

# Effects of Local Administration of Interferon- $\beta$ on Proliferation of Retinal Pigment Epithelium in Rabbit After Laser Photocoagulation

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**Purpose:** To investigate whether local administration of interferon (IFN)- $\beta$  promotes proliferation of the retinal pigment epithelium (RPE) in vivo.

**Methods:** Following local injection of IFN- $\beta$  into the sub-Tenon space of rabbit eyes, the penetration of IFN- $\beta$  into various intraocular areas was determined by means of enzyme-linked immuno-adsorbent assay. Retinal lesions were produced by laser photocoagulation (PC), and IFN- $\beta$  ( $1 \times 10^6$  IU,  $1 \times 10^5$  IU, or  $1 \times 10^4$  IU) was administered into the sub-Tenon space. Physiological saline was substituted for IFN- $\beta$  in controls. The proliferation of RPE cells was inspected histopathologically.

**Results:** After IFN- $\beta$  administration, IFN- $\beta$  was found in all intraocular areas examined, with the highest concentration detected in the choroid. After PC, profuse proliferation of RPE cells began earlier in the rabbits that received the highest dose of IFN- $\beta$  than in the control rabbits; repair of the central part of the coagulated lesion in those rabbits was complete within 7 days after PC. In control rabbits, the histopathologic wound repair process proceeded more slowly and to a limited extent. Proliferation of RPE cells in the low and medium dose IFN- $\beta$ -treated rabbits was similar to that in the control rabbits.

**Conclusion:** The present study demonstrates that repair of the PC-induced retinal lesions, particularly the proliferation of RPE cells, is promoted in vivo by local administration of IFN- $\beta$ . **Jpn J Ophthalmol 2002;46:160–169** © 2002 Japanese Ophthalmological Society

**Key Words:** Interferon- $\beta$ , laser photocoagulation, local administration, retinal pigment epithelium, wound repair.

## Introduction

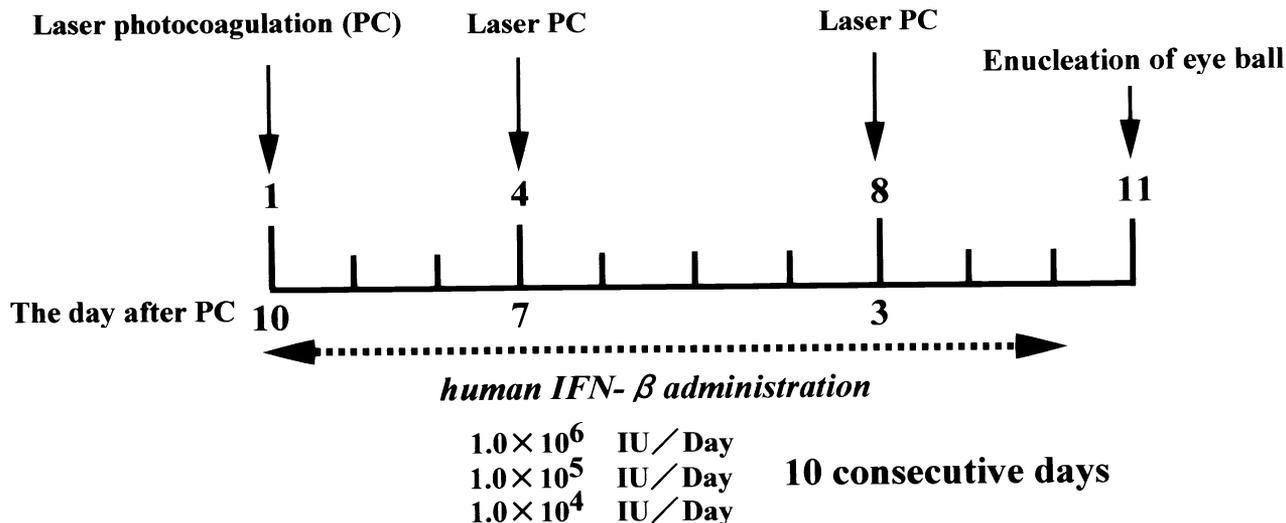
The incidence of exudative age-related macular degeneration has been increasing rapidly in recent years, and this disorder now attracts attention as a leading cause of blindness in the elderly in Western countries.<sup>1</sup> Although laser photocoagulation (PC) treatment has been shown to be beneficial for extrafoveal or juxtafoveal subretinal neovascularization, PC is applied only in select cases of subfoveal neovascularization. Because the therapeutic use of

PC is limited, other treatments have been attempted, including medication, surgery, radiotherapy, and photodynamic therapy.<sup>1–3</sup> Drugs that effectively suppress choroidal neovascularization (CNV) in various degrees have also been identified.

In recent years, interferon (IFN) has attracted attention in this respect because of its ability to suppress proliferation of vascular endothelial cells.<sup>4–6</sup> After Fung reported the therapeutic effect of IFN- $\alpha$  in the treatment of age-related macular degeneration,<sup>7</sup> other researchers performed large-scale clinical studies on IFN- $\alpha$  in Western countries.<sup>8–12</sup> The Pharmacological Therapy for Macular Degeneration Study Group recently reported that IFN- $\alpha$ -2a is ineffective for patients with CNV secondary to age-related macular degeneration.<sup>13</sup> However, IFN- $\beta$  was

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**Figure 1.** Experimental design. Rabbit retinas were moderately coagulated by laser photocoagulation (PC) on experimental days 1, 4, and 8. PC was performed at a wavelength of 595 nm, with a spot size diameter of 200  $\mu$ m, for a duration of 0.2 second, at a power of 50 mW. Because the eyeballs were enucleated on day 11, the respective histopathologic changes that were present 10 days, 7 days, and 3 days after PC were examined. IU: international unit.

shown to suppress the proliferation of vascular endothelial cells more effectively than IFN- $\alpha$ <sup>14,15</sup> and was reported to be effective in the treatment of age-related macular degeneration in Japan.<sup>16,17</sup>

Systemic administration of IFN is known to cause side effects, such as chills, fever, general malaise and neuropsychiatric symptoms,<sup>18</sup> as well as retinopathy with soft exudate.<sup>19</sup> It is also very expensive.

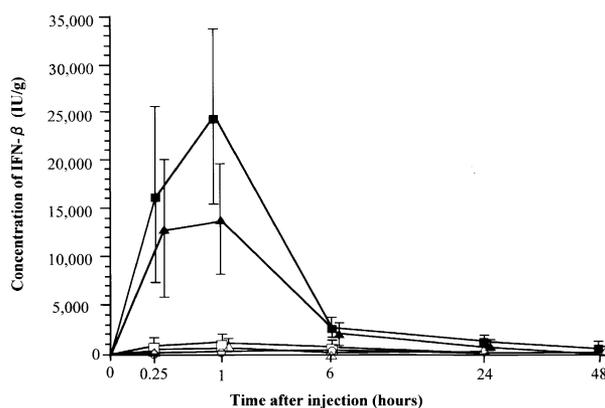
In the present study, we have attempted to apply IFN- $\beta$  locally rather than systemically to obtain a sufficiently high local concentration with a smaller dosage. The IFN- $\beta$  used was a natural product derived from human fibroblasts.<sup>20, 21</sup> After local application of IFN- $\beta$  into the sub-Tenon space of rabbit eyes, we examined its presence in various intraocular areas and its effects on the wound repair processes in areas injured by laser photocoagulation.

## Materials and Methods

### Intraocular Distribution of IFN- $\beta$ Injected Into the Sub-Tenon Space

Twenty female New Zealand white rabbits, each weighing between 2.8 and 3.1 kg, were used, and all 40 eyeballs were analyzed in this study. All procedures were carried out in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Natural human IFN- $\beta$  was supplied by Toray Industries Inc. (Kanagawa). The IFN- $\beta$  ( $5 \times 10^6$  IU in 0.5 mL of saline) was in-

jected with a 27-gauge needle into the anterior sub-Tenon space of the left eye of each rabbit. The right eye of each rabbit was untreated and served as the control. An intramuscular injection of 25 mg/kg ketamine hydrochloride was used to induce anesthesia in 4 rabbits at each of the five postinjection sampling intervals: 15 minutes and 1, 6, 24, and 48 hours after



**Figure 2.** Time course of concentration of interferon (IFN)- $\beta$  in the ocular areas after sub-Tenon administration. IFN- $\beta$  ( $5 \times 10^6$  IU in 0.5 mL of saline) was injected into the sub-Tenon space of each rabbit's left eye. The time-course for concentrations of IFN- $\beta$  in the aqueous humor ( $\Delta$ ), iris-ciliary body ( $\square$ ), vitreous body ( $\circ$ ), retina ( $\blacktriangle$ ), and choroid ( $\blacksquare$ ) are shown. The highest concentrations of IFN- $\beta$  tended to occur in the choroid. (N = 4 rabbits at each time point in each group). Bars = SD.

injection. Between 50 and 200  $\mu\text{L}$  of aqueous humor was collected from each eye by anterior chamber puncture with a 26-gauge needle. Simultaneously, 1 to 2 mL of blood was collected from each rabbit by cardiac puncture under anesthesia. The rabbits were sacrificed with an overdose of sodium pentobarbital. Both eyes of each animal were enucleated immediately after death and dissected in a cold, sterile environment. Under a dissecting microscope, the anterior segment was removed, and the vitreous and retina were aspirated. The entire choroid was scraped from the sclera with a spatula, placed in an Eppendorf tube, and weighed. All tissues were homogenized in 4.5 mL of 0.1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate in 0.5 M NaCl and centrifuged at 2,000  $g$  for 5 minutes at room temperature. Aliquots of the respective supernatants

were placed in Eppendorf sample tubes and stored frozen at  $-80^{\circ}\text{C}$  until the concentration of IFN- $\beta$  was measured. The IFN- $\beta$  concentrations in the samples were determined by means of enzyme-linked immuno-adsorbent assay.

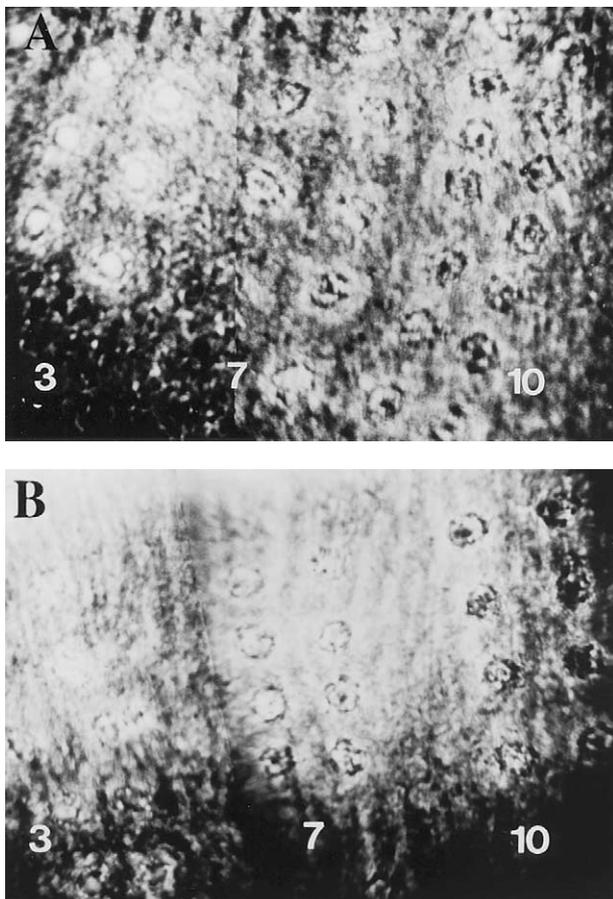
#### *Effects of Local Administration of IFN- $\beta$ on the Repair of Retinal Lesions Produced by Laser Photocoagulation*

Eight female Dutch belted pigmented rabbits, each weighing between 2.7 and 3.2 kg, were used in this experiment, and all 16 eyeballs were used. Animals were anesthetized by intramuscular injections of ketamine hydrochloride at a dose of 25 mg/kg. The pupils were dilated with 0.5% tropicamide and 0.5% phenylephrine hydrochloride, and the posterior poles of the fundus oculi were laser photocoagulated with a Dye Laser System 920 (Coherent Radiation, Santa Clara, CA, USA) at a wavelength of 595 nm, for a duration of 0.2 seconds, at a power of 50 mW, making a 200- $\mu\text{m}$  diameter spot. Eight to 12 burns were produced at the posterior pole of the retina on experimental days 1, 4, and 8, respectively. Histopathologic studies were carried out on the 24 to 36 burns produced in this manner.

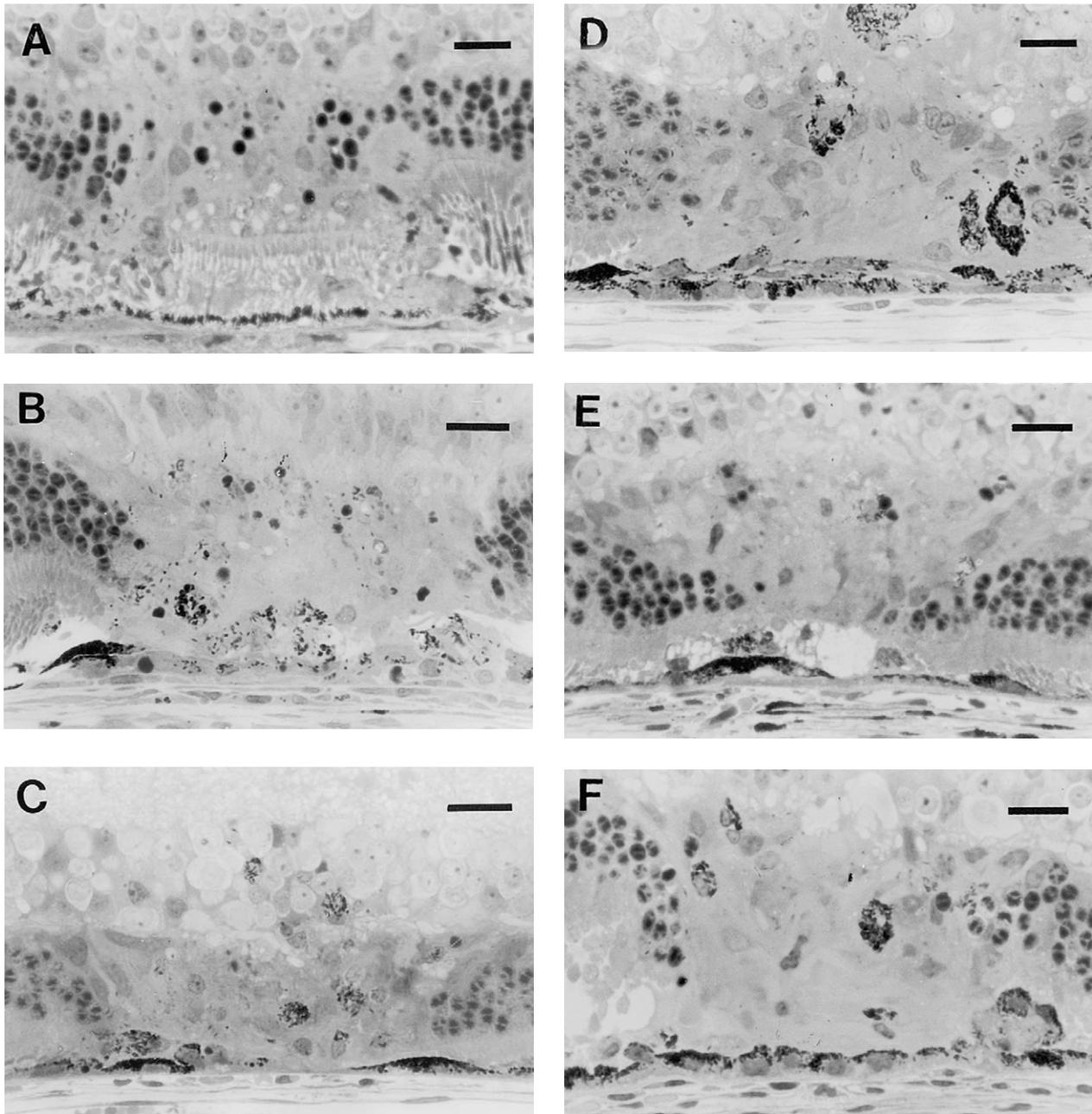
For the IFN- $\beta$ -treated group (6 rabbits, 12 eyes), IFN- $\beta$  was first dissolved in 0.5 mL of physiologic saline and injected into the bilateral sub-Tenon spaces for 10 consecutive days at a daily dose of  $1 \times 10^6$  IU (2 rabbits, 4 eyes),  $1 \times 10^5$  IU (2 rabbits, 4 eyes), or  $1 \times 10^4$  IU (2 rabbits, 4 eyes) (Figure 1). For the control group (2 rabbits, 4 eyes), physiologic saline rather than IFN- $\beta$  was injected in the same manner.

The general status of all rabbits was carefully observed after the injection of IFN- $\beta$  or saline. On day 11, funduscopy and fluorescein angiography were carried out after injection of 0.5 mL 10% fluorescein sodium (Fluorecite, Alcon, Fort Worth, TX, USA) into the ear veins. The animals were sacrificed with an overdose of pentobarbital, and the eyes were enucleated as described earlier.

Immediately after enucleation, the eyes were prefixed for 24 hours with 4% glutaraldehyde in phosphate-buffered saline (pH 7.4). Tissues were post-fixed with 1% osmium tetroxide for 2 hours. The retinochoroidal tissues, including the photocoagulated areas, were then embedded in epoxy resin 814 after dehydration in a graded alcohol series. Serial thin sections, 1  $\mu\text{m}$  in thickness, were stained with toluidine blue, and the central portion of the photocoagulated lesion was examined by light microscopy. Ultrathin sections were prepared, double-stained with



**Figure 3.** Fluorescein angiography. (A) Control eyes, (B) interferon (IFN)- $\beta$ -treated eyes ( $1 \times 10^6$  IU). In the IFN- $\beta$ -treated eyes, in contrast to the control eyes, fluorescein leakage has already ceased within 3 days of photocoagulation (PC). Numerals indicate days after PC.



**Figure 4.** Light micrographs of the coagulated areas in the control and interferon (IFN)- $\beta$ -treated eyes ( $1 \times 10^6$  IU) (stained with toluidine blue). **(A)** Control eyes 3 days after photocoagulation (PC). A marked amount of cell debris still remains in the outer retina in the coagulated area. A few retinal pigment epithelial (RPE) cells proliferate at the margin of the coagulated area. Bar = 20  $\mu$ m. **(B)** IFN- $\beta$ -treated eyes 3 days after PC. A small amount of cell debris is still found in the necrotic lesion in the coagulated area. RPE cells are irregularly distributed. They proliferate profusely and begin to migrate toward the center of the coagulated area. In the subretinal space, there are macrophages that phagocytose a large amount of cell debris. Bar = 20  $\mu$ m. **(C)** Control eyes 7 days after PC. Necrotic tissues in the outer retina have already disappeared, but repair of RPE cells on the Bruch's membrane in the center of the coagulated area is still incomplete. Bar = 20  $\mu$ m. **(D)** IFN- $\beta$ -treated eyes 7 days after PC. Proliferated RPE cells migrate to the center of the coagulated area. Almost complete repair is observed. Bar = 20  $\mu$ m. **(E)** Control eyes 10 days after PC. RPE cells containing a few melanin granules proliferate over Bruch's membrane, forming a monolayer. Bar = 20  $\mu$ m. **(F)** IFN- $\beta$ -treated rabbits 10 days after PC. Cuboidal RPE cells with abundant melanin granules completely cover Bruch's membrane. Bar = 20  $\mu$ m.

uranyl acetate and lead citrate, and examined using an electron microscope (Model H-600, Hitachi, Tokyo).

## Results

### *Intraocular Penetration of IFN- $\beta$*

The time-courses for different concentrations of IFN- $\beta$  in the aqueous humor, iris-ciliary body, vitreous body, retina, and choroid are shown in Figure 2. IFN- $\beta$  concentration peaked 1 hour after injection in all ocular tissues. Levels decreased to undetectable levels within 48 hours in all samples except those from the aqueous humor and choroid. The highest concentrations of IFN- $\beta$  most commonly occurred in the choroid, where the concentration was  $16,179 \pm 10,828$  IU/g 15 minutes after injection and  $24,662 \pm 9,535$  IU/g 1 hour after injection. The concentration of IFN- $\beta$  in the serum ( $210 \pm 191$  IU/g) was highest after 15 minutes, although it was less than 1% of that in the choroid. After 6 hours, the concentration of IFN- $\beta$  in the serum had decreased further, reaching a level lower than in any other region of the eye.

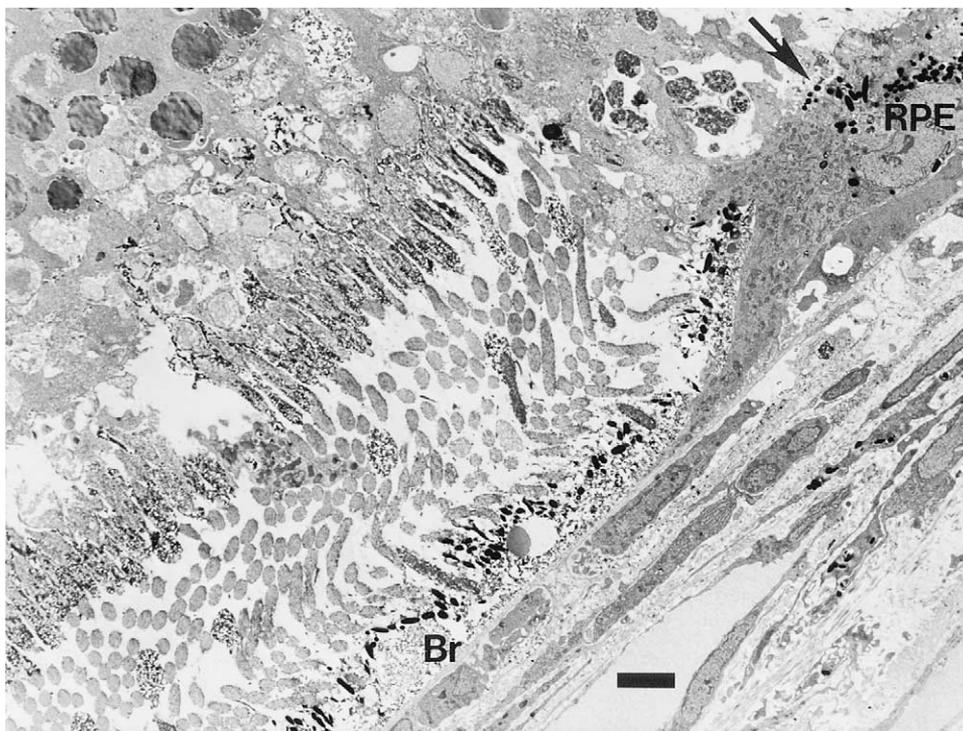
In the contralateral control eyes, the concentration of IFN- $\beta$  was  $420 \pm 272$  IU/g in the choroid and

$430 \pm 317$  IU/g in the retina 1 hour after treatment. These values in the choroid and retina were higher than in the serum. The IFN- $\beta$  concentrations in the aqueous humor ( $64 \pm 27$  IU/g), iris-ciliary body ( $74 \pm 43$  IU/g), and vitreous body ( $20 \pm 18$  IU/g) were lower than those in the serum. After 24 hours, the concentration of IFN- $\beta$  in the control eyes was below the limit of detection.

### *Effect of Local IFN- $\beta$ Administration on Repair of the Laser Photocoagulated Retina*

**Funduscopy observations.** In control eyes, the PC spots appeared as whitish retinal edema 3 days after PC. Seven days after PC, the coagulated spot took on a grayish white tinge, and by 10 days after PC, the retinal edema resolved. During fluorescein angiography, dye leaked profusely from the coagulated spots on day 3, but no leakage was observed by 7 days after PC (Figure 3A).

In rabbits that had received IFN- $\beta$  in doses of  $1 \times 10^5$  IU and  $1 \times 10^4$  IU, changes in the coagulated spots were similar to those in the control group. In those rabbits that had received IFN- $\beta$  at a dose of  $1 \times 10^6$  IU, the coagulated spots showed a grayish-whit-



**Figure 5.** Electron micrograph of the center of the coagulated area 3 days after laser photocoagulation in control eyes. A large amount of cell debris is seen in the outer retina of the photocoagulated site. Retinal pigment epithelial (RPE) cells in the center of the photocoagulated site underwent necrosis, but a small number of RPE cells (arrow) are seen proliferating in the margin of the coagulated site. Br: Bruch's membrane. Bar = 5  $\mu$ m.

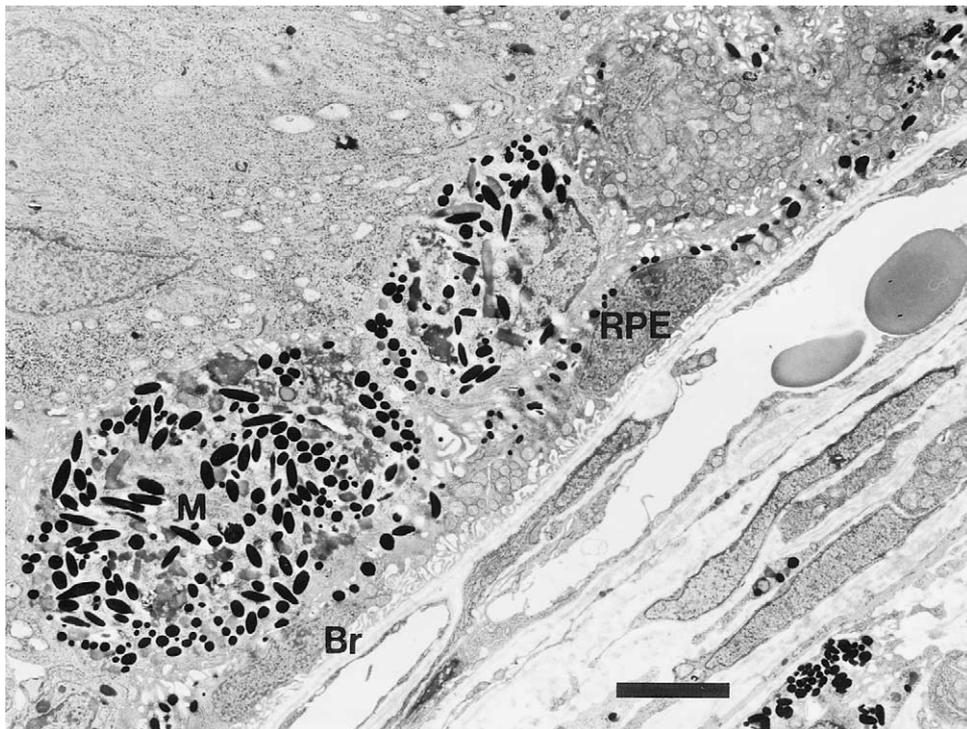
ening, and the retinal edema was already less apparent 3 days after PC. During fluorescein angiography 3 days after PC, there was minimal dye leakage from the PC spots, vague hyperfluorescence in the center of PC spots and annular hyperfluorescence in their margins (Figure 3B).

### *Histopathologic Findings*

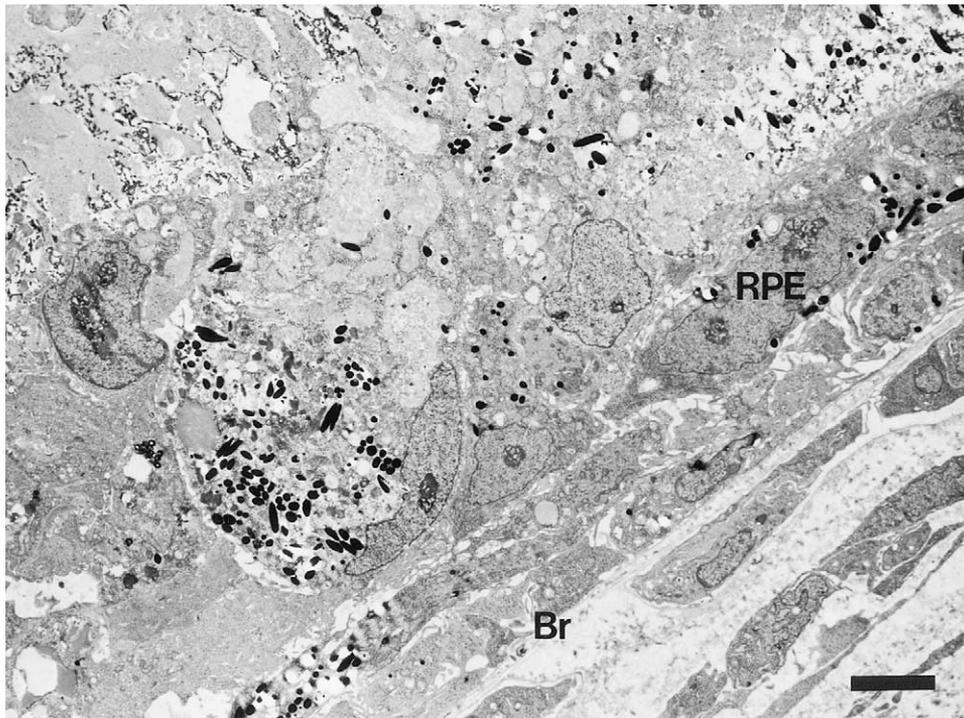
**Control rabbit eyes.** Light microscopy 3 days after PC revealed dome-shaped coagulative necrosis of cells from the retinal pigment epithelium (RPE) layer through the outer nuclear layer. A number of cells were found to be proliferating at the border of the coagulated area (Figure 4A). Electron microscopy 3 days after PC revealed some cells in the coagulated areas that exhibited coagulative necrosis, but the majority of cells in the photoreceptor layer and the outer nuclear layer of the retina survived. RPE cells showing necrotic changes remained on Bruch's membrane (Figure 5). Within 7 days after PC, necrotic cells in the outer layers of the retina had almost completely disappeared. Repairs in the RPE layer progressed only to a limited extent (Figure 4C). Ten days after PC, flat RPE cells began to proliferate from the border of the

coagulated areas toward the center, and Bruch's membrane was completely covered by a monolayer of flat RPE cells (Figure 4E). Electron microscopy revealed a monolayer of flat RPE cells with scanty melanin granules on Bruch's membrane of the coagulated lesion, and large macrophages that had phagocytosed a massive amount of melanin granules present on the RPE cells (Figure 6).

**IFN- $\beta$ -treated group.** No significant histopathologic differences were found between the control eyes and the eyes treated with IFN- $\beta$  at doses of  $1 \times 10^4$  IU and  $10 \times 10^5$  IU. In those rabbits that received IFN- $\beta$  at a dose of  $1 \times 10^6$  IU, a small number of necrotic cells remained in the coagulated areas 3 days after PC. The RPE cells proliferated vigorously and migrated from the margin of the coagulated areas toward the center (Figure 4B). Under electron microscopy, the amount of debris derived from the necrotic cells was less in the IFN- $\beta$ -treated eyes than in controls. Cells situated on Bruch's membrane had microvilli, particularly on the neuroretinal side, and a small number of melanin granules in the cytoplasm. These cells had well-defined nucleoli and a small number of immature cell junction apparatus.



**Figure 6.** Electron micrograph of the coagulated spot 10 days after laser photocoagulation in control eyes. Flat retinal pigment epithelial (RPE) cells containing scanty melanin granules, cover Bruch's membrane (Br), forming a monolayer. Macrophages (M) that have phagocytosed a massive amount of melanin pigment are seen over the RPE cells. Bar = 5  $\mu$ m.



**Figure 7.** Electron micrograph of the center of the coagulated area 3 days after laser photocoagulation in interferon (IFN)- $\beta$ -treated eyes ( $1 \times 10^6$  IU). Retinal pigment epithelial (RPE) cells with microvilli on their outer surface and a nucleus with a clear-cut nucleolus in the cytoplasm are abundant on Bruch's membrane. The cytoplasm also contains a large number of mitochondria and a small number of melanin granules. A few immature cell-adhesion apparatus are observed between the cells, making it possible to identify these as RPE cells. Bar = 5  $\mu$ m.

On the basis of these morphological characteristics, the cells were identified as RPE cells (Figure 7). Seven days after PC, RPE cells were seen to proliferate in the center of the coagulated lesion. The cells were regularly arranged in two or three layers, indicating that complete repair of the photocoagulated lesion had taken place. Necrotic debris in the outer layers of the retina was completely removed by macrophages that phagocytosed the dead cell debris together with the pigment granules (Figure 4D). Ten days after PC, a monolayer of cuboidal RPE cells was found to have covered Bruch's membrane (Figure 4F). Electron microscopy revealed that the RPE cells, which contained abundant pigment granules and intracellular organelles, formed one or two layers on Bruch's membrane (Figure 8).

### Discussion

Interferon is known to generate various biological activities, including antiviral, antitumor, and immunoregulatory effects.<sup>20–22</sup> In recent years, *in vitro*

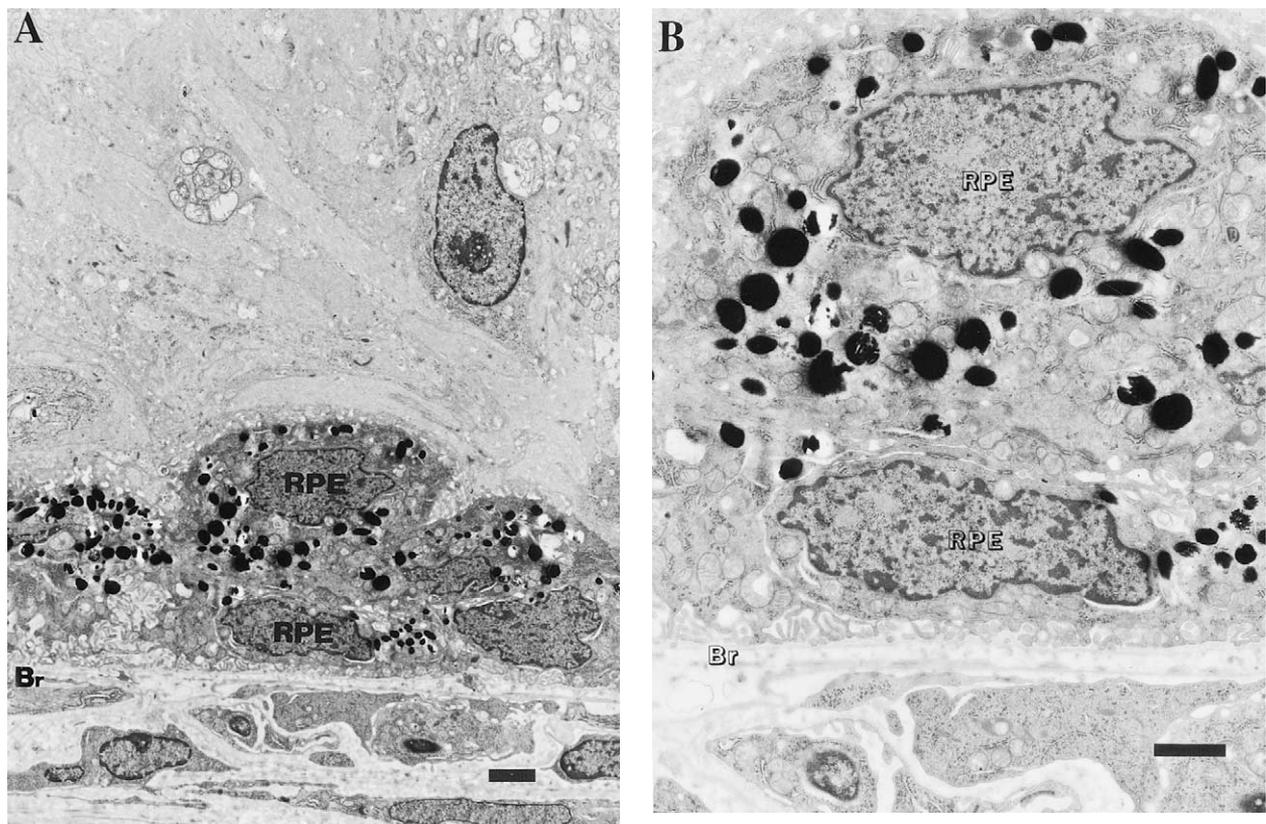
studies have demonstrated that IFN suppresses neovascularization by inhibiting the proliferation and migration of vascular endothelial cells.<sup>4–6</sup> Clinically, IFN- $\alpha$  was found to be effective for the management of hemangiomas in infants.<sup>23,24</sup> In ophthalmology, Fung<sup>7</sup> first reported that systemic administration of IFN- $\alpha$ -2a was effective in patients with CNV associated with age-related macular degeneration. These observations led to several pilot studies that used interferon for the treatment of CNV.<sup>8–12</sup> A large-scale controlled trial of IFN- $\alpha$  therapy by the Pharmacological Therapy for Macular Degeneration Study Group<sup>13</sup> suggested that IFN- $\alpha$  was not an effective treatment for exudative age-related macular degeneration. However, we noticed that the subjects enrolled in their study included patients in various stages of age-related macular degeneration, from the silent stage to very extensive CNV. In Japan, several investigators have reported the effects of systemic administration of IFN- $\beta$ <sup>14–17</sup>; these studies confirmed that IFN- $\beta$  was effective in early CNV in age-related macular degeneration. In our previous study, we

demonstrated that systemic administration of IFN- $\beta$  promotes proliferation of RPE cells and limits neovascularization, leading to the regression of CNV in monkey eyes.<sup>26</sup> We have also observed that systemic administration of IFN- $\beta$  after PC was remarkably effective in suppressing the reconstruction of the choriocapillaris,<sup>27</sup> and that systemic administration of IFN- $\beta$  after PC also promotes proliferation of RPE cells.<sup>28</sup> These results indicate that systemic administration of IFN- $\beta$  could be effective for treating clinical and experimental CNV.

Systemic administration of IFN results in various side effects;<sup>18,19</sup> therefore, we considered whether local administration, if possible, would be preferable to systemic administration. We determined the accumulation of IFN- $\beta$  in the intraocular areas after local administration. Lincoff et al<sup>25</sup> injected  $1 \times 10^6$  IU of IFN- $\alpha$ -2a retrobulbarly and measured the time course of IFN- $\alpha$ -2a accumulation in the choroid. IFN- $\alpha$ -2a detected in the choroid reached a maximum level of 32,000

IU/mg 2 hours after retrobulbar injection and then decreased to an undetectable level after 24 hours. The concentration of IFN- $\alpha$ -2a in the serum reached a maximum 4 hours after administration (227 IU/mg) but remained less than 1% of that in the choroid. Serum concentration of IFN- $\alpha$ -2a also decreased to an undetectable level after 24 hours. This observation about IFN- $\alpha$ -2a by Lincoff et al<sup>25</sup> is in line with the results we obtained with IFN- $\beta$ . Our present results with IFN- $\beta$  showed that the IFN- $\beta$  level in the choroid reached a maximum 1 hour after injection into the sub-Tenon space but decreased rapidly within 24 hours.

We carried out *in vivo* experiments with rabbits to examine the effect of IFN- $\beta$  on RPE cells. Tobe et al<sup>28</sup> reported a similar experiment in which they produced a medium-grade laser PC lesion in rabbit retina and examined the subsequent RPE cell repair process. In control eyes, the time course for the repair process in our experiment was similar to that observed by Kishimoto et al.<sup>29</sup> Three days after PC,



**Figure 8.** Electron micrograph of the coagulated area 10 days after laser photocoagulation in the interferon (IFN)- $\beta$ -treated eyes ( $1 \times 10^6$  IU). **(A)** A number of polarized cells are seen with microvilli on their outer surface, particularly on the neuroretinal side, with basal infolding towards the Bruch membrane (Br). Bar = 2  $\mu$ m. **(B)** These cells have abundant microvilli on their outer surface and a large number of mitochondria and melanin granules. These morphological features, together with the presence of many cell-adhesion apparatus and cell polarity, help to identify these as RPE cells. Bar = 2  $\mu$ m.

only a small number of RPE cells had begun to proliferate in the marginal region of the coagulated lesion (Figure 4A). Seven days after PC, there was moderate proliferation of RPE cells on Bruch's membrane (Figure 4C). By 10 days after PC, one or two layers of proliferated RPE cells had covered Bruch's membrane of the coagulated area (Figure 4E). In the IFN- $\beta$ -treated rabbits, profuse proliferation of the RPE cells was already observed 3 days after PC, and these cells were found to proliferate from the periphery toward the center of the coagulated area (Figure 4B). Seven days after PC, proliferation of the RPE cells was seen in the center of the coagulated area, and one or two layers of these cells completely covered Bruch's membrane of the same area (Figure 4D). Within 10 days after PC, RPE cells had formed a monolayer of cuboidal cells covering Bruch's membrane, with abundant adhesion apparatus expressed on the cells (Figure 4F). These results show that local administration of IFN- $\beta$  promotes proliferation of the RPE cells in the area injured by laser PC. Siren et al.<sup>30</sup> demonstrated by *in vitro* experiments that receptors to IFN- $\alpha$  and IFN- $\gamma$  are distributed in human RPE cells and that IFN- $\alpha$  and IFN- $\gamma$  suppress PAI-1 gene expression in these cells. These observations suggest the possibility of an *in vitro* action of IFN on RPE cells.

We administered IFN- $\beta$  daily at three doses (ie,  $1 \times 10^6$  IU,  $1 \times 10^5$  IU and  $1 \times 10^4$  IU). Repair did not differ between those rabbits that received low doses of IFN- $\beta$  ( $1 \times 10^5$  IU and  $1 \times 10^4$  IU) and control rabbits, indicating that only the high dose of  $1 \times 10^6$  IU induced proliferation of RPE cells. Because human IFN- $\beta$  was applied to rabbits in the present study, species specificity should also be taken into consideration. IFN- $\beta$  at a dose of  $1 \times 10^6$  IU in the rabbit corresponds to  $3.3 \times 10^5$  IU/kg body weight. In a human weighing 50 kg, this corresponds to a daily dose of  $1.7 \times 10^7$  IU, or three times the clinical dose for intravenous administration ( $6 \times 10^6$  IU). However, it is known that human IFN- $\beta$  exerts its action in a species-specific manner,<sup>31</sup> thus the activity exerted by human IFN- $\beta$  in monkeys amounted to only 12%, and in rabbits to 1%, of the activity expected in humans. If this holds true,  $1.7 \times 10^7$  IU in rabbits would correspond to  $1.7 \times 10^5$  IU in humans or about 1/30 of the systemic dose that is applied for clinical purposes. This suggests the feasibility of local administration, which can alleviate the annoying side effects that are unavoidable with systemic administration.

Our present study has proved that local administration of IFN- $\beta$  promotes proliferation of RPE cells in retinal lesions produced by laser photocoagulation.

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