Photocoagulated Human Retinal Pigment Epithelial Cells Produce an Inhibitor of Vascular Endothelial Cell Proliferation

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Purpose. To determine if conditioned culture medium of photocoagulated human retinal pigment epithelial (RPE) cells contains inhibitors for the proliferation of bovine aortic endothelial cells (BAEC) and bovine retinal endothelial cells (BREC) and to characterize the nature of these inhibitory factors.

Methods. Retinal pigment epithelial cells grown to confluence were photocoagulated (0.1 second, 50 μm, 350 mW) in serum-free medium. After 48 hours, the conditioned medium (PC-CM) was removed, and the effects of non-acid-treated and transiently acid-treated samples were determined on [3H]-thymidine uptake by BAEC and BREC. PC-CM was also subjected to size exclusion high-performance liquid chromatography (HPLC). Fractions were analyzed for the effects on the growth of the bovine endothelial cells before and after transient acid treatment.

Results. The addition of non-acid-treated PC-CM (32% vol/vol) inhibited BREC [3H]-thymidine uptake to 18.5% of the control value. With HPLC, the inhibitory activity was recovered mainly in a fraction whose apparent molecular size was 25 kd. After transient acid treatment of the fractions, there also appeared a 100-kd inhibitor. Inhibitory effects were neutralized by pretreatment of the fractions with antiserum against transforming growth factor (TGF)-β2.

Conclusions. Photocoagulated RPE cells secrete inhibitors of BAEC and BREC proliferation. Molecular size and immunologic properties of these inhibitors correspond to those of TGF-β2.


Scatter photocoagulation induces regression of the retinal neovascularization that complicates retinal capillary nonperfusion.1-3 Despite its widespread clinical use, however, the mechanism of the effect of photocoagulation is unknown. The main site of energy absorption in laser photocoagulation is melanin pigment in retinal pigment epithelial (RPE) cells and choroid. Because RPE cells are known to produce many growth factors and cytokines,4-12 it may well be that RPE cell-derived factors induce regression of neovascularization after retinal photocoagulation.

We have reported13 that photocoagulation of cultured human RPE cells induces changes in the production of growth factors and cytokines by the cells. We also have shown time-dependent accumulation of transforming growth factor (TGF)-β2 in the conditioned medium. On analysis, production of active TGF-β2 was increased from 1.0 ± 0.6 pg/cm² per 24 hours to 11.0 ± 1.0 pg/cm² per 24 hours by laser photocoagulation, and total TGF-β2 was increased from 11 ± 1.0 pg/cm² per 24 hours to 100 ± 20.1 pg/cm² per 24 hours.

In this article, we show the effects of conditioned medium of photocoagulated RPE cells on the DNA synthesis of bovine aortic endothelial cells (BAEC) and bovine retinal endothelial cells (BREC). We also describe the nature of the inhibitory factors.
MATERIALS AND METHODS

Cell Culture

Human RPE cells for culture were obtained from the eyes of aborted fetuses (17 to 22 weeks gestational age), as described. The research was approved by the Institutional Human Experiment Committee; the methods for securing human tissue were deemed to be humane, included proper consent and approval, and complied with the tenets of the Declaration of Helsinki. Cells were grown in culture media composed of Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS), penicillin G (100 IU/ml), streptomycin (100 μg/ml), and amphotericin B (0.25 mg/ml). Three independent cells from the third to sixth passages were used in these experiments. Retinal pigment epithelial cells were plated at a density of 3 × 10⁶ cells in 300 μl of medium on glass coverslips (14 mm in diameter) in 24-well cell culture plates.

Bovine aortic endothelial cells were removed from the adult bovine aortic arch by scraping, and they were cultured under the same conditions used for human RPE cells. Bovine retinal capillary endothelial cells were established as reported and were cultured with DMEM-FBS in dishes precoated with 1% gelatin. The cells formed a characteristic mosaic of nonoverlapping, polygonal cells. Positive staining of these cells with anti-factor VIII antibodies showed that they were endothelial cells. Animals were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Laser Photocoagulation and Preparation of Conditioned Medium

When RPE cells became confluent, the cells were rinsed twice with serum-free DMEM containing 0.1% bovine serum albumin (DMEM-BSA). Each well was added with 250 μl DMEM-BSA. Photocoagulation of the RPE cells was accomplished essentially as reported by a clinical slit lamp delivery system of the argon green laser (Coherent Radiation, Palo Alto, CA) using the following parameters: 350 mW of power, 0.1-second duration, 50-μm spot size, and 200 spots/coverslip. Because the RPE cells in culture became depigmented, the photocoagulation procedure required placement of black paper beneath the 24-well plates. After photocoagulation, the RPE cells were incubated for 48 hours with fresh DMEM-BSA, and the conditioned medium was recovered. Little cell debris was found in the conditioned medium. As a control, nontreated RPE cells were exposed to DMEM-BSA for 48 hours. The conditioned medium of photocoagulated RPE cells (PC-CM) and that of nontreated RPE (CM) were stored at −20°C for bioassay.

Size Exclusion Chromatography

Size exclusion chromatography was used to estimate the apparent molecular weights of the inhibitory factor(s) in the conditioned medium. Approximately 140 μg protein PC-CM was applied to a column (TSK-GEL, G3000SW, 7.5 nm × 60 cm, TOSOH, Tokyo, Japan) attached to a high-performance liquid chromatography (HPLC) system (Shimadzu, Kyoto, Japan). The column was pre-equilibrated with phosphate-buffered saline (pH 7.2), and the proteins were eluted with the same buffer with a flow rate of 0.5 ml/minute. The proteins were monitored by absorbance at 280 nm and collected in 1-ml fractions. Molecular mass markers used were immunoglobulin G (Mr 150 kd), BSA (69 kd), and chymotrypsinogen A (25 kd).

Bioassay Using Endothelial Cells

Both BAEC and BREC were viable as confirmed by trypan blue dye exclusion study and by cell number counting. The reaction was terminated by three washes in phosphate-buffered saline, and the acid-soluble pools were removed by a 20-minute exposure to ice-cold 10% trichloroacetic acid, followed by three rinses with the same solution. The cells were solubilized in 0.1 N NaOH (2 hours at room temperature), and the cell-associated radioactivity was measured in a liquid scintillation counter using Aqueous Counting Scintillant I (Amersham Japan). Triplicate determination was performed for each test condition.

High-performance liquid chromatography fractions were examined by the bioassay, PC-CM without acid treatment was applied to the TSK G3000SW column as described, and the fractions were analyzed before and after transient acid treatment. As a control, the same volume of phosphate-buffered saline was added instead of HPLC fractions. Quadruplicate determination was performed for each fraction.

Assay for Transforming Growth Factor-β2

Because photocoagulated RPE cells were considered to synthesize a considerable amount of TGF-β of latent
FIGURE 1. Inhibitory activities of retinal pigment epithelial conditioned media for vascular endothelial cell proliferation. Retinal pigment epithelial conditioned media recovered with or without laser treatment (PC-CM or CM) were evaluated for the inhibitory effect on endothelial cell proliferation. CM and PC-CM inhibited DNA synthesis by bovine aortic endothelial cells (BAEC) in a dose-dependent manner. DNA synthesis by bovine retinal endothelial cells (BREC) was inhibited more effectively than was by BAEC. Each point represents the mean ± SEM of experiments carried out in triplicate. Squares = assay on BAEC; circles = assay on BREC, open symbols = CM; closed symbols = PC-CM.

form, latent TGF-β was activated by transient acid treatment. The pH of the samples was changed to 2.5 for 30 minutes by the addition of 0.5 N HCl (2.5 μl/100 μl sample), followed by neutralization to pH 7.5 with 0.5 N NaOH (2.5 μl/100 μl sample). After the transient acid treatment, the samples were assayed for their inhibitory effect on endothelial cells. Fractions collected by size exclusion chromatography were assayed with or without this treatment.

As a positive control, highly purified porcine TGF-β2 (R & D Systems, Minneapolis, MN) was added to the assay (5 pg/ml, 10 pg/ml, 50 pg/ml, 100 pg/ml, 500 pg/ml, and 1 ng/ml). Also, 1 μg/ml or 3 μg/ml of a neutralizing antibody against TGF-β2 (R & D Systems), which has no cross-reactivity with TGF-β1, was used to test the specificity of the effect of TGF-β2 in the conditioned medium. For this assay, either 1 μl or 3 μl of 0.25 μg/μl antibody was added to a 100-μl sample. In our study, ID₅₀ of TGF-β2 on the proliferation of BAEC and BREC was approximately 200 pg/ml and 50 pg/ml, respectively. Triplicate wells were used for each concentration of the antibody.

Statistical Analysis
Statistical analysis was performed using the two-tailed Student's t-test.

RESULTS
Effects of Conditioned Medium on Endothelial Cell Proliferation
The addition of 32% (vol/vol) PC-CM decreased the proliferation of BREC to 18.5% that of the control (DMEM–BSA). CM was less effective by decreasing the value to 51.6% that of the control. The inhibitory effects were less apparent when BAEC were used as the target; still, [³H]-thymidine uptake was decreased to 36.5% or to 82.1% of the control value when 32% (vol/vol) of PC–CM or CM, respectively, was added to the assay system. The inhibitory effects on BAEC proliferation were dose dependent, as shown in Figure 1. Conditioned medium from photocoagulated coverslips (no RPE cells grown) did not inhibit proliferation of either BAEC or BREC, and brief exposure of nonphotocoagulated RPE cells to the conditioned medium from photocoagulated coverslips did not alter the production of inhibitory activities from the RPE cells.

Transient acid treatment of CM and PC-CM dramatically potentiated the inhibitory activities on BAEC proliferation. [³H]-thymidine uptake was decreased from 36.5% to 11.8% by PC–CM with the acid treatment. It suggested a possible role for TGF-β in the inhibition of endothelial cell proliferation (Fig. 2).

High-Performance Liquid Chromatography Analysis
Size exclusion chromatography of PC–CM was performed to determine the apparent molecular size of
RPE-Derived Inhibitor for Endothelial Cell Growth

FIGURE 3. High-performance liquid chromatography (HPLC) of the conditioned medium of endothelial cell proliferation. With non-acid-exposed HPLC fractions (open circles), the inhibitory activity was recovered in a fraction with a retention time 38 minutes (25 kd). In the acid-exposed sample (closed circles), a 100-kd inhibitor (retention time, 30 minutes) was detected. (A) Assay on BREC; (B) assay on bovine aortic endothelial cells. Quadruplicate determination was performed for each fraction. Molecular weight markers used were: immunoglobulin G (IgG), 150 kd; bovine serum albumin (BSA), 67 kd; chomotrypsinogen, 25 kd.

FIGURE 4. Effect of anti-transforming growth factor (TGF)-
\beta_2 antibody on the inhibitory effect of high-performance liquid chromatography fractions on endothelial cell proliferation. The inhibitory effects on acid-exposed fractions (retention times, 30 minutes and 38 minutes) are shown. Each point represents the mean ± SEM (n = 3). Empty column = no antibody; shaded column = 1 μg/ml of the neutralizing antibody against TGF-β2; filled column = 3 μg/ml of antibody. *p < 0.05; **p < 0.01; ***p < 0.001.

the inhibitors and to identify the possible activators of endothelial cell proliferation. As shown in Figure 3, no activator of endothelial cell proliferation was found by using either BAEC or BREC. With non-acid-treated HPLC fractions, a low molecular weight inhibitor was reproducibly found in the fraction collected with the 38-minute retention time (open circles). No high-molecular weight inhibitor was found by this analysis. Transient acid treatment of each fraction, however, revealed a high molecular weight inhibitor (closed circles). The inhibitor was recovered with a retention time of 30 minutes. Proliferation of BAEC and BREC was decreased by the high molecular weight inhibitor, and this inhibitor was more potent with BREC.

Effects of a Neutralizing Antibody Against Transforming Growth Factor-β

Because the apparent molecular weights of the inhibitors, i.e., 100 kd and 25 kd, corresponded to those of the latent and active forms of TGF-β, and because previous studies in this laboratory showed that RPE cells produce TGF-β2, a neutralizing antibody against TGF-β2 was added to the assay system. As shown in Figure 4, both high and low molecular weight inhibitory factors were blocked by the antibody. Statistical analysis showed that the inhibition was statistically significant (see legend, Fig. 4). Thus, TGF-β was suggested to be a potent inhibitor of endothelial cell proliferation.

DISCUSSION

In this study, we have demonstrated that the conditioned medium of photocoagulated RPE cells contains an inhibitor of DNA synthesis by both BAEC and
BREC. This inhibitory activity also was found in the conditioned medium of nontreated RPE cells, albeit in small quantity. This finding itself is not novel, but our data are supportive of the hypothesis of Glaser et al\(^a\) that preconfluent RPE cells produce an inhibitor(s) of endothelial cell growth.

We have shown also that in vitro photocoagulation upregulates production of such inhibitor(s) and that properties of the inhibitor agree well with those of TGF-\(\beta\)2. Regenerating RPE cells produce a much higher amount of TGF-\(\beta\)2 than normal quiescent RPE cells.\(^b\) If we assume that exactly 50 \(\mu\)m-diameter coagulation spots were obtained as planned, <0.3% area of a coverslip can be coagulated by the procedure. Therefore, increased cell numbers from the regeneration of RPE cells cannot explain this upregulated production of the inhibitor.

An important finding—other than the presence of TGF-\(\beta\)2 in the conditioned medium—is that we did not find any stimulator for the proliferation of vascular endothelial cells by HPLC analysis. Retinal pigment epithelial cells are known to be capable of affecting and modifying the structure of capillary endothelium.\(^c\) Activators and inhibitors of the growth of endothelial cells have been discovered in the RPE cells.\(^d\)-\(^h\) With laser photocoagulation, regenerating RPE cells show a morphology different from that of normal RPE cells.\(^i\)-\(^n\) These regenerating RPE cells behave differently from normal RPE cells in situ; thus, production of cytokines and growth factors from the cells may be altered in quantity and in quality. Under the culture conditions used, activators of endothelial cell proliferation, such as vascular endothelial growth factor (a 43-kd protein), was apparently not synthesized by RPE cells to an appreciable amount. This is not surprising because vascular endothelial growth factor is known to be synthesized mainly from anoxic cells.\(^o\) Furthermore, on analysis no inhibitory factors other than TGF-\(\beta\)2 were found.

Wong et al,\(^p\) however, described the production of a stimulator rather than an inhibitor for vascular endothelial cell proliferation in cultured RPE cells. In our experiments, however, no such activity was detected. We consider the differences to have resulted from differences in the assay system—for example, differences in cell confluency, cell density, or concentration of serum or plasma used in the cell culture. It should be noted that we used fetal rather than adult RPE cells, which may explain why we did not find a stimulator for vascular endothelial cell proliferation. Such activities were reported by several other investigators.\(^q\)-\(^t\)

By using cultured RPE cells, it may not be possible to identify all the activators and inhibitors of endothelial cell proliferation secreted from the photocoagulated RPE cells. Retinal pigment epithelial cells are known to change their state of differentiation by underlying matrix.\(^u\) At different stages of differentiation, RPE cells may secrete different species of activators or inhibitors, but we do not have any conclusive data.

Mechanisms responsible for the beneficial effects of photocoagulation on areas of retinal capillary nonperfusion are unknown. It has been hypothesized that laser treatment may release potential inhibitors of neovascularization from the scars or reduce intraocular levels of stimulators of neovascularization by destroying cells that produce these factors. Our data support the hypothesis that TGF-\(\beta\)2 is the major chemical mediator that induces regression of retinal neovascularization after photocoagulation.

Key Words
bioassay, bovine endothelial cells, cultured human retinal pigment epithelial cells, laser photocoagulation, transforming growth factor-\(\beta\)2

References
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