Retinal Pigment Epithelium Rescues Vascular Endothelium from Retinoic Acid-Induced Apoptosis

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PURPOSE. To determine whether retinoids are capable of inducing vascular endothelial cell apoptosis and whether the presence of an intact RPE monolayer can block retinoid-induced vascular endothelial cell death.

METHODS. Confluent fetal bovine aortic endothelial (FBAE) cells were incubated with various concentrations of all-trans or 9-cis retinoic acid (an analogue of 11-cis retinoic acid). Apoptosis rates were determined at 24 hours, and the effect of inhibition of protein synthesis and activation of protein kinase C on apoptosis was investigated by supplying culture medium with 0.1 mg/mL cycloheximide and 10 nM phorbol myristate acetate. To investigate the impact of RPE on retinoid-induced apoptosis, confluent FBAE cells were cultured with a confluent layer of RPE in inserts where retinoids were added to the upper compartment. A confluent bovine corneal endothelium monolayer was used as the control. The permeabilities of the RPE and bovine corneal endothelium monolayers to fluorescein (20 μg/mL) and 9-cis retinoic acid (3 × 10⁻⁴ M) were also determined.

RESULTS. 9-cis Retinoic acid induced higher rates of apoptosis in FBAE cells than did all-trans retinoic acid and the control (P = 0.004). This effect was dose-dependent, with an ED₅₀ of 1.4 μM (P = 0.99, P = 0.004). Cycloheximide did not inhibit 9-cis retinoic acid–induced apoptosis, but phorbol myristate acetate significantly decreased the apoptosis rate (P = 0.005). The presence of a confluent RPE monolayer reduced the 9-cis retinoic acid–induced apoptosis rate (P = 0.002), but the presence of a bovine corneal endothelial monolayer did not (P > 0.05). Both cell types established a similar diffusion barrier against fluorescein and 9-cis retinoic acid.

CONCLUSIONS. 9-cis Retinoic acid is an important mediator of vascular endothelial apoptosis. A confluent monolayer of RPE can prevent endothelial cell apoptosis, and this effect is not due simply to establishment of a diffusion barrier by the RPE.
MATERIALS AND METHODS

Preparation and Handling of Retinoic Acid Solutions
We used two different retinoids to investigate the effects of different retinoid receptors on vascular endothelial cell apoptosis—namely, all-trans retinoic acid, which stimulates only nuclear retinoic-acid receptors (RARs), and 9-cis retinoic acid, which is a more stable analogue of 11-cis retinoic acid and stimulates retinoid-X receptors (RXR).16 All-trans retinoic acid and 9-cis retinoic acid were purchased from Sigma-Aldrich (St. Louis, MO). Stock solutions of 0.01 M (3 mg/mL) all-trans and 9-cis retinoids were prepared in dimethyl sulfoxide (10 mM; DMSO) and absolute ethanol, respectively. Stock solutions were stored in light-protected vials at −70°C for up to 2 weeks. At the time of preparing the experiment, media aliquots were thawed and diluted to the appropriate concentrations in culture medium. The effects of all-trans and 9-cis retinoids on FBAE cell apoptosis were studied at concentrations varying between 10−8 to 10−6 M, because of the previously reported effects of retinoids on vascular endothelial cell proliferation and morphology within this range.17 Care was taken to ensure that the final solvent concentration did not exceed 0.1% (vol/vol). Because retinoids are light sensitive, they were protected from light by wrapping the stock solutions and medium with aluminum foil.

All the medium preparation and replacement procedures were performed in darkness or under dim red light (>560 nm).18 Culture medium containing the same amount of retinoid solvents (DMSO for all-trans retinoic acid and absolute ethanol for 9-cis retinoic acid) was used in control experiments.

Preparation of RPE Cell Cultures
Primary RPE cell cultures were prepared from the posterior poles of three human donor eyes (ages: 44, 72, and 66 years) obtained from the Mid-America Eye and Tissue Bank (St. Louis, MO), as described,19 and maintained according to the Declaration of Helsinki. On receipt, eyes were cleaned of extraocular tissue. The suprachoroidal space of the posterior pole was sealed with cyanoacrylate glue, and a small scleral incision was made 3 mm posterior to the limbus until the choroidal vessels were exposed. Tenotomy scissors were introduced through this incision into the subretinal space along the ora serrata. The loosened RPE vessels were exposed. Tenotomy scissors were introduced through this incision into the subretinal space, and the incision was extended circumferentially. Four radial relaxing incisions were made into the sclera and it was peeled from the periphery to the optic nerve. The posterior pole was sealed with cyanoacrylate glue, and a small scleral incision into the suprachoroidal space, and the incision was extended circumferentially. Four radial relaxing incisions were made into the sclera and it was peeled from the periphery to the optic nerve. The eye cup was then incubated with 25 U/mL Dispase (Invitrogen-Gibco, Grand Island, NY) for 30 minutes and rinsed with carbon dioxide-free medium (Invitrogen-Gibco). A circumferential incision was then made into the subretinal space along the ora serrata. The loosened RPE sheets were collected with a Pasteur pipette and plated onto bovine corneal endothelial–extracellular matrix–coated 60-mm plastic dishes (Falcon; BD Bioscience, Plymouth, UK). The cells were incubated in a humidified atmosphere of 5% CO2 and 95% air at 37°C and maintained in Dulbecco’s modified Eagle’s medium (DMEM H16; Invitrogen-Gibco) supplemented with 15% FBS, 100 IU/mL penicillin, G, 100 μg/mL streptomycin, 5 μg/mL gentamicin, 2.5 μg/mL amphotericin B, and 1 ng/mL recombinant human basic fibroblast growth factor (Invitrogen-Gibco), to promote RPE cell growth. The medium was changed every other day, and cells were observed daily. Cells became confluent in approximately 10 days, and confluent cultures were passed by trypsinization.

RPE cells were stained with a pan-tycokeratin antibody to verify they were of epithelial origin. For this purpose, harvested RPE cells were rinsed in phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde for 30 minutes, and washed again with PBS. The cells were treated for 1 hour at room temperature with 3% bovine serum albumin (Sigma-Aldrich) in PBS to block nonspecific binding sites. The cells were then incubated at 37°C for 1 hour with a fluorescein isothiocyanate (FITC)–conjugated monoclonal anti-pan cytokeratin antibody to cytokeratin-5, -6, and -8 (Sigma-Aldrich). RPE cells were washed three times with PBS and examined under a fluorescence microscope. An irrele-

vant isotypic IgG primary antibody (anti-von Willebrand antibody; Biocare Medical, Walnut Creek, CA) coupled with an FITC-conjugated secondary antibody was also used and showed no background staining. All the harvested cells were positive for pancytokeratin, indicating that the cells were of epithelial origin.20

For coculturing experiments, we used first-passage human RPE cells grown to confluence monolayers on polyethylene terephthalate cell culture inserts that fit into 24-well plates (BD-Falcon, San Jose, CA). Once cells reached confluence, growth medium was changed to a well-defined serum-free medium22 to avoid any confounding effects due to the inconsistent amounts in hormones, cytokines, and other growth factors with the serum.22,23 This chemically defined medium contained a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F12 with pyridoxine HCl, L-glutamine, and 15 mM of HEPES buffer (Invitrogen-Gibco, Grand Island, NY). The medium was supplemented with insulin, transferrin, epithelial growth factor, follicle-stimulating growth hormone, linoleic acid, putrescine, sodium selenite, hydrocortisone, triiodothyronine, streptomycin, penicillin, gentamicin, and amphotericin. Retinoic acid was excluded from the original formulation for experimental purposes.

Preparation of Fetal Bovine Aortic Endothelium Cultures
Fetal bovine aortic endothelium (FBAE) was isolated as described previously.24 Fetal bovine aorta segments were trimmed, and the lumens rinsed in calcium- and magnesium-free PBS before being filled with collagenase solution (type IV; Sigma-Aldrich) and 1 mg/mL in calcium- and magnesium-free PBS, for 5 minutes at room temperature. After removal of the collagenase solution, it was centrifuged at 1000 rpm for 5 minutes to create an FBAE pellet. The pellet was resuspended in DMEM, with 15% FBS, L-glutamine (29.2 mg/mL), gentamicin (50 μg/mL), and amphotericin B (0.25 μg/mL), and transferred to 60-mm culture dishes. We used density-inhibited second-passage endothelial monolayers 1 day after they reached confluence to control for the potential effects of transdifferentiation and cell-cycle kinetics on vascular endothelial cell susceptibility to apoptosis. These endothelial cells were synchronized in a serum-free medium containing BSA (1%), insulin (1 μM), transferrin (200 μg/mL), ascorbate (0.2 mM), and sodium selenite (6.25 ng/mL; all from Sigma-Aldrich) for 24 hours before the experiments.

The purity of the cells was determined by von Willebrand factor immunostaining. For this purpose, endothelial cells were grown to confluence in 96-well plates, washed with PBS, and fixed with 3% paraformaldehyde with 0.1% Triton X-100 for 6 hours. The cells were then washed with PBS, incubated in 10% normal goat serum with 0.1% Triton X-100 for 30 minutes, and incubated for 1 hour with a polyclonal rabbit anti-von Willebrand IgG (1:100 in PBS-albumin; Biocare Medical). Cells were washed in physiological saline and incubated with the secondary antibody (fluorescein-conjugated rabbit anti-rabbit IgG at 1:100) for 1 hour. A nonspecific isotypic IgG (anti-pig gial fibriillary acidic protein; 1:200, Sigma-Aldrich) was used as the control. Cells were then examined under a fluorescence microscope (BH2; Olympus, Tokyo, Japan), and the proportion of endothelial cells was estimated. Only cultures yielding >99% vascular endothelial cells were used in the experiments.

Cell Viability Analysis
RPE cell viability analysis was performed in a masked fashion. For this purpose, trypsinized RPE cells were washed twice with PBS, and viability was assessed by a commercial assay (Live/Dead Viability/Cytotoxicity Kit; Invitrogen-Molecular Probes, Eugene, OR). The kit contains two probes: calcein and ethidium homodimer. It relies on the intracellular esterase activity within living cells to cleave the calcein to form a green fluorescent membrane-impermeable product. In dead cells, ethidium can pass through the compromised plasma and nuclear membranes to attach to DNA, yielding red fluorescence. At least 750 cells were counted under 200× magnification. The viability of the RPE
cells was expressed as the average ratio of live cells to the total number of cells in three different areas chosen at random.

**Determination of the Apoptosis Rate**

Synchronized confluent cultures of second-passage FBAE cells were incubated in DMEM/15% FBS supplemented with various concentrations (10^{-4}–10^{-10} M) of all-trans 9-cis retinoic acid at 37°C for 24 hours. RPE cells did not exhibit apoptosis at the doses of retinoids used in this study (data not shown). Twenty-four hours after plating, triplicate wells were washed gently three times with DMEM to remove necrotic cells and were assayed for apoptosis.

The rate of apoptosis was determined by using two different methods: (1) an annexin V-affinity assay that detects the surface exposure of phosphatidylserine during apoptosis (Vybrant Apoptosis Assay; Invitrogen-Molecular Probes) and (2) the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) method, to detect DNA strand breaks (Roche Diagnostics GmbH, Penzberg, Germany).

For the annexin V-affinity assay, FBAE cells were simultaneously stained with annexin V-Alexa Fluor and propidium iodide to discriminate viable cells (annexin V + /propidium iodide -) and early apoptotic cells that had lost membrane phospholipid asymmetry (annexin V + /propidium iodide +). For this purpose, the harvested cells were incubated with annexin V-Alexa Fluor and propidium iodide (1 μg/mL) for 15 minutes in the dark in a buffer containing 50 mM HEPES, 700 mM NaCl, and 0.1% bovine serum albumin. The cell suspensions were adjusted to 1 × 10^6 cells/mL before acquisition and analysis, which were performed on a fluorescence-activated cell sorting (FACS) flow cytometer (FACSCalibur; BD Biosciences, Franklin Lakes, NJ) equipped with a 15-mW, 488-nm argon ion laser. The green and red fluorescence was collected through 530/30 band-pass and 650-nm long-pass filters, with a 15-mW, 488-nm argon ion laser.

For TUNEL staining, cells were fixed with 4% paraformaldehyde for 4 hours at 4°C and then permeabilized with 0.2% Triton-X100 in 0.2 M sodium citrate solution at 4°C for 4 minutes. Wells were washed three times with PBS and incubated with a mixture of fluorescein-labeled nucleotides and terminal deoxynucleotidyl transferase (TdT) from calf thymus for 60 minutes. TdT catalyzes the polymerization of fluorescein-labeled nucleotides to free 3'-OH terminals of DNA fragments. DNA breaks were then observed under a fluorescence microscope. Care was taken to count only the cells with bright fluorescent signals, because apoptotic cells exhibit bright fluorescence due to the large number of DNA strand breaks, in contrast to the faintly stained, necrotic cells. Also, the presence of cellular morphologic changes such as cell shrinkage was sought to confirm apoptosis on TUNEL(+) cells. The count of apoptotic cells was performed under a fluorescence microscope in a masked fashion. The total number of viable cells on that surface was also determined by trypsinizing and counting of the cells in parallel dishes (Model Z-1 counter; Coulter Scientific, Hialeah, FL), and assessing their viability with the assay described earlier. The apoptosis ratio on each well was defined as the ratio of TUNEL(+) viable cells to the total number of viable cells on that surface: apoptosis ratio = (total TUNEL(+) cells – dead cells)/total viable cells. Such combined utilization of viability and TUNEL assays is known to increase the specificity of the TUNEL test.

The dose required for a 50% maximum effect (ED_{50}) was calculated for 9-cis retinoic acid from a best-fit curve. Similar series of experiments were performed with this concentration of 9-cis retinoic acid supplemented with either 0.1 mg/mL cycloheximide or 10 mM phosphor myristate acetate, to explore the inhibition of protein synthesis and stimulation of protein kinase C (PKC) on vascular endothelial apoptosis rate, respectively.

The effect of FBAE cell density on the retinoic acid-induced apoptosis rate was also studied at plating densities that ranged from 7 to 25 viable cells/mm². For this purpose, FBAE cells were synchronized by placing them in phenol-free MEM (Invitrogen-Gibco) without serum for 24 hours before harvesting with 0.25% trypsin/0.25% EDTA in Hanks’ balanced salt solution (HBSS) for 10 minutes. Two milliliters of 0.1 mg/mL aprotinin (Sigma-Aldrich) in HEPES buffer (pH = 7.5) was added to quench the trypsin reaction, and the cell suspension was centrifuged for 5 minutes at 800 rpm. The cell pellet was washed three times and then resuspended in phenol red-free MEM without serum.

The number of cells was determined (model Z-1 counter; Coulter Scientific), and cell viability was assessed with the assay described earlier. Cells were then plated at three different densities (7, 13, and 25 viable cells) into 24-well plates and allowed to attach in DMEM (with 15% FBS, 29.2 mg/mL L-glutamine, 50 μg/mL gentamicin, and 0.25 μg/mL amphotericin B) for 6 hours. Medium was then replaced with retinoid-containing medium, and apoptosis was measured after 24 hours of incubation at 37°C. This incubation time is shorter than the reported doubling time of the vascular endothelial cells. For each plating density, 36 different wells were used to obtain the average number of apoptotic cells. Attachment rates at three different densities were calculated from parallel cultures and used to calculate the apoptosis ratio.

**RPE Rescue Effect**

Synchronized second-passage FBAE cells were cultured, either alone or in the presence of a confluent layer of first-passage RPE or bovine corneal endothelium within tissue culture inserts. 9-cis Retinoic Acid was added to the medium within the inset to a final concentration of 10^{-7} M, so that retinoic acid had to permeate either the RPE monolayer or the bovine corneal endothelial monolayer to reach the FBAE cell layer. The apoptosis rate of FBAE was determined 24 hours later.

Control experiments included FBAE cells plated within an empty insert and with RPE or bovine corneal endothelium alone in the absence of 9-cis retinoic acid.

To elucidate whether the protective effect of RPE is due to passive binding and inactivation of the retinoids by cytosolic RPE proteins, we studied the effect of RPE cytosolic extracts on the 9-cis retinoic acid-induced FBAE cell apoptosis. For this purpose, first-passage confluent human RPE cells were washed once with HBSS cooled down to 4°C. Cells were then scraped from the dish with a rubber policeman on an ice plate and centrifuged at 1000 rpm for 10 minutes. The pellet was resuspended for 20 minutes in 2 mL of hypotonic buffer containing 10 mM HEPES, 10 mM KCl, 1.5 mM MgCl₂, and 2 mM dithiothreitol (DTT) at 4°C. Cells were disrupted with microtubule pestles (USA Scientific Inc., Ocala, FL). Homogenates were then centrifuged at 2000 rpm for 10 minutes. The cytosolic fraction was collected as the supernatant. The protein concentration was determined with a Bradford assay before storage at −80°C. We were able to extract approximately 2.5 mg of proteins from 10^6 RPE cells. The amount of extracted cytosolic proteins was then corrected to the number of RPE cells and added to the 9-cis retinoic acid-supplemented FBAE medium, keeping the FBAE-to-RPE ratio used for cytosol extraction at 1:1. The rate of apoptosis was determined at 24 hours, as described earlier. FBAE cells kept in plain medium and medium supplemented with 9-cis retinoic acid were used as the control.

**Determining the Diffusion Barrier of RPE**

We also studied the diffusion of 9-cis retinoic acid through an intact RPE monolayer to determine whether RPE protection against FBAE...
FIGURE 1. Determination of FBAE apoptosis with annexin V-Alexa Fluor and propidium iodide staining. FBAE cells were synchronized overnight and exposed to 9-cis and all-trans retinoic acids at concentrations varying between $10^{-4}$ and $10^{-10}$ M within a culture medium. Twenty-four hours later, the cells were harvested, stained with annexin V-Alexa Fluor and propidium iodide, and analyzed by flow cytometry (only data from the $10^{-7}$, $10^{-6}$, and $10^{-10}$ M concentrations are shown). (A) Fluorescence microscopy of annexin V-Alexa Fluor/propidium iodide–stained FBAE cells. Viable cells (first row) do not stain with annexin V and propidium iodide; however, some may exhibit faint autofluorescence (white arrow). Early apoptotic cells (second row; black arrow) stained positively for annexin V (white arrow), because of the exposure of phosphatidylserine on the outer leaflet of the plasma membrane. At later stages of apoptosis (third row) the cell membrane ruptured and the cell underwent secondary necrosis (black arrow) resulting in a positive stain for both annexin V propidium iodide (white arrows). An apoptotic body stained with annexin V was also seen at this stage (white arrowheads). The late-stage apoptotic cell population may also be contaminated with necrotic cells and mechanically damaged cells that may also exhibit dual staining for annexin V/propidium iodide. Genuinely necrotic cells (fourth row; black arrows) lost their membrane integrity and exhibited cell swelling and mitochondrial changes ending in cell lysis. They stained positively for propidium iodide but not for annexin V (white arrows). (B) Dual-color flow cytometry of annexin V-Alexa Fluor/propidium iodide staining. In all these representative set of plots, viable cells are seen in the left lower quadrant (annexin V+/PI−), early apoptotic cells in the right lower quadrant (annexin V+/PI−), late apoptotic/necrotic cells in the right upper quadrant (annexin V+/PI+), and necrotic cells in the left upper quadrant (annexin V−/PI+). Early and late apoptotic cell population increased with increasing concentrations of 9-cis retinoic acid (middle row). A mild increase in FBAE apoptosis was also evident with all-trans retinoic acid. However, this effect did not vary within the concentration range used in this study. (continues)
apoptosis is provided simply by the establishment of a diffusion barrier. For this purpose, first‐passage RPE monolayers were established on tissue culture well inserts in a serum‐free medium. We used phenol red‐free Dulbecco’s modified Eagle’s medium and Ham’s F12 as a base solution and omitted retinoic acid from the original recipe.21 The transepithelial resistance was checked daily with an epithelial voltohmometer (Millipore, Bedford, MA) until it was stable. Readings were corrected for background resistance contributed by the blank filter and culture medium.21 The integrity of the RPE monolayer was also tested by determining its diffusion barrier against sodium fluorescein. For this purpose, the medium within the inserts was supplied with sodium fluorescein (0.5 mL; 20 μg/mL; Altaire Pharmaceuticals, Inc., Aquebogue, NY). Inserts were then placed within the wells containing the medium without fluorescein (1.2 mL) and incubated in a humidified atmosphere of 5% CO2 and 95% air at 37°C. At different time points (5 minutes to 3 days) the concentrations of sodium fluorescein in the lower and upper chambers were quantified by spectrophotometry (490 nm; Cary 50 Bio; Varian, Inc., Palo Alto, CA) with the use of a standard curve. Empty inserts, bovine corneal endothelial monolayers, and EDTA (6.5 mM)‐treated RPE and bovine corneal endothelial monolayers were used as the control.

Similarly, the permeability of human RPE and bovine corneal endothelial layers to 9‐cis retinoic acid was determined by absorbance spectrophotometry.32 For this reason, the medium within the insert (0.5 mL) was supplied with 3 × 10−4 M 9‐cis retinoic acid, placed into wells containing the medium without 9‐cis retinoic acid (1.2 mL) and cultured for up to 3 days. At different time points, the amount of 9‐cis retinoic acid on both sides of the RPE monolayer was determined by ultraviolet (330 nm) absorbance spectrophotometry with a standard curve. In positive control experiments, the RPE and bovine corneal endothelium, RPE cytosolic extract, cycloheximide, and phosphor myristate acetate were corrected to the apoptosis rate of FBAE in the presence of absolute ethanol, which was used as the solvent for 9‐cis retinoic acid.

Average standardized FBAE apoptosis rates in each experimental group were compared pair‐wise by using the Bonferroni multiple‐comparison procedure.35

**RESULTS**

**Effect of Retinoids on the FBAE Apoptosis Rate**

Under the fluorescence microscope, live cells exhibited weak annexin V staining, whereas a strong fluorescent signal was emitted on the apoptotic cell membranes. Late apoptotic–necrotic cells showed a strong nuclear staining for propidium iodide in addition to the annexin V signal (Fig. 1A). Only 0.9% ± 0.8% of the FBAE cells in the control group exhibited early apoptosis after 5‐cis retinoic acid, which became significant at concentrations higher than 10−6 M (5.2% ± 0.3% for 10−6 M, P = 0.048; 5.8% ± 0.8% for 10−5 M, P = 0.001; Fig. 1B, C). A similar dose‐dependent increase was also observed in the total apoptosis rate of FBAE cells treated with 9‐cis retinoic acid, which became significant at concentrations higher than 10−6 M (2.0% ± 1.5% for control; 9.2% ± 2.2% for 10−6 M, P = 0.044; 11.5% ± 2.3% for 10−5 M; P = 0.038). The all‐trans retinoic acid mildly elevated early and total apoptosis rates; however, this increase remained nonsignificant at all concentrations used in the study. Similar to annexin V/propidium iodide staining, TUNEL assay showed a dose‐dependent increase in FBAE apoptosis after

**FIGURE 1 (Continued). (C) Apoptosis rates determined with annexin V/propidium iodide. [[Early apoptosis rates; total apoptosis rate (early apoptosis + late apoptosis). Only 0.9% ± 0.8% of the control FBAE cells exhibited early apoptosis (annexin V+/PI−). In the concentrations of all‐trans and 9‐cis retinoic acids increased the FBAE cell apoptosis rate. However, this increase reached a statistically significant level only at concentrations of 9‐cis retinoic acid higher than 10−6 M (5.2% ± 0.3% for 10−6 M, P = 0.048; 5.8% ± 0.8% for 10−5 M, P = 0.001). Similarly, a total apoptosis ratio for the control population (2.0% ± 1.5%) increased significantly at concentrations of 9‐cis retinoic acid higher than 10−6 M (9.2% ± 2.2% for 10−6 M, P = 0.044; 11.5% ± 2.3% for 10−5 M; P = 0.038). The increase in early and total apoptosis rates with all‐trans retinoic acid remained nonsignificant at all concentrations used in the study. Data represent the mean ± SD of results in three separate experiments.**
being treated with 9-cis retinoic acid. The average ratio of TUNEL(+) FBAE cells in culture without retinoid addition was 0.78 ± 0.51 cells per 100,000 cells (Fig. 2A, gray line). The 9-cis retinoic acid increased the apoptosis rate significantly (P = 0.004) in a dose-dependent manner, particularly at concentrations higher than 10^{-6} M. The dose–response curve for 9-cis retinoic acid fit to a Chapman four-parameter sigmoidal curve in the form of f = (γ₀ + a)[1 − exp(−b · x)]^y (r = 0.99; P = 0.004) with an ED₅₀ of 1.4 µM (Fig. 1B). The all-trans retinoic acid did not increase the apoptosis rate of FBAE significantly (P = 0.85, Fig. 2A). None of the retinoid solvents had a significant effect on FBAE apoptosis (Fig. 2A; P > 0.05).

**Effect of FBAE Cell Density on Apoptosis Rate**

The reattachment rate of FBAE plated at densities of 7 (43.3% ± 2.8%), 15 (45.1% ± 4.8%) and 25 viable cells/mm² (48.7% ± 4.2%) did not differ (P = 0.2). However, the ratio of apoptotic cells within the FBAE population after exposure to 9-cis retinoic acid was dependent on the cell density (Fig. 5). The apoptosis rate of FBAE was 23.6 ± 8.0 cells per 100,000 cells at a plating density of 7 cells/mm², and decreased to 18.1 ± 7.1 cells per 100,000 cells at a plating density of 13 cells/mm² and 4.8 ± 2.1 cells per 100,000 cells at a plating density of 25 cells/mm² (P = 0.03). The decrease in apoptosis rate as a function of FBAE density was statistically significant (r = −0.99; P = 0.03).

**Modulators of 9-cis Retinoic-Acid–Induced FBAE Apoptosis**

9-cis retinoic acid (10^{-6} M) alone increased TUNEL(+) FBAE cells by 2.0 ± 0.5 times over baseline (P < 0.001). A slight increase in apoptosis by the addition of cycloheximide was not significant (3.0 ± 1.0; P = 0.11, Fig. 4). Addition of phorbol myristate acetate alone decreased the rate of apoptosis back to baseline (1.0 ± 0.2; P = 0.005). We then added RPE cytosolic extract to determine whether this effect was simply due to cytosolic proteins that bind and neutralize the biological effects of retinoids. The addition of RPE cytosol inhibited the 9-cis retinoic acid–induced FBAE apoptosis (0.9 ± 0.3; P = 0.02). Similarly, the presence of an intact RPE monolayer rescued FBAE cells from apoptosis (1.1 ± 0.4; P = 0.001). However, a confluent layer of bovine corneal endothelium failed to protect FBAE from 9-cis retinoic acid–induced apoptosis (1.7 ± 0.6; P > 0.05). In the absence of 9-cis retinoic acid, neither RPE nor bovine corneal endothelium had any effect on the baseline apoptosis rate of FBAE cells (0.9 ± 0.3 and 1.0 ± 0.1, respectively, P < 0.05).

**9-cis Retinoic Acid Permeability of RPE and Bovine Corneal Endothelium**

Absorbance spectrophotometry showed an excellent linear correlation between the concentration and peak absorbance of fluorescein at 490 nm (r = 0.99, P < 0.0001) and 9-cis retinoic acid at 330 nm (r = 0.99, P < 0.0001; Fig. 5A).

Without any cellular barrier fluorescein placed in the upper compartments of the inserts diffused rapidly into the lower compartment and reached equilibrium within 1 hour (Fig. 5B). An intact RPE monolayer created a diffusion barrier to the free movement of fluorescein (P = 0.002, Fig. 5B). Although fluorescein gradually leaked, an RPE monolayer was able to maintain the fluorescein within the insert at a higher concentration than the equilibrium point, for up to 3 hours (P = 0.049). The barrier function of bovine corneal endothelium was comparable to RPE (P = 0.65). RPE lost its barrier function once the integrity of the epithelial monolayer was disrupted by adding to the medium EDTA, a calcium chelating agent that is known to disrupt cell–cell junctions (P = 0.08).

Free diffusion of 9-cis retinoic acid through the polyethyl-ene terephthalate membrane of the tissue culture insert was slower than fluorescein (P = 0.01, Fig. 5C). However, on day 3, the concentrations in the upper and lower compartments were equal. Presence of an RPE monolayer resulted in a significant retention of 9-cis retinoic acid in the upper compartment. This effect was obvious at 2 hours (P < 0.001) and continued throughout the experiment (P < 0.001). A similar barrier effect was also created with bovine corneal endothelial cells (P = 0.04). Addition of EDTA abolished the barrier function of both the RPE (P = 0.03) and bovine corneal endothelium (P = 0.02).

**DISCUSSION**

In the present study, we demonstrate that 9-cis retinoic acid, an analogue of 11-cis retinoic acid, is capable of inducing vascular endothelial cell apoptosis in the absence of an intact RPE monolayer. Metabolites of vitamin A can be found in virtually all cells of the body and play essential roles in immunity, cellular differentiation, control of gene expression, and development in vertebrates. Despite the demonstration that retinoids induce apoptosis in developing animals and cancer cells, there is less evidence that endogenous retinoids regulate apoptosis in physiological conditions. However, retinoids can inhibit human umbilical vein endothelial cell growth in vitro, decrease cell proliferation, and increase apoptosis in the intima of a healing vein bypass graft. Most effects of vitamin A derivatives are exerted by all-trans- and 9-cis retinoic acids. These retinoids act via activation of two distinct families of nuclear receptors: retinoic acid receptors (RAR) that can bind both 9-cis retinoic acid and all-trans retinoic acid and retinoid X receptors (RXR) that solely bind 9-cis retinoic acid. In the present study, the observation that only 9-cis retinoic acid can induce FBAE apoptosis in vitro suggests that activation of retinoid X receptors is necessary to avoid this effect.

Retinoids play an important part in the visual cycle in the human eye and in the normal physiological state there is active metabolism of retinoids in the RPE, photoreceptor outer segments, and Müller cells. Rod and cone opsins contain 11-cis retinal, a metabolite of vitamin A, which is isomerized to all-trans retinal by light. For the visual cycle to be reintiated, the retinal chromophore must be reisomerized to 11-cis retinal. For this purpose, all-trans retinoic acid is transported from the photoreceptor outer segment to the RPE, where it is reisomerized to 1-cis retinal. Recent evidence suggests that in the cone-dominated retina, outer retinal cells and Müller cells can maintain the visual cycle in the absence of RPE. Cone cells express enzymes required for the alternative visual cycle, such as 11-cis retinol dehydrogenase, all-trans retinol isomerase, and even RPE65, an enzyme essential for the formation of 11-cis retinol. This dual pathway for reisomerization of the visual chromophore is important for understanding the implications of the present study. Under normal conditions light isomerizes 11-cis retinal to all-trans retinal, and thus leads to a high level of all-trans retinoic acid reaching the RPE and Müller cells. In the absence of an intact RPE, reisomerization will be performed by Müller cells in cone dominated regions of the retina. This alternative pathway is able to supply the cones with the 11-cis isomer and may explain why in age-related maculopathy the kinetic aspects of rod visual adaptation are affected first with relative sparing of the cones. In this setting, the choriocapillaris vascular endothelium is exposed to high levels of 11-cis retinoids due to free diffusion of 11-cis retinoids either within shedding photoreceptor outer segments or bound to interphotoreceptor retinol binding protein (IRBP), which is not
FIGURE 2. (A) Effect of retinoids on FBAE cell apoptosis rate. Average apoptosis rate of FBAE in culture is 0.78 ± 0.51 cells per 100,000 cells (gray line). all-trans Retinoic acid and solvents for 9-cis retinoid and all-trans retinoid acids (DMSO and absolute ethanol, respectively) did not change the apoptosis rates significantly (P > 0.05). However, 9-cis retinoid acid, especially at concentrations higher than 10⁻⁷ M, increased the apoptosis rate (P = 0.004). (B) Dose-response curve for 9-cis retinoid acid-induced apoptosis. The curve fits into a Chapman four-parameter sigmoidal curve in the form of $f = (y_0 + a)(1 - \exp(-b \cdot x))^c$ ($r = 0.99; P = 0.004$), with an $ED_{50}$ of 1.4 μM. The maximum apoptosis rate (14/100,000 cells within 24 hours at a 750-μM concentration of 9-cis retinoid acid) was 18 times the physiologic rate in vitro.
compartmentalized within the subretinal space, as in the presence of the RPE. However, the protective effect of the RPE is not due simply to the presence of a diffusion barrier presented by these cells, since bovine corneal endothelium provided a similar diffusion barrier to both fluorescein and 9-cis retinoic acid, but did not protect the endothelium from retinoic acid-induced apoptosis. It is likely that the protective effect of the RPE is related to its superior ability to reisomerize all-trans retinoic acid and prevent abnormal concentrations of 11-cis retinoids from reaching the underlying vascular endothelium. The delayed cone pigment regeneration time in age-related maculopathy is also an indication that efficient reisomerization of cone visual pigments requires an intact RPE.

Because of the approximately 2000 times higher demand for rhodopsin regeneration in daylight vision, the human fovea has evolved to contain highly packed Müller and cone cell populations that are capable of recycling retinoids in addition to RPE. In the absence or dysfunction of the RPE, the foveal choriocapillaris vascular endothelium is exposed to relatively high amounts of 11-cis retinoid derivatives and becomes more vulnerable to retinoic acid-induced apoptosis. This process may explain the frequent clinical observation of subclinical foveal choriocapillaris perfusion defects in age-related macular degeneration that often precede the development of overt geographic atrophy.

Several clinical and experimental facts support our theory. Previous workers have noted that after macular translocation, choriocapillaris atrophy recurs in a region that is now located under the new foveal position. In contrast, in retinitis pigmentosa, there can be patches of preserved choriocapillaris in regions of absent RPE, indicating that prior loss of photoreceptors may protect the choriocapillaris from atrophy once the native RPE is lost. Of interest, in the rd mouse, photoreceptor degeneration leads to RPE loss at the eighth week, and subsequent choriocapillaris atrophy develops mainly in the cone-rich ventral and dorsal peripapillary area. Knocking out the visual retinoid renewal cycle in RXRα−/− mice results in complete preservation of the choriocapillaris despite RPE and photoreceptor degeneration. Finally, surgical excision of sensory retina before mechanical debridement of the RPE has been shown to preserve the underlying choriocapillaris for up to 8 weeks.

The techniques we used for detecting apoptosis involve different biochemical hallmarks of apoptosis and have well-documented sensitivity differences. Despite this fact, both assays indicated a strong and significant proapoptotic effect of 9-cis retinoic acid at concentrations as low as 10⁻⁶ M. The low ED₅₀ (1.4 μM) of 9-cis retinoic acid-induced apoptosis also indicates the high susceptibility of choriocapillaris vascular endothelium to its toxic effect. There are approximately 10⁸ rhodopsin molecules in the outer segment, and 10⁵ of these are circulated through the visual cycle per minute. In the absence of RPE, the burden of 11-cis retinoid regeneration for highly demanding cone photoreceptors will be performed by Müller cells possibly explaining the hypertrophy of these cells in age-related macular degeneration. Müller cells are 20 times faster in photoreisomerization than the RPE; however, the lack of RPE contribution may slow down the generation of 11-cis retinoids and thereby delay development of clinically overt choriocapillaris atrophy. There are no published data about the in vivo retinoid levels to which choriocapillaris is exposed in the absence of RPE cells; however, using known biometric parameters one may estimate that theoretically this amount would focally exceed 10⁵ times the calculated ED₅₀ within 17 hours. Several other inhibiting factors must have been playing a role in prolonging the development of choriocapillaris atrophy for up to 1 to 2 weeks after RPE loss. These may include diffusible survival factors from the neighboring RPE cells, decreased 11-cis retinoid production due to the decline in rhodopsin level in the macula of the eyes with...
age-related macular degeneration,69 partial metabolism of retinoids within the choroid70 and sensory retina,42,71 and binding and neutralization of the retinoids by several retinoid binding proteins, including serum retinol-binding protein that can freely diffuse into the subretinal space in the absence of RPE, or interphotoreceptor retinol binding which can neutralize up to 12% of this extra retinoid load.72

A unique feature of the human retina is that it contains an excess of retinoids (2.5 fold) over the quantity of rhodopsin. Most of this is stored in the form of retinyl ester in the RPE73 and increases with age.74 However, release of these excess retinoids within the RPE does not appear to contribute to choriocapillaris atrophy, because our results indicate that RPE cytosol exerts a protective effect rather than a proapoptotic effect on FBAE cells. The protective effect of RPE cytosol can be attributed to large amounts of retinoid-binding proteins, which are capable of neutralizing the biological effects of free retinoids.47

We observed that cell proximity is inversely proportional to the susceptibility of vascular endothelium to retinoic acid–induced apoptosis. Similarly, proliferative vascular endothelium, which participates in tumor angiogenesis in vivo, is more sensitive to apoptotic cell death than is nonproliferating vascular endothelium in vitro.75 Age-related cellular loss in the choriocapillaris is highest in the macula68 which may preferentially predispose the choriocapillaris endothelium of the aged macula more vulnerable to retinoic acid-induced apoptosis. It is apparent that the proliferative state of the cells enables resistance to retinoic acid–induced apoptosis or generates a paracrine protective effect. The inhibitory effect of phorbol myristate acetate on FBAE apoptosis indicates an important role for protein kinase C activation in an antiapoptotic pathway. Phorbol ester–mediated inhibition of FBAE apoptosis may be a complex process involving both transcriptional and posttranscriptional controls,76 mediated by both calcium-dependent and atypical protein kinase C isoenzymes.77

On the basis of animal studies, Korte et al.8 hypothesized that the RPE supplies a trophic factor for the underlying choriocapillaris.8 The results of the present study do not refute this hypothesis. However, our results provide some evidence in support of an alternative proposal—that at least some of the trophic effect of the RPE on maintaining choriocapillaris integrity is due to the RPE monolayer’s protecting the choriocapillaris from damage by retinoids ordinarily released by the overlying outer segments. Loss or damage to the RPE precedes the loss of the choriocapillaris in many diseases.9 In animal studies RPE damage due to intravenous administration of sodium iodate8 or intravitreous injection of ornithine7 damages the RPE and leads to secondary choriocapillaris atrophy. In severe nonexudative AMD, patients lose vision due to atrophy of the RPE, choriocapillaris, and outer retina.78 RPE loss may be the initiating event, with secondary changes in the underlying vasculature. In all these examples, loss of an intact RPE monolayer may exert an indirect, toxic effect on the underlying vascular endothelium, in which RPE loss leads to alterations in retinoid metabolism and apoptosis of the underlying vascular endothelium. Paradoxically, loss of RPE over an area of geographic atrophy may result in direct exposure of the adjacent choriocapillaris to proangiogenic all-trans retinoic acid from the neighboring photoreceptors and subsequent development of subretinal choroidal neovascularization.79,80 The delicate balance between the production of all-trans retinoic acid and the capacity of residual dysfunctional RPE, Müller, and cone cells to reisomerize it to 11-cis isomer may determine the clinical
phenotype of the age-related macular degeneration. If the reisomerization machinery works sufficiently the dominant isoform is 11-cis retinoic that results in geographic atrophy. In contrast, ineffective reisomerization yields excess all-trans retinoic acid, which induces subretinal choroidal neovascularization. In Scandinavian countries, where cumulative light exposure is low, geographic atrophy is the more frequently occurring form of age-related macular degeneration, in contrast to countries where higher light exposure levels result in the predominantly exudative form. Although our data do not provide any direct evidence to support this hypothesis, several clinical and experimental observations indicate a possible relationship between age-related macular degeneration and visual cycle end-products. For example, patients with age-related macular degeneration are exposed to significantly higher cumulative levels of visible light throughout their lives, and their RPE cells exhibit progressive dysfunction of retinoic acid reisomerization machinery. Blocking the forma-

**FIGURE 5.** (A) Absorbance spectrophotometry. Optic absorbance at 330 and 490 nm showed excellent linear correlations with concentrations of 9-cis retinoic acid and fluorescein, respectively. (B) Barrier against fluorescein. In control wells fluorescein diffuses freely and reaches the equilibrium (gray line) in both sides of the insert within 1 hour. Presence of an intact RPE or bovine corneal endothelial monolayer creates a similar barrier against free diffusion of fluorescein. Although this barrier was not absolute, the presence of an RPE monolayer maintained a significant difference in the concentration of fluorescein up to 3 days. Breaking intercellular attachments with EDTA disrupted the barrier effect of the RPE. (C) Barrier against 9-cis retinoic acid. It takes 3 days for hydrophobic 9-cis retinoic acid to equilibrate between the upper and lower chamber of the insert. Monolayers of RPE or bovine corneal endothelium (BCE) caused significant retention of 9-cis retinoic acid within the upper chamber. Disruption of intercellular connections with EDTA increased the permeability across the RPE monolayer.
tion of excess free 11-cis retinoic acid with 13-cis retinoic acid can decrease the cellular damage from light exposure.89

In summary, we have demonstrated that 9-cis retinoic acid (an analogue of the 11-cis isomer) is an important mediator of vascular endothelial apoptosis. These observations suggest that the relationship between the RPE and the choriocapillaris, in which a functioning RPE is necessary to maintain choriocapillaris perfusion may be modulated partially by a simple mechanism in which altered retinoid metabolism due to RPE loss may induce vascular endothelial death. Further work is needed to elucidate the full significance of this apoptotic pathway and to determine the contribution of this pathway to the balance between the RPE and choriocapillaris in vivo.

References


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