Cellular Response in Subretinal Neovascularization Induced by bFGF-Impregnated Microspheres

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PURPOSE. To determine the sequence of cellular changes associated with a new rabbit model of subretinal neovascularization (SRN) induced by subretinal injection of basic fibroblast growth factor (bFGF)-impregnated microspheres.

METHODS. bFGF-impregnated gelatin microspheres, prepared by forming a polyanion complex between gelatin and bFGF, were subretinally implanted into rabbit eyes. The eyes were studied by immunohistochemistry at 3 days to 8 weeks after implantation. Antibodies to CD4, CD8, cytokeratin, CD31, glial fibrillary acidic protein (GFAP), and RAM11 were used.

RESULTS. Cytokeratin-positive retinal pigment epithelial (RPE) cells appeared on day 3 and continued to increase in number in the subretinal space throughout the growth of the SRN membrane, becoming the predominant cell type. Macrophages (RAM11-positive) appeared early, but most disappeared within 7 days. GFAP-positive Müller cells were evident early in the retina but migrated into the subretinal space after 7 days; the gliotic adhesion formed between the retina and the SRN membrane was prominent at 8 weeks. CD31-positive endothelial cells were first evident at 14 days and formed neovascular channels that were still present for up to 8 weeks. CD4- and CD8-positive lymphocytes appeared in the early stages but were few in number.

CONCLUSIONS. SRN membranes are primarily composed of RPE cells and vascular endothelial cells. The membrane adheres to the retina by a gliotic band. The cellular components involved in the membrane of this model resemble those found in SRN membranes removed from patients with age-related macular degeneration. (Invest Ophthalmol Vis Sci. 1999;40:524–528)

Subretinal neovascularization (SRN) is often associated with severe visual impairment, especially in age-related macular degeneration (AMD). The pathogenesis of SRN is not fully understood; however, the new vessels of SRN are of choroidal origin. Recent studies of surgically excised choroidal neovascular membranes (CNVMs) have provided some pathologic information on the mechanism of CNVM formation.1–3 However, data obtained from these studies have often been limited to late stages in the evolution of the CNVMs. Early stages of CNVMs can only be studied in an animal model.

We have developed a new model of SRN in the rabbit based on implanting basic fibroblast growth factor (bFGF)-impregnated gelatin microspheres under the retina.4 Approximately 80% of eyes that receive bFGF-impregnated microspheres show fluorescein leakage from the CNVM 2 weeks after implantation. These CNVMs are largely involved within 8 weeks after microsphere implantation.

In this study we examined the time course of cellular response into the subretinal space after initiation of the bFGF SRN model using cell-specific antibodies. The results provide insight into pathogenic mechanisms involved in this lesion and demonstrate relevance to human CNVMs.

METHODS

Preparation of bFGF-Impregnated Microsphere Suspension

bFGF-impregnated microspheres were prepared by forming a polyanion complex as previously described.4 Briefly, 2.5 mg of cross-linked gelatin microspheres were placed in 100 μl of distilled water containing 25 μg of bFGF at 37°C for 1 hour, after which 400 μl of phosphate-buffered solution (PBS; pH...
**TABLE 1. Time Course of Cellular Response in the Subretinal Space**

<table>
<thead>
<tr>
<th>Marker</th>
<th>3 Days</th>
<th>1 Week</th>
<th>2 Weeks</th>
<th>4 Weeks</th>
<th>8 Weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokeratin</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>CD31</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RAM11</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>GFAP</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
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<tr>
<td>CD4</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
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<tr>
<td>CD8</td>
<td>-</td>
<td>-</td>
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Quantified by counting the number of positive cells in an average of three ×40 objective fields: ±, <1; +, 1-10; ++, >10.

7.4) was added to prepare the 0.5% (wt/vol) microsphere suspension for in vivo experiments. Gelatin (isoelectric point [pI] of 4.9, molecular weight of 99,000, Nitta Gelatin, Osaka, Japan) and human recombinant bFGF (R&D Systems, Minneapolis, MN) were used.

**Subretinal Implantation of bFGF-Impregnated Microspheres**

One eye each of 18 pigmented rabbits of both sexes, weighing 2.5 to 3.5 kg, was used. All procedures were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The animals were anesthetized with a mixture (4:1) of ketamine hydrochloride (16 mg/kg) and xylazine hydrochloride (4 mg/kg), and the pupils were dilated with 1% tropicamide and 2.5% phenylephrine hydrochloride eyedrops. The ocular surface was then additionally anesthetized with topical instillation of 0.5% proparacaine hydrochloride.

bFGF-impregnated microspheres were implanted under the retina with a glass micropipette as previously described. Each eye received 50 μL of bFGF-impregnated microsphere suspension (2.5 μg of bFGF). The eyes were examined by indirect ophthalmoscopy, photographed, and studied by fluorescein angiography weekly for up to 8 weeks.

**Figure 1.** Light micrograph of the lesion 3 days after implantation. Many RAM11-positive cells (arrows) infiltrate under the retina-surrounding microspheres. Original magnification, ×500.

**Figure 2.** Light micrograph of the lesion 2 weeks after implantation. Note CD31-positive cells (arrows) arising from the choriocapillaris are seen within the membrane. Original magnification, ×500.
Immunohistochemical Examinations

Three eyes each were examined by immunohistochemistry at 3 days and at 1 week, and four eyes each were examined by immunohistochemistry at 2, 4, and 8 weeks after implantation. Briefly, each eye was enucleated, and its cornea, lens, and vitreous were removed. The eye was bisected, and the site of the lesion was delimited by sharp cuts. One half of the lesion was fixed in half-strength Karnovsky's solution (2.5% glutaraldehyde and 2% formaldehyde) in 0.1 M cacodylate buffer (pH 7.4). The tissues were dehydrated in a series of graded alcohols and embedded in glycol methacrylate; 2- to 3-μm sections were stained with periodic acid-Schiff or hematoxylin-eosin. For immunohistochemical examinations, the other half of the tissue was embedded in optimal cutting temperature (O.C.T.) compound (Tissue-Tek; Miles, Elkhart, IN) and frozen in liquid nitrogen. Cryosections (8 μm) were cut and stored at —70°C. Thawed sections were air-dried, fixed with acetone for 5 minutes, and washed with PBS (pH 7.4). The sections were blocked for 15 minutes with 1% bovine serum albumin (Sigma, St. Louis, MO) in PBS after blocking of endogenous peroxidase by 0.3% hydrogen peroxide. The specimens were incubated for 30 minutes with the primary antibody and then washed for 15 minutes with PBS. Sections were stained using the avidin-biotin complex (Vectastain ABC Elite Kit; Vector Laboratories, Burlingame, CA), and aminoethyl-carbazole (Vector) was used to give a red color reaction. Sections were counterstained with Mayer's modified hematoxylin.

The primary monoclonal antibodies used were cytokeratin (mouse anti-human; Dako, Carpinteria, CA), CD4 and CD8 (mouse anti-rabbit; Spring Valley, Woodbine, MD), CD31 (mouse anti-human; Dako), glial fibrillary acidic protein (GFAP; Sigma), and RAM11 (mouse anti-rabbit macrophage; Dako).

RESULTS

All experimental animals developed SRN and showed a similar sequence of histologic and immunohistochemical findings, which are summarized in Table 1. The eyes showed no fluorescein leakage at 3 days or 1 week. Fluorescein leakage was visible from 2 weeks to 8 weeks after implantation; after 8 weeks the eyes showed no more leakage.

Three days after implantation, the microspheres were observed clearly under the retina. Many retinal pigment epithelial (RPE) cells (cytokeratin-positive) and macrophages (RAM11-positive) infiltrated the subretinal space surrounding the microspheres (Fig. 1). Adjacent Müller cells were GFAP-positive but did not infiltrate the subretinal space. Few CD4-positive

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**FIGURE 3.** Light micrograph of the lesion 2 weeks after implantation. The membrane is mainly composed of cytokeratin-positive cells. Original magnification, ×500.

**FIGURE 4.** Light micrograph of the lesion 4 weeks after implantation. GFAP-positive Müller cells are seen traversing the retina. A GFAP-positive plaque is seen between the retina and the neovascular membrane. Original magnification, ×500.
mononuclear cells and no CD8-positive cells were seen. No CD31-positive endothelial cells were observed in the subretinal space at this time.

One week after implantation, the microspheres were mainly surrounded by cytokeratin-positive RPE cells and RAM11-positive macrophages. GFAP-positive Müller cells extended occasional processes into the subretinal space. A few CD4- or CD8-positive cells were observed. CD31-positive endothelial cells were not seen in the subretinal space.

Two weeks after implantation, the microspheres were still observed but were decreased in number. CD31-positive endothelial cells were now seen in the subretinal space and formed neovascular channels (Fig. 2). The multilayered RPE (cytokeratin-positive) cells (Fig. 3) and endothelial cells formed a membrane-like structure. RAM11-positive macrophages were seen but were decreased in number compared with 1 week.

Four weeks after implantation, many RPE (cytokeratin-positive) cells and some endothelial (CD31-positive) cells were observed in subretinal membranes. Müller (GFAP-positive) cells were adherent to the retinal surface of the membrane and only rarely extended into the subretinal membrane (Fig. 4). A few RAM11-positive macrophages were seen, but CD4- or CD8-positive cells were sparse. A few microspheres were still present in some areas.

Eight weeks after implantation, CD31-positive endothelial cells were still seen in a membrane that was largely covered with cytokeratin-positive RPE cells. In some areas, gliosis was observed on the membranes.

**DISCUSSION**

In this study, we examined the time course of cellular infiltration into the subretinal space caused by implantation of bFGF-impregnated gelatin microspheres. The time course can be divided into two phases: an early phase (foreign body reaction) and a late phase (neovascularization). During the early phase, within 1 week of implantation, microspheres were surrounded by many RPE cells and macrophages and did not appear to be degraded. No neovascularization was observed. It is likely that the microspheres did not release a large amount of bFGF during this phase because bFGF is physically immobilized in the matrix of the cross-linked gelatin and is released as a biologically active form only after enzymatic degradation of the cross-linked gelatin. During the late phase, starting 2 weeks after implantation, the number of microspheres decreased, and endothelial cells migrated from the choroid forming with RPE cells a neovascular membrane. It seems likely that the microspheres were enzymatically degraded by RPE cells or macrophages and released bFGF, resulting in proliferation of RPE cells and choroidal endothelial cells. At 8 weeks after implantation, the neovascular membranes were covered with RPE cells and appeared to be adherent to the retina by a gliotic band. The microspheres were largely degraded.

Injection of microspheres alone was also associated with a mild cellular reaction composed of macrophages and RPE cells; however, this was not associated with neovascularization. The microspheres used in this study were relatively large, with an average diameter of 40 μm, and may have caused a foreign body reaction. Subretinal injection itself may also result in damage to outer segments of the photoreceptors or partial dispersion of RPE cells that could incite RPE proliferation or a gliotic response.

During the late phase, neovascularization occurs, and the new vessels show an association with many RPE cells. Gelatin itself enhances phagocytosis of microspheres by macrophages through its opsonizing ability. Some microspheres may be ingested and degraded by intracellular lysosomal enzymes, and others may be degraded by gelatinases in the extracellular space. RPE and endothelial cells both have been shown to be capable of gelatinase (matrix metalloproteinase-2 and -9) secretion in vitro.

The neovascularization may be a result of several soluble factors. A direct or indirect effect of bFGF itself on choroidal vessels is most likely. bFGF is a prominent promoter of endothelial cell migration and proliferation and stimulates the angiogenic process. Release of bFGF from the microspheres requires microsphere degradation that may occur by phagocytosis by macrophages or RPE or by proteolytic digestion. A second source of angiogenic factors is from soluble products of infiltrating macrophages and RPE. In particular, migrating RPE cells have been shown to be a source of the strongly angiogenic vascular endothelial growth factor (VEGF) in human CNVM. The presence of VEGF or other secondary angiogenic factors derived from either RPE or macrophages will be an important focus of future study. bFGF may also promote the development of the SRN lesion by stimulating growth of the nonendothelial cells. bFGF is mitogenic for both RPE and glial cells and thus may have an important role in promoting the relatively high cellularity found in the lesions. Therefore, although it is clear that the presence of bFGF in the microspheres was the determinant directly associated with the strong subretinal angiogenic response, it is possible that the cellular or physical reactions induced by the microspheres themselves may contribute to the stimulus.

Choroidal neovascular membranes in this model show immunohistochemical characteristics similar to those of CNVMs surgically excised from patients with ARMD. In these membranes, the stromal cells are predominantly composed of RPE and vascular endothelial cells; however, small numbers of macrophages are also seen. Most CNVMs in ARMD include prominent extracellular matrices (ECMs) rich in collagen, laminin, and fibronectin. CNVMs in this model appear to be composed primarily of cellular components with less prominent ECMs. The strong proliferative response of RPE cells to wound healing in the rabbit may explain why RPE cells are especially prominent in the membranes in this model. Identification of ECM components in this model may require the increased sensitivity of immunohistochemical methods. It is possible that the CNVMs of ARMD contain more ECM than in this model because the angiogenic process in ARMD is more protracted.

GFAP-positive Müller cells migrated into the subretinal space after 7 days and formed a gliotic adhesion between the retina and the SRN membrane that was most prominent at 8 weeks. The photoreceptors in the area in which microspheres accumulated were mostly degenerated and were replaced with proliferated Müller cells. It seems likely that the mechanical damage of microsphere injection or the interference of the normal metabolism of RPE cells by the accumulated microspheres induced the degeneration of photoreceptors. Müller cells tend to proliferate into the subretinal space and form multiple layers after the degeneration of photoreceptors. bFGF from the microspheres may also stimulate the proliferation of Müller cells.
A Possible Role for p16INK4 in Neuronal Cell Death after Retinal Ischemia–Reperfusion Injury

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PURPOSE. To study whether cell type-specific death occurs in retinal ischemia-reperfusion injury and the possible roles of p16INK4 in the determination of cell death. METHODS. Retinal ischemia-reperfusion injury was induced in rats by a ligation method. After 1 hour of ischemia and a time of reperfusion that varied, rat eyes were enucleated. Cell death in the retina was studied by the TdT-dUTP terminal nick-end labeling method and propidium iodide (PI) staining. Electron microscopic observation of the retina was also performed. Immunohistochemical studies using antibodies against syntaxin and calbindin were performed to detect amacrine cells and horizontal cells, respectively, and immunohistochemical studies using an antibody against p16INK4 were performed to study whether this cell cycle-related protein was expressed in dying cells. RESULTS. Most of the calbindin-positive horizontal cells in the outer aspect of the inner nuclear layer (INL) showed morphologic features of necrosis. In contrast, syntaxin-positive amacrine cells in the inner aspect of the INL showed features of apoptosis. Of 320 calbindin-positive horizontal cells, only 11 (3.4%) showed positive PI staining. Those calbindin-positive, horizontal cells were p16INK4-positive. In contrast, 746 of 910 (82.0%) syntaxin-positive amacrine cells showed condensed PI staining, and none were p16INK4-positive. CONCLUSIONS. Expression of p16INK4 may regulate the fate of retinal neurons in ischemia-reperfusion injury, and cell type-specific death thus occurs in the retina after such injury. (Invest Ophthalmol Vis Sci. 1999;40:528–533)