Effect of Focal X-ray Irradiation on Experimental Choroidal Neovascularization

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PURPOSE. Radiation therapy has been used to treat choroidal neovascularization (CNV) in patients with age-related macular degeneration. The in vivo effect of applying focal x-ray irradiation to the eye of rabbits with experimental CNV was investigated.

METHODS. CNV was induced in the rabbit eyes by subretinal implantation of gelatin hydrogel microspheres impregnated with basic fibroblast growth factor. Three weeks after implantation, 17 of 34 eyes with CNV lesions accompanied by fluorescein leakage were irradiated with a single dose of 20 Gy; the other 17 eyes were not irradiated and served as the controls. The eyes were examined before irradiation and 1, 2, and 4 weeks after irradiation, by indirect ophthalmoscopy and fluorescein angiography. The degree of a decreasing amount of fluorescein leakage from the CNV lesions after irradiation was graded using a computerized image analysis system and was compared in the irradiated and nonirradiated eyes. These eyes were also examined histologically and immunohistochemically.

RESULTS. Fluorescein leakage from the CNV lesions had significantly decreased in the eyes irradiated with 20 Gy compared with the control eyes, throughout the study period ($P < 0.05$). Histologic and immunohistochemical studies at 4 weeks after irradiation demonstrated that the degree of vascular formation and the number of vascular endothelial cells in the subretinal membrane of the irradiated eyes were less than those of the control eyes.

CONCLUSIONS. Focal x-ray irradiation at the ocular region effectively reduced experimental CNV activity. These results support the possibility that radiation therapy may be beneficial in treating CNV. (*Invest Ophthalmol Vis Sci.* 1999;40:1496–1502)

Radiation therapy has recently been introduced to treat subfoveal choroidal neovascularization (CNV) in patients with age-related macular degeneration. Chakravarthy et al.[1] treated 19 patients with age-related macular degeneration with a dose of irradiation of 10 Gy or 15 Gy in their pilot study. At 6 and 12 months after irradiation, visual acuity was either maintained or had improved in 63% to 78% of the patients. Other studies have administered a total dose of between 5 Gy and 24 Gy in 1 to 10 fractions and have supported this finding.[2-5] On the other hand, it was reported that radiation therapy had no beneficial effect on patients with age-related macular degeneration.[6-8] Thus, it is still controversial whether focal radiation therapy is actually effective in treating subfoveal CNV associated with age-related macular degeneration. Despite these advances, experimental studies on the in vivo effects of focal irradiation on CNV have not been performed. It is therefore valuable and necessary to determine whether focal irradiation directed at ocular regions is effective in suppressing CNV activity.

Basic fibroblast growth factor (bFGF) is a potent mitogen of capillary endothelial cells and is capable of inducing angiogenesis in vivo.[9-15] Implanting gelatin hydrogels impregnated with bFGF induces neovascularization in vivo by means of a continuous release of bioactive bFGF, with eventual degradation of the gelatin hydrogel.[16] A new rabbit model of CNV has recently been developed, in which CNV is induced by the subretinal implantation of bFGF-impregnated gelatin hydrogel microspheres.[17] In that study, 83% of the eyes that received bFGF-impregnated gelatin microspheres developed CNV accompanied by definite fluorescein leakage.

In this study, using this rabbit model of CNV, we evaluated the effect of focal x-ray irradiation of the rabbit eye on the level of CNV activity by indirect ophthalmoscopy, fluorescein angiography, light microscopy, and immunohistochemistry.

MATERIALS AND METHODS

Preparation of bFGF-Impregnated Gelatin Hydrogel Microspheres

CNV was induced in the rabbit according to a slight modification of the method described by Kimura et al.[17] Gelatin (Nitta

Gelatin, Osaka, Japan) was heated in distilled water at 40°C for 30 minutes, resulting in a 10% (wt/vol) aqueous solution of gelatin, with an isoelectric point of 4.9 and a molecular weight of 99,000 kDa. Ten milliliters of the gelatin aqueous solution was added to 375 ml of olive oil (Wako Pure Chemical, Osaka, Japan) while stirring at 420 rpm for 10 minutes at 40°C to yield a water-oil emulsion. The temperature of the emulsion was decreased to 15°C. The mixture was continuously stirred for 30 minutes to complete the gelation of gelatin in the water phase. After the addition of 100 ml of acetone (Wako Pure Chemical), the emulsion was further stirred for 1 hour. The resultant microspheres were washed twice with acetone and isopropyl alcohol (Wako Pure Chemical). They were then centrifuged at 5000 rpm for 5 minutes, and suspended in isopropyl alcohol. The microspheres were passed through sieves with apertures of 20 μm and 45 μm. They were then centrifuged again at 5000 rpm for 5 minutes and dried.

The non-cross-linked gelatin microspheres were placed in a 10 mM glutaraldehyde aqueous solution (Wako Pure Chemical) containing 0.1% (wt/vol) Tween 80 (Wako Pure Chemical); this solution was stirred at 4°C for 15 hours to facilitate their cross-linking. The microspheres were further agitated by placing them in 100 ml of 10 mM glycin aqueous solution (Wako Pure Chemical), at 37°C for 1 hour. The resultant microspheres were washed with distilled water, centrifuged at 5000 rpm for 5 minutes, and lyophilized.

Human recombinant bFGF, having an isoelectric point of 9.6 (Kaken Pharmaceutical, Tokyo, Japan), was impregnated into the gelatin hydrogel microspheres by placing the dried microspheres in an aqueous solution of bFGF. Five milligrams of the cross-linked microspheres was placed in 100 μl of distilled water containing 100 μg of bFGF at room temperature for 1 hour. Thereafter, 400 μl of phosphate-buffered saline (PBS, pH 7.4) was added to result in a 1% (wt/vol) microsphere suspension for the following in vivo experiments.

Induction of CNV

Forty-four rabbits of either sex, weighing between 2.5 kg and 3.5 kg, were used. All procedures were carried out in accordance with the ARVO Statement of the Use of Animals in Ophthalmic and Vision Research. The rabbits were anesthetized with an intramuscular injection of a solution consisting of ketamine hydrochloride (24 mg/kg) and xylazine hydrochloride (6 mg/kg). The pupil of the right eye of each rabbit was dilated with 1% tropicamide and 2.5% phenylephrine hydrochloride eye drops. The ocular surface was anesthetized with a topical application of 0.4% oxybuprocaine hydrochloride eye drops.

A paralimbal conjunctival incision was made 4 mm from the temporal limbus of the right eye. The sclera was exposed, and a 1-mm sclerotomy was made with a microsurgical vitreal retinal blade 2 mm from the limbus, using an operating microscope. A glass micropipette with an 80-μm tip was introduced through the sclerotomy and was advanced into the retina. Fifty microliters of the 1% (wt/vol) gelatin microsphere–PBS suspension that contained 10 μg of bFGF was injected into the subretinal space. This resulted in serous retinal detachment. The sclerotomy and overlying conjunctiva were closed with 7-0 vicryl sutures. A topical antibiotic ointment was applied to the eye of each animal at the end of the procedure to reduce the risk of postoperative infection.
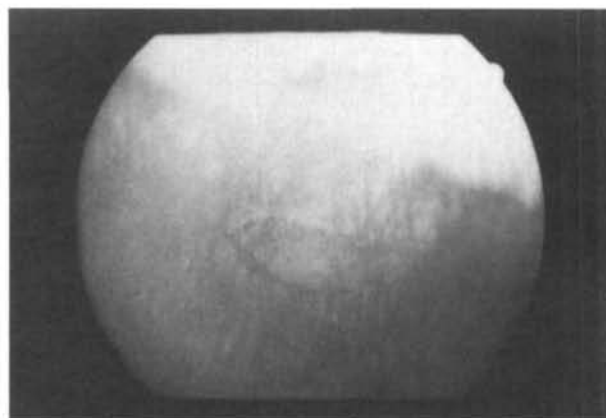

FIGURE 1. A fundus photograph of a rabbit eye 3 weeks after implantation of the bFGF-impregnated gelatin microspheres. A whitish lesion with hyperpigmentation can be seen at the lower part of the area of implantation.

The 44 eyes into which bFGF-impregnated gelatin microspheres had been implanted were examined weekly by indirect ophthalmoscopy and fluorescein angiography. By gross observation, the serous retinal detachment flattened spontaneously within 2 days after subretinal implantation of the microspheres. The subretinally administered microspheres accumulated at the lower part of the area of implantation. Although leakage of the microspheres from the area of implantation into the vitreous cavity was observed in some eyes, no significant inflammation occurred in the vitreous cavity of any eyes. No choroidal hemorrhage was seen during or after surgery in any eyes. Whitish lesions with hyperpigmentation were observed at the lower part of the implanted area, 2 to 3 weeks after implantation of the microspheres, in 34 of the 44 eyes (77% ; Fig. 1). These lesions were associated with definite fluorescein leakage and suggest the development of CNV. These 34 eyes were used for the following studies.
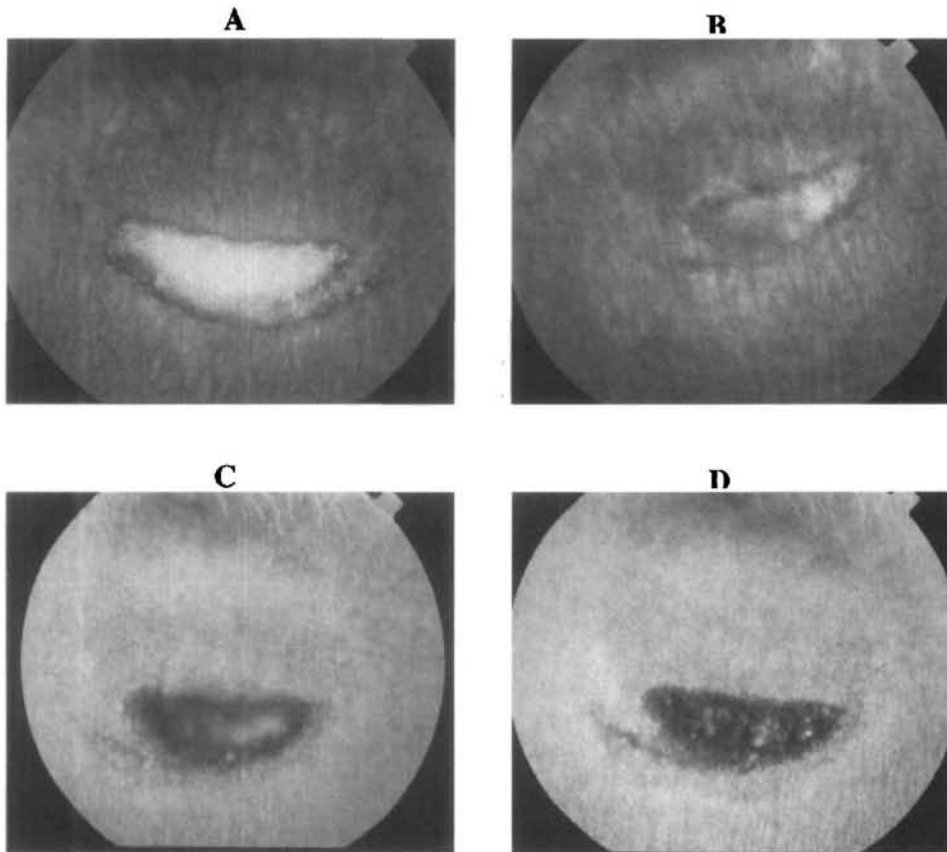
X-ray Irradiation

The right eye of the anesthetized rabbit was centered in a circular opening (12 mm in diameter) of a lead shield that had a thickness of 6 mm. The rest of the body of the rabbit was also shielded with the lead shield. An MI-201 x-ray unit equipped with a 0.5-mm copper filter (Shimadzu, Shiga, Japan), operated at 250 kV(p) and 16 mA, was used to apply radiation to the eyeball. The x-ray irradiation was delivered at a rate of 1.8 Gy/min from a distance of 50 cm.

To determine whether irradiation affects the angiogenic ability of bFGF-impregnated gelatin hydrogel microspheres, other bFGF-impregnated gelatin hydrogel microspheres were pretreated with a single irradiation dose of 20 Gy before implantation. These hydrogel microspheres were implanted into the subretinal space of four other animals.

Clinical Studies

The 34 eyes showing fluorescein leakage from the CNV lesions were divided into two groups, each containing 17 eyes. The eyes in the first group were irradiated with a single dose of 20 Gy 3 weeks after implantation of the bFGF-impregnated microspheres. The eyes in the second group were not irradiated and served as the controls. Eight of the 17 eyes in each group were

FIGURE 2. Representative fluorescein angiograms of a rabbit eye irradiated with a single dose of 20 Gy taken before irradiation and 1, 2, and 4 weeks after irradiation are shown. (A) The angiogram taken before irradiation (3 weeks after implantation of the bFGF-impregnated gelatin microspheres) shows actively leaking fluorescein in the hypofluorescent lesion. (B) Fluorescein leakage 1 week after irradiation had decreased by a mild amount from that before irradiation (decrease rate 42%, grade 1). (C) Fluorescein leakage 2 weeks after irradiation had decreased by a moderate amount from that before irradiation (decrease rate 71%, grade 2). (D) Fluorescein leakage 4 weeks after irradiation had decreased by a marked amount from that before irradiation (decrease rate 84%, grade 3).

examined by indirect ophthalmoscopy and fluorescein angiography before irradiation and 1, 2, and 4 weeks after irradiation. Fluorescein angiography was performed with intravenous administration of a solution (0.2 ml) containing 40 mg sodium fluorescein, and the eyes were examined with a TRC 50IA fundus camera (Topcon, Tokyo, Japan). The decrease in area of fluorescein leakage from the CNV lesions after irradiation was quantified in a masked fashion by computerized image analysis using image software (Image 1.44; National Institutes of Health, Bethesda, MD) on a Power Macintosh 8500/120 computer. Each measurement was graded as follows: Grade 0, no decrease (decrease rate in area <10%) or increase; grade 1, mild decrease (decrease rate in area 10%-50%); grade 2, moderate decrease (decrease rate in area 51%-75%); or grade 3, marked decrease (decrease rate in area >75%). Representative photographs of each grade are shown in Figures 2 and 3. Figures 2A and 3A show CNV lesions before irradiation, with actively leaking fluorescein. The CNV lesions in Figures 2B, 2C, and 2D show decreased rates of 42% (grade 1), 71% (grade 2), and 84% (grade 3), respectively. The CNV lesions in Figures 3B, 3C, and 3D show a slight increase (increased rate in area 3%, 6%, and 10%, respectively), and all these eyes were graded as grade 0.

Statistical Analysis

Data obtained from the eyes of the irradiated group and the control group were compared using the Mann–Whitney *U* test. A difference at a level of $P < 0.05$ was considered to be statistically significant.

Histologic Studies

The right eyes of six of the remaining rabbits in each study group were subjected to histologic examination. These rabbits were killed by intravenous administration of a lethal dose of pentobarbital at 3, 4, and 7 weeks after implantation of the microspheres (i.e., before irradiation and 1 and 4 weeks after irradiation; $n = 2$ at each time point). The eye was enucleated and immediately placed in 2.5% glutaraldehyde and 2% formaldehyde in 0.1 M PBS (pH 7.4) for 15 minutes. The cornea, lens, and vitreous were carefully removed from the eye and placed in the fixative for an additional 24 hours at 4°C. From each eye, the area where the hydrogel had been implanted was resected under the dissecting microscope. Each specimen was embedded in paraffin. Sections of 2 μm to 3 μm were made and stained with hematoxylin-eosin or trichrome, for light microscopy.

Immunohistochemical Studies

The right eye of the other three rabbits in each study group were subjected to immunohistochemical examination. These rabbits were killed by the method mentioned above at 4 weeks after irradiation. The eye was enucleated and its cornea, lens, and vitreous were removed. The tissue was fixed in 4% paraformaldehyde, embedded in OCT compound (Tissue-Tek; Sakura Seiki, Tokyo, Japan), 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 (Wako Pure Chemical) in PBS after blocking of endogenous peroxidase by 0.3% hy-
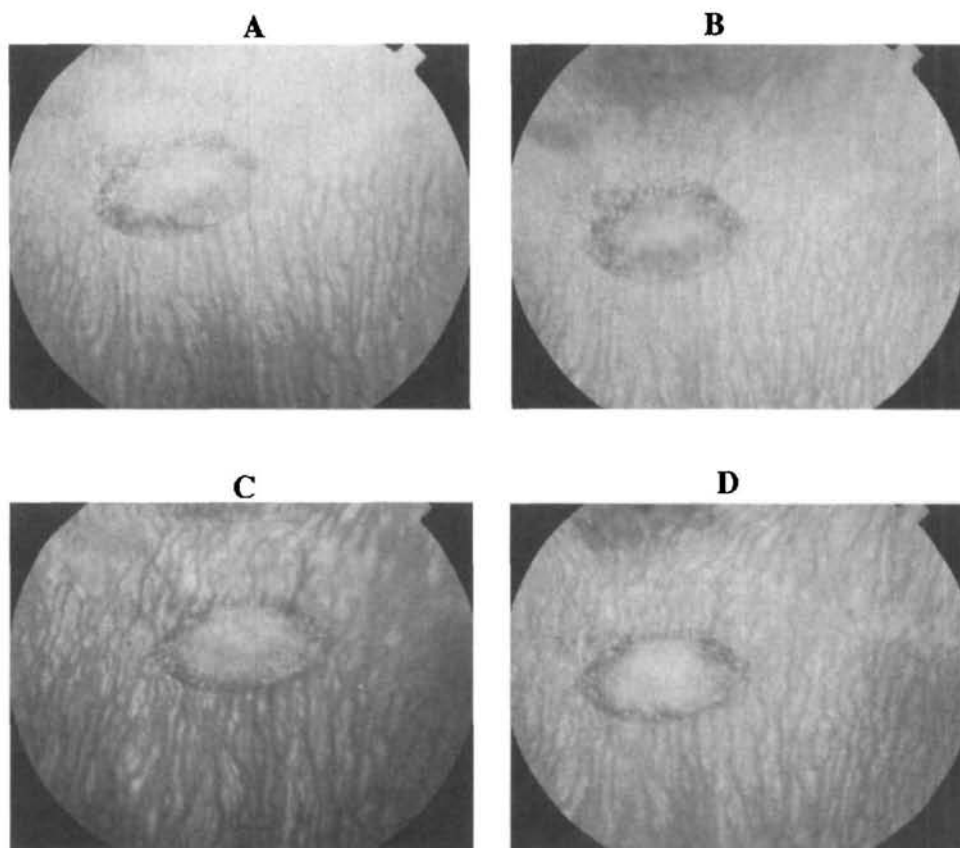
FIGURE 3. Representative fluorescein angiograms of a nonirradiated rabbit eye. A The angiogram taken 3 weeks after implantation of the bFGF-impregnated gelatin microspheres shows actively leaking fluorescein in the hypofluorescent lesion. (B, C, D) Angiograms taken 4, 5, and 7 weeks, respectively, after implantation of the bFGF-impregnated microspheres (the same time points at which the angiograms were obtained in the irradiated rabbits) reveal a slight increase (increase rate in area; **B**, 3%; **C**, 6%; **D**, 10%) rather than a decrease in the level of fluorescein leakage, compared to the angiogram taken 3 weeks after implantation (A). All these eyes were graded as grade 0.

drogen 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 (Pathostain ABC-POD(M) Kit; Wako Pure Chemical), and aminoethyl-carbazole (AEC; Dako, Carpinteria, CA) 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); CD31 (mouse anti-human; Dako); and RAM11 (mouse anti-rabbit macrophage; Dako).

RESULTS

Clinical Studies

Figure 4 shows the grade of decrease of fluorescein leakage from the CNV lesions in the nonirradiated control eyes and in the eyes irradiated with a single dose of 20 Gy. In the control eyes, grades 2 and 3 were not noted at each time point. In contrast, in the irradiated eyes, 2 of 8 eyes (25%) were graded as grade 3, and 3 of 8 eyes (37.5%) were graded as grade 2 by 4 weeks after irradiation. A significant difference in grade was observed between the irradiated eyes and the nonirradiated eyes, throughout the follow-up period (Mann–Whitney U test, $P < 0.05$).

Ophthalmoscopically, the whitish lesions of CNV in the eyes of both study groups turned into hyperpigmented lesions over time. Hyperpigmentation of the CNV lesions in the irradiated eyes seemed to occur earlier than in the control eyes.

In three of the four eyes (75%) into which preirradiated hydrogel microspheres impregnated with bFGF had been im-

planted, definite fluorescein leakage from the CNV lesions was observed. This indicates that irradiation does not affect the angiogenic ability of the bFGF-impregnated hydrogel microspheres.

Histologic Studies

Three weeks after the hydrogel microspheres were implanted (before irradiation), vessels that had newly formed from the choriocapillaris were seen beneath the retina of all the eyes that showed fluorescein leakage. At this point, many microspheres were still present and were surrounded by retinal pigment epithelial (RPE)-like cells.

One week after irradiation, a membranelike structure (which was mainly composed of RPE-like cells, an extracellular matrix, and new vessels) was observed in the nonirradiated eyes. The lesions in the irradiated eyes demonstrated nearly the same histologic features as those in the nonirradiated eyes.

Four weeks after irradiation, there was a striking difference between the irradiated and nonirradiated eyes. In the nonirradiated eyes, fibrovascular membranes were observed beneath the retina (Fig. 5). Numerous new vessels extended from the choriocapillaris. A few microspheres were still present beneath the retina. In contrast, the subretinal membrane of the irradiated eyes consisted mainly of a large number of RPE-like cells that showed pseudocinar structures (Fig. 6A). A few new vessels, some of which were surrounded by RPE-like cells, were observed in the membrane (Fig. 6B). Moderate outer retinal degeneration and mild choroidal atrophy were seen only in the areas where microspheres were implanted, not only in the irradiated eyes but also in the nonirradiated eyes (Figs. 5, 6A, 6B).
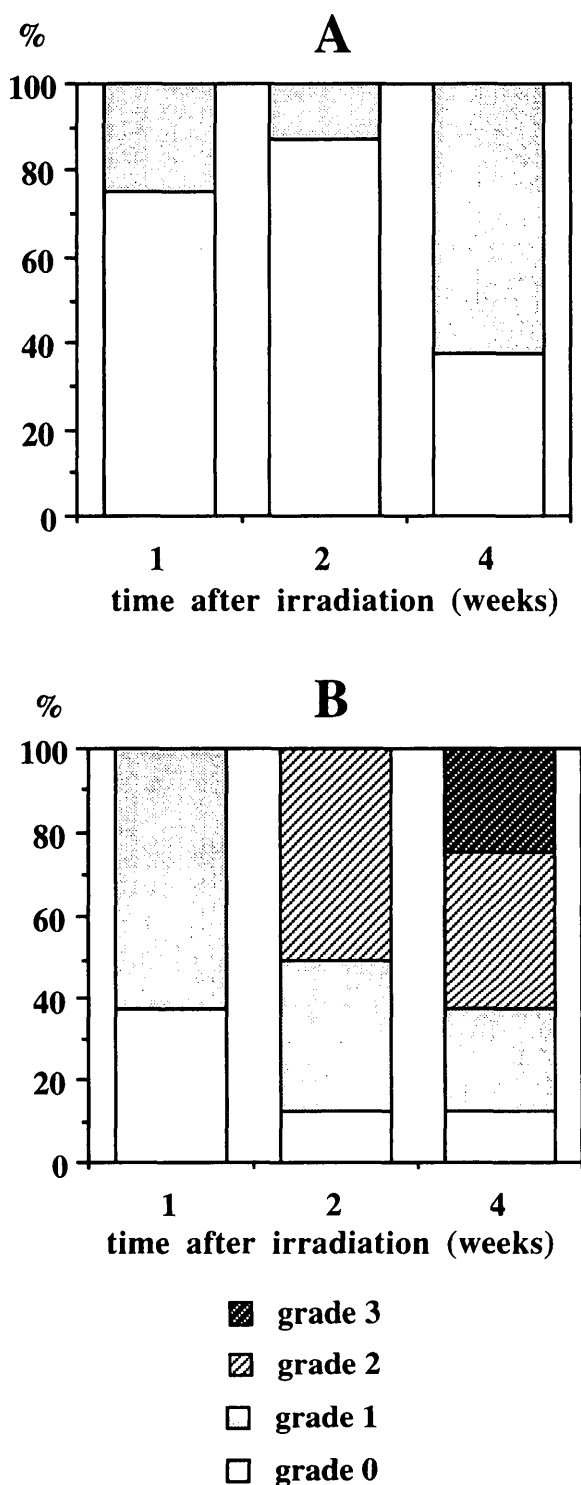
%   A

%   B

■ grade 3

▨ grade 2

□ grade 1

□ grade 0

Figure 4. Grade of decrease in fluorescein leakage at each indicated time point of the eyes in the nonirradiated control group (A) and the eyes in the group irradiated with a single dose of 20 Gy (B). The gelatin hydrogel microspheres were implanted 3 weeks before irradiation. There is a significant difference in grade between the irradiated and nonirradiated eyes throughout the follow-up period (Mann-Whitney U test, $P < 0.05$).

Immunohistochemical Studies

The eyes studied showed a similar sequence of immunohisto-chemical findings, which are summarized in Table 1. Seven

weeks after implantation of the microspheres (4 weeks after irradiation), endothelial cells (CD31-positive), RPE cells (cytok-eratin-positive), and macrophages (RAM11-positive) infiltrated the subretinal space of nonirradiated eyes. The number of cytokeratin-positive RPE cells in the subretinal space was in-creased more in the irradiated eyes than in the nonirradiated eyes. In contrast, the number of RAM11-positive macrophages and CD31-positive endothelial cells was much lower in the irradiated eyes than in the nonirradiated eyes.

DISCUSSION

The present study demonstrated that a single fraction of 20 Gy of focal x-ray irradiation directed at the rabbit eye significantly decreased fluorescein leakage from CNV lesions.

The CNV lesions in this model were induced by subretinal implantation of bFGF-impregnated gelatin hydrogel micro-spheres. Cross-linked gelatin is degradable only in the presence of protein hydrolases. The bFGF-impregnated gelatin micro-spheres released biologically active bFGF in the subretinal space, presumably as a result of enzymatic degradation of the cross-linked gelatin. Possible mechanisms of CNV induction in this model are that bFGF released from the microsphere may continuously stimulate the proliferation and migration of cho-roidal endothelial cells and that RPE cells stimulated by bFGF may release other angiogenic factors.[17] The preirradiated bFGF-impregnated gelatin microspheres showed a capability of inducing angiogenesis similar to that of the bFGF-impregnated gelatin microspheres, which were not previously irradiated. This rules out the possibility that radiation alters the release profile of bFGF from the cross-linked gelatin hydrogels and, consequently, delays the onset of angiogenesis.

Radiation is most effective in suppressing cell proliferation if it is applied while a cell is undergoing mitosis or early in the S phase. Radiation exerts its effects by damaging DNA, causing these cells to die during the next mitotic cell division.[18] An early event of neovascularization is the migration and prolifer-ation of capillary endothelial cells into the surrounding tissues. X-ray irradiation inhibits the proliferation of vascular endothe-lial cells in vitro.[19-23] Furthermore, focal irradiation inhibits tissue neovascularization in vivo. Using a polymer sheet im-planted in the epigastric vascular pedicle of rats, Doyle et al.[24] studied the effect of irradiation given in different doses and at different times, on angiogenesis. Prionas et al.[25] showed that a single 15 Gy-dose of radiation inhibited neovascularization of mesenchymal tissue. Endothelial cells play an important role in angiogenesis and are thought to be the most radiosensitive elements of the vasculature. Focal irradiation may decrease the leakage of CNV lesions by directly inhibiting the proliferation of endothelial cells in the choriocapillaris that were specifically activated by exogenous bFGF or by altering the biological activity of the endothelial cells.

Ophthalmoscopically, scarring of CNV lesions associated with hyperpigmentation occurred earlier in the irradiated eyes than in the nonirradiated eyes. Histologic findings 4 weeks after irradiation showed that the subretinal membrane of the irradiated eyes consisted mainly of RPE-like cells and that the degree of vascular formation was much less in the irradiated eyes than in the nonirradiated eyes. Immunohistochemical studies demonstrated that there were much less CD31-positive endothelial cells and much more cytokeratin-positive RPE cells
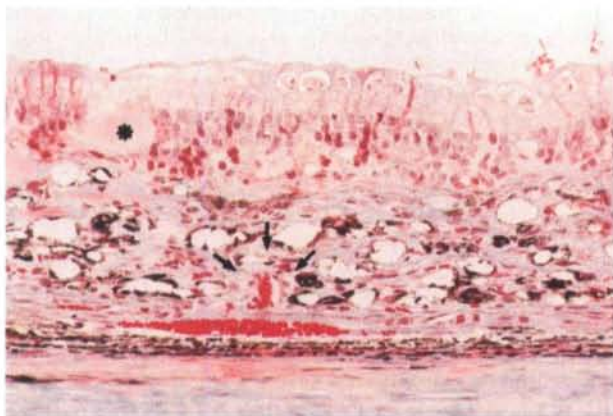
FIGURE 5. Light micrograph of a nonirradiated eye taken 7 weeks after implantation of the microspheres. A subretinal neovascular membrane extends from the underlying choriocapillaris through a break in Bruch's membrane (*arrows*). This neovascular membrane consists of RPE-like cells, an extracellular matrix, and vascular formation. A few microspheres are still present beneath the retina (*asterisk*). Trichrome; original magnification, ×80.

seen in the subretinal membrane of the irradiated eyes than in that of the nonirradiated eyes. In this study, radiation was administered 3 weeks after microsphere implantation, at a time when vascular endothelial cells in the CNV lesion may be most actively proliferating. Radiation suppressed the proliferation of vascular endothelial cells and may cause vascular occlusion in the subretinal membrane. Thereafter, bFGF released from microspheres and still present in the subretinal space stimulated the proliferation of RPE cells, and, consequently, the subretinal membrane was surrounded by proliferating RPE-like cells.

bFGF has been found in choroidal neovascular membranes surgically removed from patients with age-related macular degeneration.[9,26] This suggests that bFGF is possibly related to the development of CNV in humans. The pathogenesis of CNV includes a break in Bruch's membrane and dysfunction of RPE cells. The histologic findings in this study demonstrated that new vessel formation extended from the choriocapillaris to the subretinal space. This feature is also seen in CNV associated with age-related macular degeneration. Therefore, our rabbit model of CNV may be useful in understanding the effect of irradiation on CNV associated with age-related macular degeneration. However, the angiogenic response in this model is not the same as that in humans with age-related macular degeneration. CNV in this model is developed with relatively acute angiogenic reactions and lacks a relationship to the degenerative changes, which are seen in CNV secondary to age-related macular degeneration. CNV induced in this study may represent a model of wound healing. The wound healing aspects may bring along an inflammatory component.

Immunohistochemical studies 4 weeks after irradiation demonstrated that there were very few RAM11-positive macrophages in the irradiated eyes. Because these cells are also radiosensitive, it is possible that the radiation affected not only the vascular endothelial cells but also the inflammatory cells and their associated effects.

Mild choroidal atrophy and outer retinal degeneration were observed histologically only in the areas where microspheres were implanted, in both the irradiated and nonirradiated eyes. No apparent histologic findings of retinal or choroi-

dal toxicity caused by irradiation were noted. It is possible that the mechanical damage of microsphere injection or the interference of the normal metabolism of RPE cells by the accumulated microspheres induced the retinal degeneration and choroidal atrophy.[27]

Much attention has been paid to the feasibility of radiation therapy in preventing CNV in patients with age-related macular degeneration, because the preliminary findings of Chakravarthy et al.[1] However, whether radiation therapy is effective in preventing CNV associated with age-related macular degeneration is still controversial. In addition, the in vivo effect of focal irradiation on CNV has not been studied. To the best of our knowledge, this is the first report that shows the inhibitory effect of focal irradiation on experimental CNV. In this study, we used a single fraction of 20 Gy of x-ray irradiation. In most published studies examining the effects of CNV treated with irradiation the doses used range between 10 Gy and 20 Gy in several fractions. These fractionated doses are biologically lower than a single dose of 20 Gy. The dose used in this study, therefore, is relatively high for clinical application. Further study is needed to determine the optimal dose of radiation therapy in treating CNV without damaging ocular tissues.

In conclusion, focal irradiation in 1 fraction of 20 Gy at ocular regions is effective in inhibiting CNV activity in rabbits with bFGF-impregnated gelatin microspheres. This study and
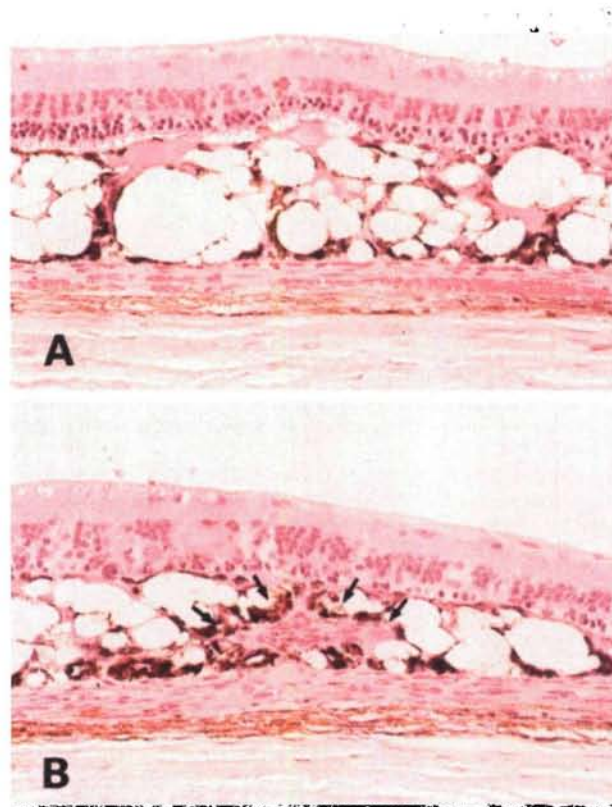
FIGURE 6. Light micrographs of an eye irradiated with a single dose of 20 Gy taken 7 weeks after implantation of the microspheres (4 weeks after irradiation). (A) The subretinal membrane consists mainly of a large number of RPE-like cells with pseudocinar structures. (B) A few new vessels (*arrows*), surrounded by RPE-like cells, can be seen in the membrane. Hematoxylin-eosin; original magnification, ×80.

TABLE 1. Cellular Response in the Subretinal Space

	Irradiated	Nonirradiated
Cytokeratin	+ +	+
CD31	±	+
RAM11	±	+

Quantified by counting the number of positive cells in an average of three ×40 objective field: (±) = <1; (+) = 1-10; (++) = >10.

its results suggest that focal radiation therapy may be beneficial in treating CNV.

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