesis of extracellular matrix proteins.

Our results demonstrate that an inert polymer of
blown microfiber configuration will support fibro-
plasia in vitro. This is a critical step in the develop-
ment of a keratoprosthesis that will heal to and be-
come an integral part of the surrounding host cornea.
Short- and long-term experiments are being con-
ducted to determine the optimal conditions for in
vivo fibroplasia in the cornea.

Key words: fibroplasia, cornea, polymer, connective tissue, keratoprosthesis

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