Synergistic Suppression of Retinal Pigment Epithelial Cell Proliferation in Culture by Radiation and Hyperthermia

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**Purpose.** To study combination effects of radiation and hyperthermia on the in vitro cell proliferation of cultured human retinal pigment epithelial cells; to explore possible application of the combination treatment for proliferative vitreoretinopathy.

**Method.** Cultured human retinal pigment epithelial cells were treated by radiation, hyperthermia, or a combination of the two. Cell proliferation was evaluated by counting the cell number and by the uptake of bromodeoxyuridine.

**Results.** X-ray irradiation of 100 cGy or 300 cGy was not effective in suppressing proliferation of the retinal pigment epithelial cells. Similarly, heat treatment at 42°C or 43°C for 30 min did not suppress proliferation of the retinal pigment epithelial cells. However, combination of hyperthermia at 42°C for 30 min with 300 cGy irradiation suppressed cellular growth of the retinal pigment epithelial cells to 35.7% of the control as estimated by the cell counting and to 48.3% by bromodeoxyuridine uptake study. Combination treatment of 43°C, 30 min hyperthermia and 300 cGy irradiation was more effective.

**Conclusions.** A combination of low-dose radiation and mild hyperthermia is effective in the suppression of growth of cultured human retinal pigment epithelial cells, and the effects were found to be synergistic. It is expected that the synergistic effects will lower the radiation dose and also reduce the possible side effects of radiation in the treatment of proliferative vitreoretinopathy. Invest Ophthalmol Vis Sci 1993; 34:2068-2073.

Proliferative vitreoretinopathy (PVR) is a major cause of failure of retinal detachment surgery. This disorder is characterized by the formation of cellular membranes on the detached retina and also in the vitreous. The cellular membrane is composed of retinal pigment epithelial (RPE) cells, fibroblasts, glial cells and macrophages. Although the cellular components of the periretinal and intravitreal membrane differ from one case to another, the RPE cells are generally believed to be the key component that induces PVR.

Recent advances in the vitreous surgery have changed the treatment and prognosis of PVR. However, reproliferation of the cells hinders reattachment of the retina. Many pharmacologic agents have been tested to inhibit the proliferation of cells in the eye. For example, trials of fluorouracil, colchicine, and daunorubicin have been shown to effectively suppress cellular growth in experimental models of PVR. Immediate clinical use of such drugs, however, is not practical because of the rapid disappearance of the drugs from the eye and also because of their possible toxicities.

Ionizing irradiation therapy of PVR has also been advocated by several laboratories using animal PVR models. We have studied the possible application of irradiation or hyperthermia on the treatment of exper-
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MATERIALS AND METHODS

Preparation of RPE Cells

RPE cells were obtained from human eye bank eyes. The research was approved by the Institutional Human Experimentation Committee, and in the authors' opinion, methods for securing human tissue were humane, included proper consent and approval, and complied with the tenets of the Declaration of Helsinki. Human eyes were washed in calcium- and magnesium-free phosphate-buffered saline solution containing penicillin G potassium (1,000 IU/ml), streptomycin (1 mg/ml) and amphotericin B (2.5 mg/l). The RPE cells were removed from the choroid gently with a pipette after 1 hr of treatment with 0.25% trypsin. Freed RPE cells were recovered by centrifugation at 1,000 rpm for 5 min and then resuspended in Dulbecco’s modified Eagle’s medium supplemented with 16% fetal bovine serum, penicillin G (100 IU/ml), streptomycin (0.1 mg/ml), and amphotericin B (0.25 mg/l). RPE cells were cultured in an incubator under a humidified atmosphere (5% carbon dioxide, 95% air) at 37°C. The cells of passages 3–5 were used for the experiments.

Radiation and Hyperthermia

RPE cells were plated on coverglasses 14 mm in diameter (5,000 cells/plate) and in 25-cm² flasks (50,000 cells/flask) at 48 hr before the treatment. Coverglasses were used for the evaluation of DNA synthesis and flasks for the determination of cell number. The RPE cells were divided into four groups. Group A cells received x-ray irradiation, group B was treated by hyperthermia, group C was treated by both irradiation and hyperthermia, and group D was designated as the control group for cell proliferation without any treatment.

On the day of treatment, group A cells were irradiated at room temperature using a linear accelerator. Radiation doses of 100, 300, or 500 cGy were delivered to the cells on coverglasses and in flasks. After the irradiation, the cells were returned to the incubator at 37°C with fresh medium. Group B cells were treated by hyperthermia at 42°C or 43°C. Cells on a coverglass were transferred into a bottle filled with preheated medium. The bottles were immersed in a water bath for 30 min. The flasks of group B cells were filled with warmed medium, and were treated similarly. After the hyperthermia, the cells were incubated in fresh medium. Group C cells received hyperthermia of 42°C or 43°C, 2 hr after the irradiation of 100 cGy or 300 cGy. After irradiation and hyperthermia, the medium was replaced with fresh medium. Group D cells, the control group, received no treatment. But the cell was cultured for 30 min using the medium that was 500 cGy irradiated and once heated to 43°C on the treatment day. After that fresh medium was used to culture the RPE cells. Quadruplicate evaluation by direct cell counts and DNA synthesis was performed for each dose and temperature, each pretreatment and posttreatment day.

Evaluation of Cell Proliferation

On days 1, 3, and 7 after the treatment, cell proliferation was evaluated by cell count and by measurement of DNA synthesis rate. The cell number in flasks was quantitated with a Coulter counter (Coulter Electronics, Luton, England). DNA synthesis was evaluated by the incorporation of bromodeoxyuridine by using the Cell proliferation kit (Amersham, Little Chalfont, England). DNA synthesis was expressed as the ratio of nuclear-staining cells (cells incorporated bromodeoxyuridine) to the total cell number compared with that in the control group.

Statistical Analysis

Statistical analysis was performed using Student’s t test and the analysis of variance was used for the evaluation of synergistic effects.

RESULTS

Cell Number

The cell number is represented in Figure 1. X-ray irradiation of 100 cGy or 300 cGy induced suppression of cellular growth, but the changes were not statistically significant. However, 500 cGy of irradiation suppressed cellular growth on the seventh day after treatment. The change was statistically significant according to the t test (P < 0.01). Hyperthermia of 42°C or 43°C for 30 min suppressed the increase of cellular number only slightly (Fig. 1A). Combination therapy of irradiation and hyperthermia, conversely, was more effective than irradiation of 500 cGy (Fig. 1B). As shown more clearly in Figure 2, on the seventh day after the treatment, combination treatment decreased the cell count. The cell counts were: 42°C and 100 cGy, 35.7 ± 2.2% (mean ± SEM, n = 4); 42°C and 300 cGy, 24.2 ± 1.0%; 43°C and 100 cGy, 35.3 ± 2.7%; 43°C and 500 cGy, 20.5 ± 1.3% of the control. Radiation of 500 cGy suppressed the cell number to 40.1 ± 2.1%. Statistically significant differences and synergistic effects by the analysis of variance were found between single therapy and combined therapy in each dose (P < 0.01) (Table 1).
DNA Synthesis

Without combination treatment, mild hyperthermia or low-dose irradiation showed little inhibition of DNA synthesis. However, even with 100 cGy or 42°C for 30 min, some cells showed bizarre shape with giant cell formation. In the combined therapy with the delivery of 100 cGy, DNA synthesis was decreased to 28.7 ± 1.8% (n=4) of the control by 42°C hyperthermia and to 33.7 ± 1.7% by 43°C heat-treatment on the first day. But the DNA synthesis recovered gradually to 114.6% and 85.0% of the control, on the seventh day (Fig. 3). Conversely, the combined therapy with the delivery of 300 cGy produced less prominent recovery of DNA synthesis. On the seventh day, 48.3 ± 14.6% DNA synthesis by 42°C hyperthermia and 26.0 ± 9.7% by 43°C heat-treatment were observed (Fig. 3). The changes were statistically significant compared to

TABLE 1. Summary of Statistical Analysis

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Student’s t-test: (P < 0.05) 0, (P < 0.01) 0
Analysis of Variance: (P < 0.05) *, (P < 0.01) †
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Figure 3. DNA synthesis of retinal pigment epithelial cells after the treatment. Combination of 100 cGy radiation and hyperthermia (42°C or 43°C) decreased the DNA synthesis only transiently. Combination of 300 cGy with hyperthermia were more effective than radiation of 500 cGy during the follow-up period (mean + SEM, n = 4).

Figure 4. DNA synthesis of the retinal pigment epithelial cells on the seventh day after the treatment. A combination of 300 cGy and hyperthermia effectively suppressed the DNA synthesis of the cultured human RPE cells (gray columns). (A) radiation, (B) hyperthermia, (C) combination of radiation and hyperthermia, (D) control.

DISCUSSION
Although many types of cells contribute to the formation of PVR, proliferation of the RPE cells is generally believed to be the main cause. RPE cells not only proliferate and form cellular membrane in the eye, they also release substances that recruit other types of cells in the process of PVR. Rowen and Glaser showed that RPE cell-conditioned medium stimulates chemotaxis of astrocytes, one of the glial cells, and induces chemokinesis of fibroblasts. If we can suppress the proliferation of RPE cells, which are dispersed on or beneath the retinal surface, it means we could also minimize the recruitment of other cells that play important roles in the pathogenesis of PVR. Recently, along with many drugs, irradiation has been regarded as a potential new therapy for PVR. Some studies demonstrated that radiation therapies using x-rays, beta-electron, and 60Co can suppress cellular proliferation of fibroblasts and RPE cells in vitro as well as reduce the incidence of experimental traction retinal detachment in vivo. Ocular side effects of radiation are well known, but less than 1,000 cGy is generally believed to be free of complications. Even so, the radiation dose should be as low as possible.

Hyperthermia, in combination with radiation, is an increasingly popular therapeutic modality for the treatment of malignant tumors. Hyperthermia has been tried not only for malignant diseases but also for nonmalignant hyperplasia such as benign prostatic hyperplasia recently. The mechanism of the effects at the cellular level of hyperthermia is believed to be membrane damage, DNA damage, and creation of chromosomal aberrations. It is now generally agreed that the difference in heat sensitivity among tumor cells and normal cells is small at the cellular level. Differences in the reaction to hyperthermia at the tissue level may explain the differences in the heat sensitivities among normal and tumor tissues. When the tissue temperature reaches above 42°C, the immature tumor blood flow is reduced and the heat is retained, whereas the normal tissue is rapidly cooled by the active, efficient, and increasing blood circulation. In addition, hyperthermia is known to be preferentially cytotoxic toward hypoxic cells and S-phase cells, which are radioresistant. Hyperthermia therefore enhances the radiation effect. Although PVR is not a neoplastic disease state, it is possible that cells in the PVR membrane, like a tumor tissue, are damaged more easily by hyperthermia than the cells in the normal retina, because PVR membranes have few blood vessels and there are more S-phase cells in the detached retina than in the attached retina. Combined therapy for PVR possibly lowers the radiation dose to achieve the suppressive effects and lowers the potential side effects of radiation.

In this study, we chose radiation doses (100 cGy and 300 cGy) and thermal doses (42°C and 43°C for 30 min) that by themselves are not sufficient to suppress proliferation of RPE cells. As shown in Figures 1 and 2, a combination of 100 cGy or 300 cGy...
B

FIGURE 5. Light microgram of the human retinal pigment epithelial cells (original magnification, X100). (A) Non-treated human retinal pigment epithelial cells. (B) Human retinal pigment epithelial cells on the seventh day after combined therapy of 300 cGy irradiation and 43°C, 30 min hyperthermia. There are few nuclear stained cells, and bizarre-shaped giant cells were observed.

irradiation and 42°C or 43°C heat-treatment suppressed RPE cell proliferation as estimated from the cell number. Also, DNA synthesis studies showed that combination of 300 cGy irradiation and 42°C or 43°C hyperthermia were more effective than irradiation of 500 cGy (Fig. 3, Fig. 4). Furthermore, a synergism was found between radiation and hyperthermia. Although there was a slight recovery in the DNA synthesis on the seventh day (Fig. 3), bizarre-shaped giant cells observed in the combination treatment groups (Fig. 5) mean that the possibility of these cells regaining normal growth capability is small. Although we studied the effects of combination treatment only by using RPE cells, there is a report that shows a greater in vitro heat and radiation sensitivity of human skin fibroblast than lung and colon carcinoma cells, which are good candidates for the combination therapy. Therefore, it is probable that the combination therapy will effectively suppress the proliferation of other cell types in PVR.

The time interval between irradiation and hyperthermia may be important in modifying the efficiency of combination therapy. The thermal enhancement ratio of normal tissue decreases promptly by 2 hr after hyperthermia. Conversely, the thermal enhancement ratio of proliferative tissue decreases slowly and maintains a medium level for several hours. Malignant tumors are treated by combined therapy as simultaneous as possible, because at that time the thermal enhancement ratio of radiation damage is maximal. But because PVR is not really a malignant proliferation, the safeguard of the normal tissue surrounding the PVR membranes should be considered. Therefore, we chose the time interval of 2 hr when the difference of thermal enhancement ratio between normal and proliferative tissues is greatest, and the combination therapy is still effective.

A possibility of side effects associated with combined therapy cannot be denied. With combined therapy of 45°C or more for malignant tumors in the rabbit model, mild intraretinal and subretinal hemorrhage and retinal edema are reported. One available report, however, shows that the incidence of ocular side effects does not increase with the combination therapy compared to the radiation therapy of the same doses. According to Liggett, in the normal rabbit eye, hyperthermia of 45°C or less for 45 min combined with radiation is the maximum allowable threshold without causing diffuse retinal damage by clinical examination, fluorescein angiography, electroretinography, and histopathology. If we adopt the results, the combination therapy studied in this report should be free of ocular complication.

In this study, we have clearly demonstrated that a combination of low-dose radiation and mild hyperthermia is effective in the suppression of growth of cultured human RPE cells. Moreover, it is suggested that the use of hyperthermia may possibly reduce radiation dose and the incidence of radiation side effects without losing the suppressive effects on RPE cell proliferation. In human PVR, more complex factors modulate proliferation of cells than in the model studied here. We should therefore be careful to apply results of the study to human situation directly. More studies are needed before this combination therapy can be considered a possible treatment for PVR. However, this new experimental treatment has the potential for providing a therapeutic tool for the treatment of PVR.

Key Words
hyperthermia, x-ray irradiation, human retinal pigment epithelial cells, proliferative vitreoretinopathy

References
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