HUMAN RPE CELL APOPTOSIS INDUCED BY ACTIVATED MONOCYTES IS MEDIATED BY CASPASE-3 ACTIVATION

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ABSTRACT

Purpose: To determine the effects of activated monocytes on the induction of human retinal pigment epithelial (HRPE) cell reactive oxygen metabolite (ROM) production and apoptosis.

Methods: HRPE cells were co-cultured with interferon-γ (IFN-γ)–stimulated human monocytes. HRPE apoptosis was detected by propidium iodide, proliferating cell nuclear antigen (PCNA) and TdT-mediated dUTP nick end labeling (TUNEL) staining, caspase-3 activation, and Western blot analysis. HRPE cell ROMs were imaged using the fluorescent marker dihydrotetramethylrosamine (H_2TMRos).

Results: IFN-γ–activated monocytes in direct contact with HRPE cells elicited significant increases in TUNEL-positive ($P<.0001$) and decreases in PCNA-positive ($P<.0001$) HRPE cells. The activated monocytes also induced HRPE cell caspase-3 activation, which was inhibited by inhibitor Z-DEVD-fmk. Co-incubations, in which monocytes were either prevented from direct contact with HRPE cells or separated from HRPE cells after 30 minutes of direct contact, did not induce significant HRPE cell apoptosis. Anti-CD18 and anti-ICAM-1 antibodies significantly reduced activated monocyte-induced TUNEL-positive HRPE cells, by 48% ($P = .0051$) and 38% ($P = .046$), respectively, and caspase-3 activity by 56% ($P<.0001$) and 45% ($P<.0001$), respectively. Overlay of monocytes induced HRPE cell ROM that was inhibited by anti-CD18 and anti-ICAM-1 antibodies, but not by superoxide dismutase (SOD) or nitric oxide (NO) inhibitors. Accordingly, neither SOD nor NO inhibitors had significant effects on HRPE cell apoptosis or caspase-3 activation.

Conclusions: We demonstrated that IFN-γ–activated monocytes may induce ROM in HRPE cells through cell-to-cell contact, in part via CD18 and ICAM-1, and promote HRPE cell apoptosis via caspase-3 activation. These mechanisms may compromise HRPE cell function and survival in retinal diseases in which mononuclear phagocyte infiltration at the HRPE interface is observed.

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INTRODUCTION

Human retinal pigment epithelial (HRPE) cells form the outer blood-retina barrier and serve supportive functions for the overlying neural retina. Dysfunction and/or loss of HRPE cells can lead to retinal dysfunction and visual loss. HRPE cell alterations are a major component of ocular diseases such as proliferative vitreoretinopathy (PVR), age-related macular degeneration (ARMD), and uveitis. PVR is characterized by formation of epiretinal membranes in which HRPE cell proliferation and extracellular matrix production predominate in the early stages of the disease, while the number of HRPE cells decreases in late stages of the disease,[1] with apoptosis of HRPE cells observed in these membranes.[2] Advanced cases of ARMD and uveitis frequently show loss and degeneration of HRPE cells. Because breakdown of the blood-retina barrier is frequently seen in these diseases, HRPE cells may be exposed to humoral factors, inflammatory mediators, and infiltrating leukocytes. These factors not only alter HRPE cell function but also may influence the survival and integrity of HRPE cells. Antagonistic or synergistic signals acting on HRPE cells may be important in bringing about HRPE cell death or promoting cell survival. Epidermal growth factor, basic fibroblast growth factor, platelet-derived growth factor, and tumor necrosis factor a (TNF-α), for example, induce HRPE cell proliferation, while transforming growth factor-ß (TGF-ß) has been known to cause HRPE cell apoptosis.[2-6]

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Direct cell-to-cell contact has also been shown to influence cellular response, function, and survival in many kinds of cell types.[7-9] HRPE cells are frequently associated with monocytes in PVR, ARMD, and uveitis.[10-15] We previously found that direct interactions between HRPE cells and monocytes result in chemokine induction, and that HRPE cell responses to cytokines differ when they are co-cultured with monocytes.[9,16] Because inflammation can cause tissue destruction as well as proliferation, mononuclear phagocytes might also influence HRPE death and survival.

Activated monocytes and macrophages have the capacity to direct apoptosis of various cell types, including glomerular mesangial cells, fibroblasts, neurons, smooth-muscle cells, lung epithelial cells, T lymphocytes, neutrophils, endothelial cells, and tumor cells.[7,17-22] Although quiescent macrophages can induce neuronal apoptosis,[17] stimulation with inflammatory mediators, including interferon-γ (IFN-γ), which is known to be increased in eyes with PVR and uveitis, is required for cytotoxic activity of monocytes and macrophages with most cells.[20,22] Therefore, activated monocytes and macrophages may be expected to have effects on HRPE cell death and survival.

The balance between cell apoptosis and survival is dependent upon the relative expression of specific genes whose products interact to determine the final outcome of apoptotic signals. Among a series of cellular events, the activation of caspases appears to be a major factor for the execution of apoptosis.[23] Caspases are synthesized as inactive proenzymes that are proteolytically cleaved to an active form. Activated caspases then cleave specific target proteins at aspartic acid residues, inactivating or damaging essential cellular proteins, including enzymes involved in DNA repair, structural components of the cytoplasm and nucleus, and various protein kinases.[24,25] Of the caspase family members, caspase-3 appears to be a central effector of the caspase cascade and is considered by many as a final executioner of apoptotic cell death program.[26] Previous studies have shown that in HRPE cell apoptosis, caspase-3 activation is involved in hydrogen peroxide-, blue light exposure-, and zinc chelator-induced apoptosis, while it does not participate in ischemia-induced HRPE cell apoptosis.[27-30] However, there is no information about caspase-3 activity in HRPE cells exposed to contact with monocytes.

In this study, we examined whether IFN-γ–activated monocytes can trigger HRPE cell apoptosis. In addition, we tested the involvement of caspase-3 and characterized the cellular interactions leading to activated monocyte-induced HRPE cell apoptosis.

MATERIALS AND METHODS

REAGENTS

Recombinant human (rh) IFN-γ, antibodies against ICAM-1, VCAM-1, TNF-α, IL-1ß, and TNF-related apoptosis-inducing ligand (TRAIL) were purchased from R&D Systems (Minneapolis, Minn). Anti-CD18 antibody was purchased from Immunotech, Inc (Westbrook, Me). Rabbit polyclonal anti-caspase-3 antibody was purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, Calif). Dulbecco's Modified Essential Medium (DMEM), Ca^{2+}, Mg^{2+} free phosphate-buffered saline (PBS), fetal bovine serum (FBS), triton X-100, sodium chloride, glycerol, magnesium chloride, EDTA, sodium orthovanadate, sodium pyrophosphate, AEBSF [4-(2-aminoethyl)-benzenesulfonyl fluoride], sodium fluoride, aprotinin, propidium iodide, superoxide dismutase (SOD), and catalase were purchased from Sigma Chemical Co (St Louis, Mo). In Situ Cell Death Detection Kit, Peroxidase, was purchased from Roche Molecular Biochemicals (Indianapolis, In). Caspase-3 Cellular Activity Assay Kit was purchased from Biomol (Plymouth Meeting, Pa). Z-DEVD-fmk, N^G-monomethyl-L-arginine (L-NMMA), and N^G-monomethyl-D-arginine (D-NMMA) were purchased from Calbiochem (San Diego, Calif). CellTracker Green CMFDA and dihydrotetramethylrosamine (H_2TMRos) were purchased from Molecular Probes, Inc (Eugene, Ore). Penicillin G, streptomycin sulfate, amphotericin B, and Ficoll-Paque Plus were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden), and Fico-Lite monocytes were purchased from Atlanta Biologics (Atlanta, Ga). Diff-Quick was purchased from Baxter (McGaw, Ill), and limulus amoebocyte lysate (LAL) assay was purchased from BioWhittaker (Walkersville, Md).

HRPE CELL CULTURE

HRPE cells were isolated from healthy donors within 24 hours of death as previously described, in accordance with the Helsinki agreement.[31] In brief, the sensory retina was separated gently from the HRPE monolayer, and the HRPE cells were removed from Bruch's membrane using 1-hour incubation with papain (5 µg/mL). Isolated HRPE cells were seeded into Falcon Primaria flasks in DMEM containing 15% FBS, penicillin G (100 U/mL), streptomycin sulfate (100 µg/mL), and amphotericin B (0.25 µg/mL). The HRPE monolayers exhibited uniform immunohistochemical staining for fibronectin, laminin, and type IV collagen in a "chicken wire" distribution, characteristic for these epithelial cells. Cells, grown in culture up to six passages, were used for all experiments.

MONOCYTE CULTURE

Human monocytes were isolated as previously described,

in accordance with the Helsinki agreement.[9,16,32] Peripheral blood was drawn from healthy volunteers into a heparinized syringe and diluted 1:1 in normal saline, and mononuclear cells were separated by density gradient centrifugation (Ficoll-Paque Plus). The cells were washed and layered onto density gradient (1.068 g/mL) to enrich the monocytes (Fico-Lite monocytes). The isolated cells were washed, cytospun onto a glass slide, stained with Diff-Quick, and differentially counted.

CELL STIMULATION

HRPE cells and monocytes were either left unstimulated or stimulated with rhIFN-γ for 24 and 12 hours, respectively, before co-culture. In some experiments, HRPE cells were prelabeled with CellTracker Green CMFDA before co-culture, as described previously.[19,33] Cell cultures were washed with serum-free medium, incubated 1 hour in serum-free medium containing 3 µM CMFDA, and washed in medium containing 10% FBS to remove unbound CMFDA. Experiments were conducted in DMEM/F12 containing 10% FBS. Enriched monocyte populations were layered onto confluent HRPE monolayers. Co-cultures were incubated in control medium or medium also containing rhIFN-γ (500 U/mL) for 48 hours. In experiments in which blocking monoclonal antibodies directed against ICAM-1 (30 µg/mL), VCAM-1 (25 µg/mL), CD18 (10 µg/mL), TNF-α_(1 µg/mL)_ IL-1ß_(1 µg/mL), and TRAIL (100 ng/mL) were used, antibodies were incubated with HRPE cells and monocytes 1 hour before addition of monocytes and maintained during stimulation. L-NMMA (1 mM), D-NMMA (1 mM), and SOD (100 and 1,000 U/mL) were incubated with HRPE cells and monocytes for 30 minutes before co-culture. To determine whether cell contact was necessary for induction of apoptosis, HRPE cells and monocytes were co-incubated in the same culture media but separated by porous polycarbonate filters. Cold Ca^{2+}, Mg^{2+} free PBS containing 0.5% EDTA was used to remove monocytes from HRPE cells for caspase-3 activity analysis and Western blot analysis, as previously described.[9] The isolated cells were cytospun onto a glass slide, stained with Diff-Quick, and counted. The purity of the cells was more than 95%. Cytokines and reagents were negative for endotoxin contamination as determined by the LAL assay method (<0.05 endotoxin units/mL).

ASSESSMENT OF APOPTOSIS

Morphological Criteria

At the end of the experiment, cultures in wells were fixed by adding 4% paraformaldehyde, stored at 4ºC for 24 hours to ensure firm adherence of the fixed apoptotic cells to the cultures. Subsequently, medium was removed and propidium iodide in PBS (5 µg/mL) was added for 5 minutes to stain both monocytes and RPE cells. This reagent was then discarded and wells were covered with a fluorescent mountant. Using inverted fluorescent microscopy, five fields were randomly and blindly selected from each well so that at least 500 RPE cells were counted in each well. RPE cells were identified as cells that were labeled with green fluorescence and larger than monocytes. Apoptotic cells were clearly distinguishable by characteristic cytoplasmic blebbing, cell shrinkage, nuclear condensation, and fragmentation.

TUNEL Staining

Staining with TdT-mediated dUTP nick end labeling (TUNEL) was performed according to the manufacturer's protocol. Briefly, cultures were fixed and incubated with TUNEL mixtures containing TdT and fluorescein-labeled dNTP for 1 hour at 37ºC. Incorporated fluorescein was detected by anti-fluorescein antibody from sheep conjugated with horseradish peroxidase using the substrate diaminobenzidine. HRPE cells were distinguished by the subsequent labeling with anti-vimentin antibody, alkaline phosphatase labeled polymer (DAKO EnVision Labelled Polymer, DAKO Co, Carpinteria, Calif), and New Fuchsin substrate (DAKO Co). Stained cells were analyzed by light microscopy.

Immunohistochemistry

Fixed cells prelabeled with CellTracker Green CMFDA were incubated for 1 hour at 37ºC with monoclonal anti–proliferating cell nuclear antigen (anti-PCNA) antibody. Bound antibodies were distinguished by the subsequent incubation with rhodamine conjugated anti-mouse IgG (ICN Pharmaceuticals, Inc, Aurora, Ohio).

CASPASE-3 ACTIVITY

Caspase-3 activity was assayed using a caspase-3 cellular activity assay kit (Biomol, Plymouth Meeting, Pa) according to the manufacturer's protocol. Briefly, cell extracts were added to the microtiter wells, and reaction was initiated by adding 200 µM Ac-DEVD-pNA substrate. In parallel, the samples were reacted with this substrate in the presence of 0.1 µM Ac-DEVD-CHO, a specific caspase-3 inhibitor, to measure the nonspecific hydrolysis of the substrate. Absorbance was read at 405 nm in a microtiter plate reader.

WESTERN BLOT ANALYSIS

HRPE cells were lysed with buffer containing 50 mM Hepes (pH 7.4), 1% triton X-100, 0.15 M sodium chloride, 10% glycerol, 1.5 mM magnesium chloride, 1 mM EDTA, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 1 mM AEBSF, 10 mM sodium fluoride, 10 µg/mL aprotinin, and 10 µg/mL leupeptin. Lysates

were incubated on ice for 15 minutes with shaking, then centrifuged at 15,000 rpm for 15 minutes at 4ºC.

Western blot analysis of HRPE extracts followed the manufacturer's procedure. Briefly, samples containing 20 µg of protein were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and then were electrotransferred to nitrocellulose membranes. For signal protein detection, samples were blocked with a solution of tris-buffered saline containing 5% dry nonfat milk and 0.1 % Tween-20 (TBST) at room temperature for 1 hour, probed with antibodies to caspase-3, and washed three times in TBST. The membranes were incubated with horseradish peroxidase–conjugated secondary antibody for 1 hour at room temperature, washed with TBST, and visualized using an enhanced chemiluminescent technique.

ROM PRODUCTION DETERMINATION

HRPE cells were incubated in phenol red–free media to prevent interference with the fluorescence measurements. H_2TMRos was used as a fluorescence marker for reactive oxygen metabolite (ROM) in HRPE cells. HRPE cells were incubated for 2 hours in media containing 1 µg/mL H_2TMRos. Before co-incubation, this media was replaced with H_2TMRos-free media. Fluorescence was measured using a PTI Photomultiplier Detection System connected to a Zeiss Axiovert-inverted fluorescent microscope, enhanced by quartz optical elements. 530-nm excitation and 590-emission wavelengths were used. Measurements were performed in a circle (d = 100 µm) area of the activated monocyte-HRPE contact (n = 10). When SOD was used, the media containing SOD was replaced with SOD-free media immediately prior to microscopy to eliminate the "quenching" effect of SOD on emitted fluorescence.

STATISTICAL ANALYSIS

Individual experiments were done three times using a different HRPE cell line, with each experimental condition performed in triplicate. Human monocytes used in experiments were isolated from the blood of three different donors on separate days. Each cell line displayed similar increases or decreases over control levels. Data are expressed as mean ± SD. Various assay conditions were evaluated using ANOVA test with a post hoc analysis (Scheff multiple comparison test); P values <.05 were considered to be statistically significant.

RESULTS

APOPTOTIC INDEX OF HRPE CELLS CO-CULTURED WITH ACTIVATED MONOCYTES

We investigated whether activated monocytes could induce HRPE cell apoptosis. Prelabeling of HRPE cells with CellTracker Green CMFDA and staining of fixed cultures with propidium iodide enabled selective assessment of HRPE cells. We could see the shape and size of HRPE cells (Figure 1). Because apoptotic cells remain intact and closely associated within the cultures, it is possible to definitively assay apoptotic cell death without disturbing the cultures. This method reduces the risk of underestimating apoptosis, an inherent problem with certain assays of apoptosis.[19] In addition, we performed TUNEL staining. When HRPE cells were overlaid with IFN-γ–activated monocytes, and the co-cultures stimulated with IFN-γ, we found similar percentages of apoptotic HRPE cells exhibiting nuclear condensation (Figure 1A) and TUNEL staining (Figure 1E), 25.3 ± 4.1 and 23.7 ± 3.4%, respectively. PCNA, a useful marker for identifying proliferating cells, was also assessed after monocytes/HRPE co-culture. When monocytes were activated with IFN-γ, and the subsequent co-cultures stimulated with IFN-γ, significant increases in TUNEL-positive (P<.0001) and decreases of PCNA-positive HRPE cells (P<.0001) were observed, compared to IFN-γ–stimulated control HRPE cells without monocyte co-culture (Figure 2). When unstimulated HRPE cells were co-cultured with unstimulated monocytes, TUNEL-positive HRPE cells were not induced and PCNA-positive HRPE cells were

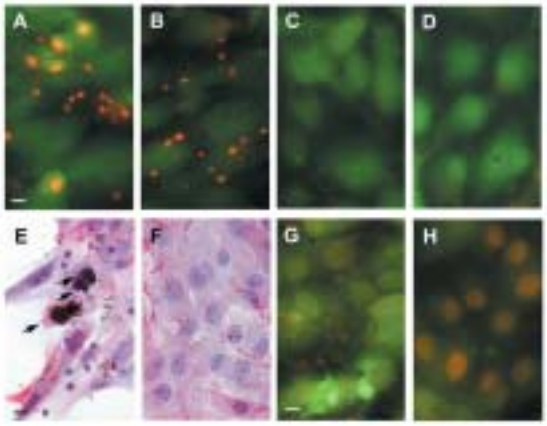

FIGURE 1

Apoptosis, TUNEL staining, and PCNA immunostaining of HRPE cell: monocyte co-cultures. A, Propidium iodide staining of 48-hour-old HRPE cell: activated monocyte co-cultures stimulated with IFN-γ, (activated co-cultures). Green HRPE cells, prelabeled with CellTracker Green CMFDA, are distinguished from monocytes, which are seen as red cells with red nuclei. HRPE cells with nuclear condensation and cell shrinkage can be seen in the activated co-cultures. B, Unstimulated HRPE cell: monocyte co-cultures. C, IFN-γ–stimulated HRPE cells. D, Unstimulated HRPE cells. E, TUNEL staining (brown) of activated co-cultures. HRPE cells were also stained for vimentin (red). F, TUNEL staining of HRPE cells alone stimulated with IFN-γ. G, PCNA immunostaining (red) of HRPE cells in activated co-cultures is reduced when compared to IFN-γ–stimulated HRPE cells alone, seen in H. HRPE cells were prelabeled with CellTracker Green CMFDA prior to co-culture. Bar = 20 µm.

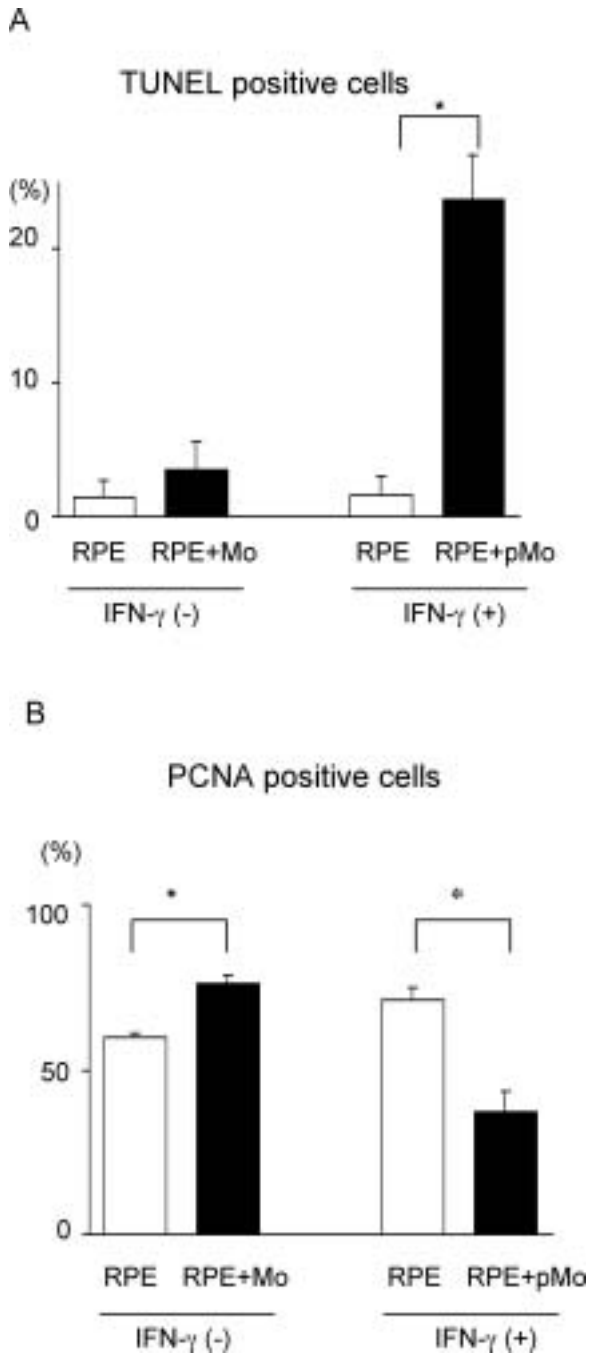

FIGURE 2

Quantitation of effects of co-culture of HRPE cells with activated monocytes on HRPE cell TUNEL and PCNA staining. Monocytes were primed with IFN-γ (pMo) or left unstimulated (Mo) prior to co-culture established as described in the "Materials and Methods" section. In activated co-cultures, co-cultures were also stimulated with IFN-γ, A, TUNEL staining of HRPE cells and monocytes. B, PCNA immunostaining of HRPE cells and monocytes. Data are expressed as percentage of TUNEL-positive HRPE cells and PCNA-positive HRPE cells. Values represent means ± SD. *, $P<.05$.

increased compared with unstimulated HRPE cell cultures (Figure 2). IFN-γ–stimulated HRPE cells, not co-cultured with monocytes, showed no apoptosis and demonstrated significant increases in PCNA-positive cells ($P = .0025$), compared with unstimulated HRPE cell cultures.

IFN-γ priming of both HRPE cells and monocytes prior to co-culture did not result in significant increases in TUNEL-positive HRPE cells, compared to monocyte priming alone (Table I). When neither HRPE cells nor monocytes were primed or when only HRPE cells were primed, co-cultures stimulated with IFN-γ induced only 48% and 52%, respectively, of the HRPE cell apoptosis produced when only monocytes were primed.

CASPASE-3 ACTIVATION IN HRPE CELLS INDUCED BY ACTIVATED MONOCYTES

We examined the involvement of caspase-3 in IFN-γ–activated monocyte-induced HRPE cell apoptosis. We quantitated caspase-3 activity in HRPE cell lysates by measuring the cleavage of the caspase-3–specific substrate Ac-DEVD-pNA. Caspase-3 activity was not detected in HRPE cells alone, with or without IFN-γ stimulation (Figure 3A). Unstimulated co-cultures did not show significant differences in HRPE cell caspase-3 activity compared with control HRPE cells (Figure 3A). When monocytes were primed, however, co-cultures stimulated with IFN-γ exhibited significant ($P<.0001$), time-dependent increases of caspase-3 activity in HRPE cells. Immunoblotting analysis also showed that the cleavage of procaspase-3 (p20) was detected in HRPE cells in activated co-cultures (Figure 3B). When the co-cultures were incubated for 24 hours with Z-DEVD-fmk, a caspase-3 inhibitor, caspase-3 activation was significantly inhibited, by 80% (Figure 3C). This inhibitor also significantly decreased TUNEL-positive cells, by 71% (Figure 3C). When both HRPE cells and monocytes were primed with IFN-γ, caspase-3 activity of HRPE cells in co-cultures was not significantly different from that with only monocyte priming (Table II). When neither HRPE cells nor monocytes, or only HRPE cells, were primed, caspase-3 activity

TABLE I: EFFECTS OF IFN-γ PRETREATMENT ON HRPE CELL TUNEL STAINING*

	TUNEL-POSITIVE HRPE CELL (%)
RPE+Mo	11.4 ± 4.8
pRPE+Mo	12.4 ± 3.0
pRPE+pMo	20.83 ± 4.8
RPE+pMo	23.7 ± 3.4

pMo, monocytes primed with IFN-g; PRPE, HRPE cells primed with IFN-γ.

*All co-cultures were incubated with IFN-γ for 48 hours.

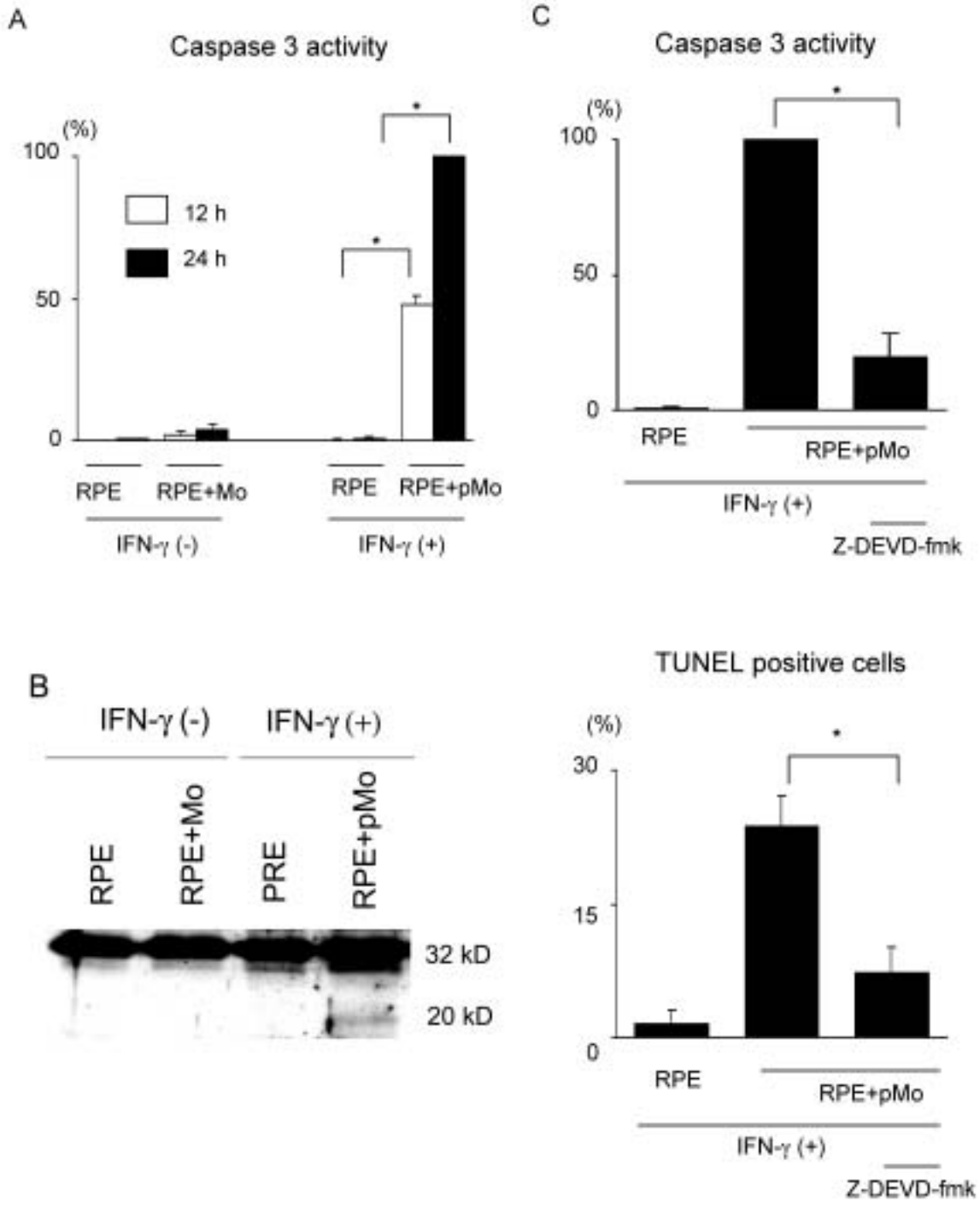

FIGURE 3

Effects of co-culture of HRPE cells with activated monocytes on caspase-3 activation. Monocytes were primed with IFN-γ (pMo) or left unstimulated (Mo) prior to co-culture established as described in the "Materials and Methods" section. In activated co-cultures, co-cultures were also stimulated with IFN-γ. A, Caspase-3 activity was measured after 12 and 24 hours of co-culture. Activity is expressed as percent of maximal activity occurring in activated 24-hour co-cultures. B, Cell lysates were prepared after 24 hours of co-culture, and Western blot analysis was performed. C, Z-DEVD-fmk (100 nmol/mL) was incubated with HRPE cells for 1 hour before addition of monocytes. Caspase-3 activity was measured after 24 hours of co-culture and is expressed as the percent activity compared with activated co-cultures without treatment with Z-DEVD-fmk (top). TUNEL staining was performed (bottom). Percentage of TUNEL-positive HRPE cells was counted. Values represent means ± SD. °, $P<.05$.

TABLE II: EFFECTS OF IFN-γ PRETREATMENT ON HRPE CELL CASPASE-3 ACTIVITY

	HRPE CELL CASPASE-3 ACTIVITY (%)	
	12 HR	**24 HR**
RPE+Mo	7.3 ± 1.2	22.3 ± 2.0
pRPE+Mo	14.7 ± 1.9	23.8 ± 4.8
pRPE+pMo	42.8 ± 5.7	91.2 ± 7.1
RPE+pMo	48.1 ± 2.6	100

pMo, monocytes primed with IFN-γ; pRPE, HRPE cells primed with IFN-γ.
All co-cultures were incubated with IFN-γ.

was only 22% and 24% of that in co-cultures with monocyte priming, respectively.

By increasing the ratios of monocytes to HRPE cells in activated co-cultures, a progressively greater proportion of HRPE cells showed apoptosis (Figure 4A). Consistent with this result, HRPE cell caspase-3 activity increased as the ratio of IFN-γ–activated monocytes to HRPE cells increased (Figure 4B).

ROLE OF CELL-TO-CELL CONTACT IN ACTIVATED MONOCYTE-INDUCED HRPE CELL APOPTOSIS

We examined whether cell contact was obligatory for HRPE cell apoptosis in activated co-cultures. Co-incubation of HRPE cells and activated monocytes in the same cultures, but separated by porous polycarbonate filters, did not significantly induce apoptosis or caspase-3 activation of HRPE cells (Figure 5). When HRPE cells and activated monocytes were co-cultured for 30 minutes, and then monocytes were removed, no significant induction of apoptosis or caspase-3 activation of HRPE cells was measured.

INVOLVEMENT OF CD18 AND ICAM-1 IN HRPE CELL CASPASE-3 ACTIVATION AND APOPTOSIS CAUSED BY ACIVATED MONOCYTES

We investigated whether adhesion or cytokine pathways contributed to the apoptosis and caspase-3 activation induced in activated co-cultures. When function-blocking anti-CD18 and anti-ICAM-1 antibodies were included in activated co-cultures, HRPE cell apoptosis was significantly decreased, by 48% ($P = .0051$) and 38% ($P = .046$), respectively (Figure 6A). Anti-CD18 and anti-ICAM-1 antibodies also significantly inhibited HRPE cell caspase-3 activity, by 56% ($P < .0001$) and 45% ($P < .0001$), respectively (Figure 6B). These inhibitory effects were not significantly enhanced by preincubation of the HRPE cells and/or monocytes with the antibodies prior to co-culture (data not shown). When these antibodies were

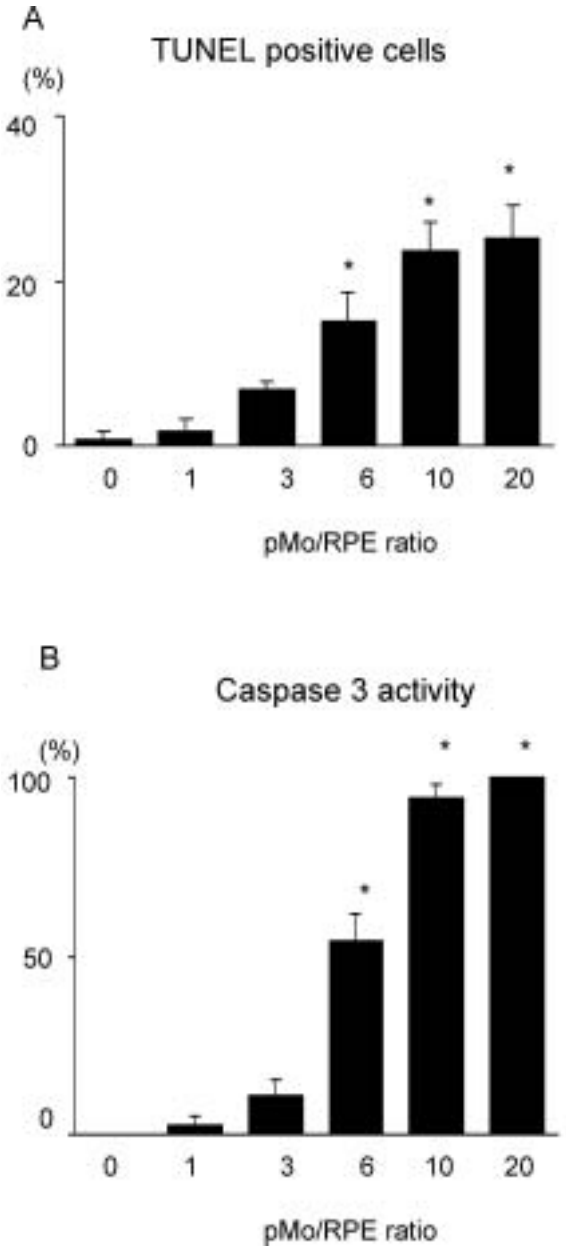

FIGURE 4
Effects of ratios of monocytes to HRPE cells in co-cultures on TUNEL positivity and caspase-3 activity. Monocytes were primed with IFN-γ for 12 hours (pMo), and then co-cultures were stimulated with IFN-γ. A, After 48 hours of co-culture, percentage of TUNEL-positive HRPE cells was counted. B, Caspase-3 activity was measured after 24 hours of co-culture and is expressed as percent activity compared with maximum activity (monocytes/HRPE cells = 20). Values represent means ± SD. *, $P<.05$, compared with control.

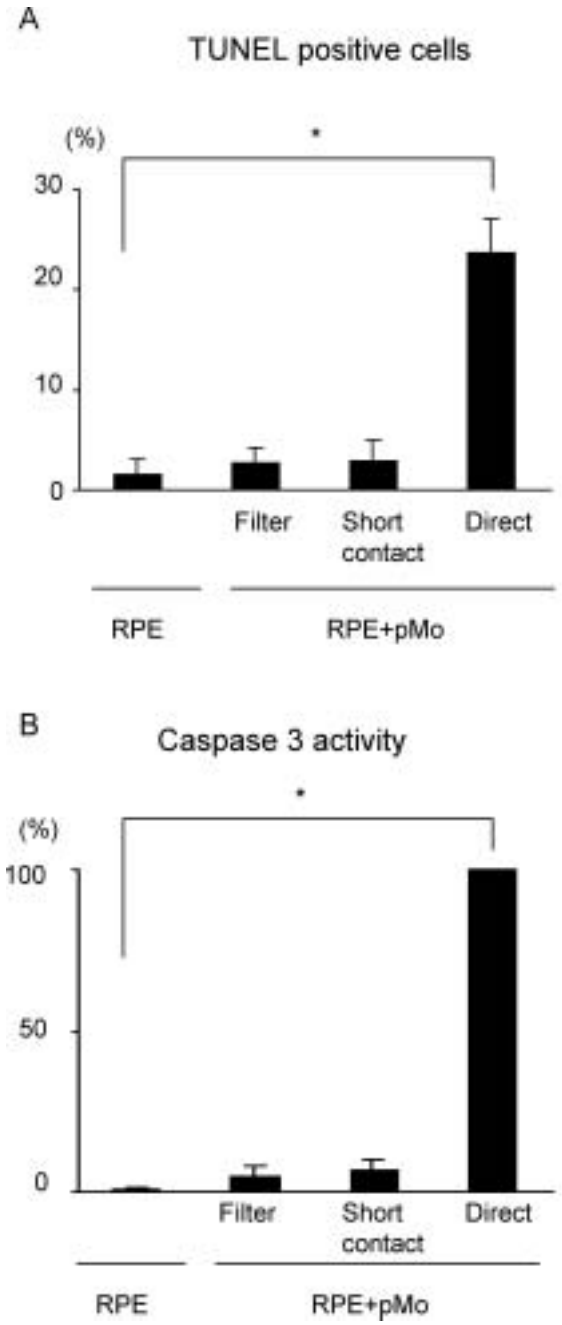

FIGURE 5

Role of cell-to-cell contact in activated monocyte-induced HRPE cell apoptosis. Monocytes were overlaid directly onto HRPE cells (direct), or co-incubated with HRPE cells in the same cultures, but separated by porous polycarbonate filters (filter), or overlaid directly onto HRPE cells for 30 minutes and then detached from HRPE cells (short contact). A, After 48 hours of co-culture, percentage of TUNEL-positive HRPE cells was counted. B, Caspase-3 activity was measured after 24 hours of co-culture, and expressed as percent activity compared with co-cultures in which monocytes were directly overlaid onto HRPE cells for the entire 24 hour period. Values represent means ± SD. *, $P<.05$.

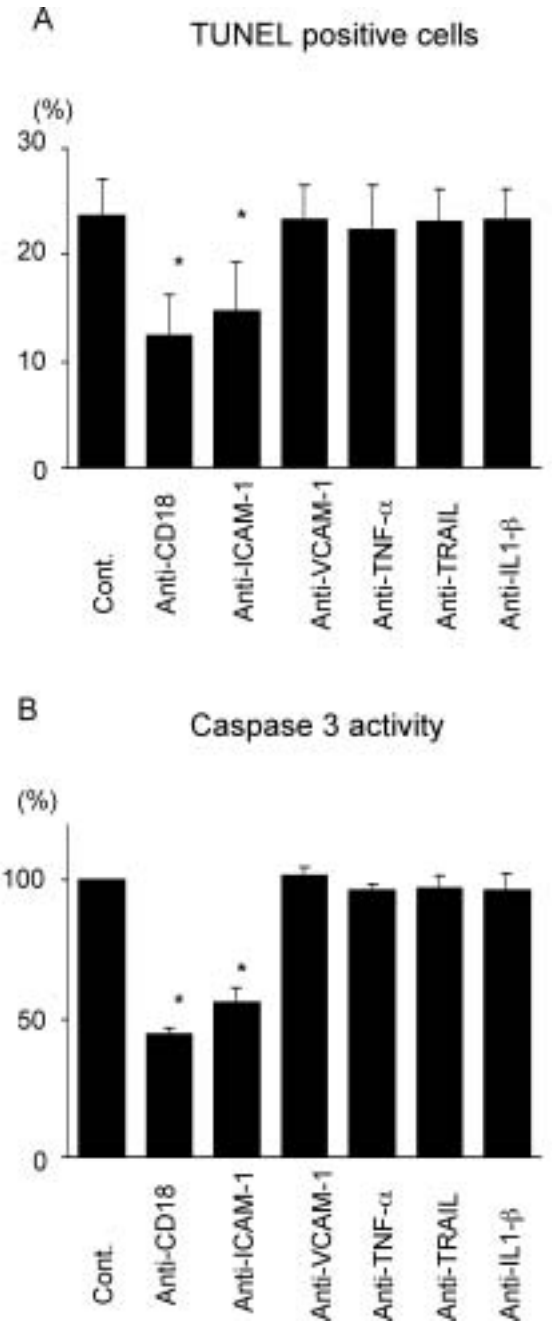

FIGURE 6

Role of adhesion molecules and cytokines in activated monocyte-induced HRPE cell apoptosis. Monocytes were primed with IFN-γ, and then co-cultures were stimulated with IFN-γ. Antibodies directed against CD18, ICAM-1, VCAM-1, TNF-α, TRAIL, and IL-1ß were incubated with HRPE cells and monocytes. A, Percentage of TUNEL-positive HRPE cells was counted. B, Caspase-3 activity was measured after 24 hours of co-culture, and expressed as percent activity compared with control co-cultures. Values represent means ± SD. *, $P<.05$, compared with control.

removed following preincubation with activated monocytes and HRPE cells but prior to co-culture, none of these antibodies significantly inhibited the caspase-3 activation (data not shown). Function-blocking antibodies to VCAM-1, TNF-α, IL-1ß, or TRAIL did not inhibit the caspase-3 activation or apoptosis.

INTRACELLULAR REACTIVE OXYGEN METABOLITE PRODUCTION IN HRPE CELLS CO-INCUBATED WITH ACTIVATED MONOCYTES

To assess whether ROMs may be associated with IFN-γ–activated monocyte-induced HRPE cell apoptosis, we visualized ROMs in HRPE cells following activated monocyte contact. We observed intracellular ROM production in HRPE cells co-incubated with activated monocytes (Figure 7). This intracellular ROM production was inhibited by anti-CD18 and anti-ICAM-1 antibodies by 61% and 35%, respectively (Figure 7B). In contrast, the antioxidant, SOD (100 U/mL and 1,000 U/mL), did not affect this HRPE cell intracellular fluorescence, presumably owing to its failure to penetrate into the cells. Likewise, SOD did not have significant effects on apoptosis or caspase-3 activation of HRPE cells (data not shown). We also found no involvement of nitric oxide (NO) in activated monocyte-induced HRPE cell apoptosis. The NO inhibitor, L-NMMA, did not affect HRPE apoptosis or caspase-3 activation (data not shown).

DISCUSSION

The blood-retina barrier limits access of inflammatory cells and molecules into the eye. Breakdown of the outer blood-retina barrier and monocytic infiltration are prominent features of PVR, ARMD, and uveitis. HRPE cells and monocytes are closely associated in the histopathologic lesions of eyes with these diseases.[10-15] There is increasing evidence that both monocytes and HRPE cells have critical regulatory functions in these disease processes and that monocytes and monocyte-derived cytokines can modulate HRPE functions and integrity.[31,34,35]

In eyes with PVR and uveitis, IFN-γ is known to be upregulated, while activated T lymphocytes, a producer of IFN-γ, have been identified in lesions of ARMD.[36-38] IFN-γ has effects on cell survival and death.[39,40] This is the first study, to our knowledge, in which IFN-γ–activated monocytes have been shown to induce HRPE cell apoptosis. When HRPE cells were co-cultured with monocytes activated with IFN-γ, we observed HRPE cell shrinkage, nuclear condensation, and cytoplasmic blebbing.

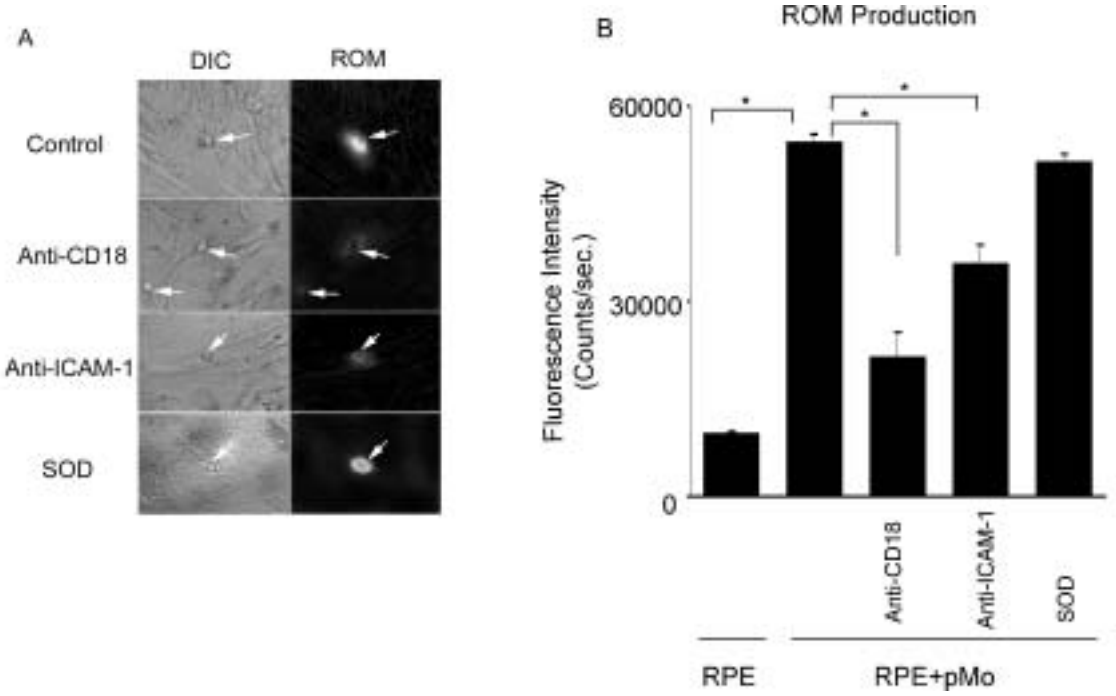

FIGURE 7

Production of reactive oxygen metabolites (ROM) in HRPE cells co-cultured with activated monocytes. Monocytes were primed with IFN-γ, and then co-cultures were stimulated with IFN-γ. Anti-CD18 and anti-ICAM-1 antibodies and SOD (100 U/mL) were incubated with HRPE cells and monocytes. A, Differential image contrast (DIC) photomicrographs (left) and fluorescence photomicrographs (ROM) (right) of co-cultures in which HRPE cells were preincubated with dihydrotetramethylrosamine (H_2TMRos). B, Fluorescence intensity was quantitated. Values represent mean fluorescence/cell ± SD. °, $P<.05$.

Activated monocytes also induced increases in TUNEL-positive HRPE cells and decreased percentages of PCNA-positive cells. These effects were enhanced by increasing the ratios of activated monocytes to HRPE cells in the co-cultures. In contrast, unstimulated monocytes did not induce HRPE cell apoptosis. In addition, increases in PCNA-positive HRPE cells were observed during co-culture with unstimulated monocytes, suggesting that unstimulated monocytes may actually enhance HRPE cell proliferation, which is consistent with previous observations.[41] These data suggest that activated monocytes could cause HRPE cell apoptosis and that the tissue milieu, including the presence of cytokines and numbers of infiltrating cells, might also change how monocytes function to modulate HRPE cell proliferation or demise.

Previous studies have reported the varied effects of mononuclear phagocytes on cell death and survival. Lang and Bishop[42] demonstrated a key role for macrophage-directed apoptosis in the elimination of unwanted capillaries in neonatal development of the mouse and rat eye. Monocytes activated with IFN-γ and lipopolysaccharide have shown cytotoxicity to vascular endothelial cells,[43] whereas unstimulated monocytes have been reported to prevent apoptosis in serum-starved vascular endothelial cells and, in fact, induce proliferation of the cells in vitro.[44] In this study, activated monocytes induced HRPE cell apoptosis, while quiescent monocytes induced HRPE cell proliferation. Therefore, a critical prerequisite for how monocytes participate in sustaining or eliminating resident cells, including HRPE cells, appears to be state of monocyte activation. Such monocyte-mediated regulation of resident cell populations is probably important in numerous diseases and over a wide range of disease activity or quiescence.

Apoptosis is mediated by multiple pathways that involve a complex array of biochemical regulators and molecular interactions. Among them, caspase-3 activation in apoptosis of various cells has been reported, implicating caspase-3 as a major execution protease. The cleavage of caspase-3 from its proactive form to its active form has been shown to be critical for its role in apoptosis.[45] The caspase-3–dependent pathway is observed in tumor cell–induced apoptosis of T lymphocytes and endothelial cells.[46,47] In this study, caspase-3 activity in HRPE cells increased as co-incubation times with activated monocytes increased. Consistent with this result, Western blot analysis of HRPE cell lysates revealed cleaved caspase-3 induction after co-culture with activated monocytes. The caspase-3 activation and apoptosis in HRPE cells were prevented by caspase-3 inhibitor, Z-DEVD-fmk, suggesting that caspase-3 may play a dominant role during activated monocyte-induced HRPE apoptosis.

Activated monocyte-induced HRPE cell apoptosis also appears to be dependent on cell-to-cell contact, since HRPE cell apoptosis was not observed when co-cultured HRPE cells and monocytes were separated by porous polycarbonate filters. Short contact (30 minutes) also did not induce HRPE cell apoptosis, suggesting that continuous contact is important in the apoptosis induction. There is increasing evidence that integrins have an important role in regulation of cell death and survival.[48] In this study, activated monocyte-induced HRPE cell apoptosis was reduced by anti-CD18 ($ß_2$ integrin) antibody. Our result is in agreement with previous reports that have shown the involvement of $ß_2$ integrins in apoptosis of smooth-muscle cells, monocytes, T lymphocytes, and neutrophils.[8,49-52] Previous studies have shown that the CD18–ICAM-1 adhesion pathway is important in the recognition of target cells and in monocyte-induced cytotoxicity of vascular endothelial cells and tumor cells.[53,54] In contrast, Noble and associates[44] demonstrated that the contact-mediated monocyte signaling that protects serum-starved endothelial cells from apoptosis is not inhibited by anti-CD18 antibody, suggesting that different signals are mediated by distinct pathways. In this study, we demonstrated that anti-ICAM-1 antibody also reduced caspase-3 activation and apoptosis of HRPE cells in response to co-incubation with IFN-γ–activated monocytes. Our data indicate that $ß_2$ integrin and ICAM-1 binding are likely to be important adhesive interactions in contact-dependent HRPE cell apoptosis.

The susceptibility of the target cells, as well as the type of triggering signal, dictate apoptotic mechanisms. IFN-γ has been demonstrated to increase susceptibility of mesangial cells and placental syncytiotrophoblasts to activated monocytes.[19,55] The increased susceptibility of placental syncytiotrophoblasts caused by IFN-γ is due to increase of ICAM-1 expression. ICAM-1 transfected melanoma cells also show augmented susceptibility to cytotoxicity by activated monocytes. ICAM-1 is present on HRPE cells in vitro and in the eyes of experimental uveitis and ARMD.[56-58] Although we have demonstrated that IFN-γ enhances ICAM-1 expression on HRPE cells,[56] priming HRPE cells with IFN-γ in this study did not show significant effects on caspase-3 activation or apoptosis in HRPE cells co-cultured with activated monocytes. Therefore, monocyte activation rather than HRPE cell susceptibility appears to be more important in CD18/ICAM-1 involvement in HRPE cell apoptosis induced by activated monocytes.

In previous studies, TNF-α has been considered to play a major role for activated monocyte- and macrophage-induced apoptosis in many cells, including neutrophils, mesangial cells, and placental syncytiotrophoblasts.[7,19,55,59] Griffith and associates[20] reported that activated monocyte-induced cytotoxicity of tumor cells is

mediated by TRAIL. However, in this study, function-blocking anti-TNF-α, anti-IL-1ß, or anti-TRAIL antibodies did not inhibit the caspase-3 activation or apoptosis of HRPE cells induced by activated monocytes. Therefore, these factors do not appear to be involved in activated monocyte-induced HRPE cell apoptosis. Our data is supported by the report that TNF-α alone did not induce HRPE cell apoptosis, even though it possesses cytotoxic activity to many other cell types.[60]

ROMs are also considered to be the major mediator of neutrophil-induced cytotoxicity of corneal epithelial cells, monocyte-induced apoptosis in natural killer cells, TGF-ß–induced apoptosis of fibroblasts, and IFN-α– and IFN-γ–activated monocyte cytotoxicity of tumor cells.[61-64] ROMs are known to induce HRPE cell apoptosis and to be implicated in the development of uveitis, ARMD, and PVR.[65-68] In our study, activated monocytes induced ROM production in HRPE cells prior to apoptosis induction. Anti-CD18 and anti-ICAM-1 antibodies significantly reduced the intracellular ROM induction. However, SOD did not affect intracellular ROM induced by activated monocytes, presumably owing to its failure to penetrate into HRPE cells.[69,70] SOD also did not have significant effects on apoptosis or caspase-3 activity in HRPE cells induced by activated monocytes. Intracellular formation of ROM is known to be more important to apoptosis induction than extracellular addition of ROM.[71] Therefore, although IFN-γ induces monocyte ROM release,[72] this is unlikely to have a significant role in HRPE cell apoptosis, since SOD did not inhibit HRPE cell apoptosis in our study. In contrast, intracellular ROM in HRPE cells induced by the contact of activated monocytes via adhesion molecules, not susceptible to SOD, may well be involved in HRPE cell apoptosis.

Involvement of NO has also been shown in activated monocyte cytotoxicity.[19] However, in this experiment L-NMMA, a NO inhibitor, did not inhibit HRPE cell apoptosis induced by activated monocytes. Although NO has been shown to inhibit HRPE cell growth and cause retinal damage, NO produced by RPE cells stimulated with IFN-γ and lipopolysaccharide could not induce RPE cell apoptosis.[73,74] Our data, together with these observations, suggest that NO does not play a significant role in the monocyte cytotoxic activity to HRPE cells.

Our data indicate that there are specific mechanisms in activated monocyte-induced HRPE cell apoptosis which exhibit some properties in common with apoptosis of other cell types. Our observations provide evidence for a new concept in blood-retina barrier breakdown: monocyte-directed regulation of HRPE cell populations by the coordinated induction of apoptosis. In other tissues, new investigations have shown monocyte/macrophage-directed regulation of mesangial cells in glomerulonephritis and of smooth-muscle cells in atherosclerotic plaques.[8,19] Monocyte-induced HRPE cell survival and death also play a pivotal role in the outcome of inflammatory responses seen in numerous retinal diseases. This study may provide a novel molecular mechanism for the regulation of HRPE monolayer integrity in ocular diseases.

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DISCUSSION

Dr Steven E. Wilson. Elner and coworkers are to be congratulated for design and implementation of an outstanding study demonstrating in vitro that activated monocytes have the capacity to promote human retinal pigmented epithelial (RPE) cell apoptosis. They further demonstrated that this process is mediated via direct cell-cell contact involving CD18 and ICAM-1.

There are few significant criticisms that can be offered regarding this important study that strongly implicates infiltrating monocytes in the death of RPE cells in numerous retinal diseases where RPE cell death has been shown to play an important role in pathophysiology. This includes age-related macular degeneration,[1-5] proliferative vitreoretinopathy,[1] and many retinal degenerations.[1,5] Inflammatory cells have been associated with the pathophysiology of many of these retinal diseases.[6,7]

There is another potential role for monocytes in the pathophysiophysiology of diseases of the retina. This

hypothesized role is based on recent developments in the understanding of osteoclast generation in bone tissue. Recent seminal studies have demonstrated that osteoclast cells are derived from monocyte cells or their precursors through cell-cell interactions with osteoblast cells.[8,9] These interactions are mediated by receptor activator of NF-kappa B (RANK) expressed by the monocyte and RANK ligand (RANKL), monocyte chemotactic and stimulating factor (M-CSF), and osteoprotegerin (OPG) expressed by the osteoblast (Figure 1).[8,9] Recently, studies in our laboratory have demonstrated that monocyte cells or their precursors may interact with activated keratocyte cells in the cornea to form "keratoclasts" in processes also involving RANK/RANKL, M-CSF, and OPG (Wilson SE, Mohan RR, Alekseev A. Expression of monocyte-regulatory cytokine and receptor mRNAs and proteins by corneal fibroblasts. Association for Research in Vision and Ophthalmology. No. 4223. Ft. Lauderdale, May, 2003). These keratoclast cells are hypothesized to participate with the activated keratocytes in generation and remodeling of the corneal stroma in wound healing and corneal disease.

Following from these paradigms, it would be interesting and potentially of great importance to the pathophysiology of many retinal diseases where fibrovascular tissue is present to determine whether monocyte cells that gain entry to the retinal tissues can undergo transition to a "retinoclast" that participates in the laying down, organization, and dissolution of pathological fibrous tissue. Thus, it would be interesting to determine whether RPE or other cells that comprise the normal or diseased retina express RANKL, M-CSF, and OPG in vitro and in situ. If so, future studies should be directed at identifying monocyte-derived cells in the retinal tissues and studying their role in pathophysiology. Finally, if a retinoclast-type cell participates in breakdown of fibrous tissue analogous to the role of the osteoclast in bone, then treatments could be envisioned in which pharmacological mediators are developed to enhance this activity to therapeutic benefit.

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Dr Ronald Klein. Would you speculate on the role of inflammation in the pathogenesis of AMD and whether it precedes the appearance of late AMD or it is a reaction to late AMD? Were your human retinal pigment epithelial cells derived from normal human eyes or eyes with signs of drusen or other changes associated with age-related macular degeneration?

Dr Marilyn B. Mets. What about hereditary retinal diseases? Do you think that there may be an inflammatory process in them as well? In Retinitis Pigmentosa or other diseases of a hereditary retinal nature where the basic biochemical track is affected because of a genetic abnormality, do you think inflammation may play a secondary role?

Dr Susan G. Elner. First, in response to Dr Klein: Our retinal pigment epithelial cell cultures are established from either healthy donor eyes obtained from the

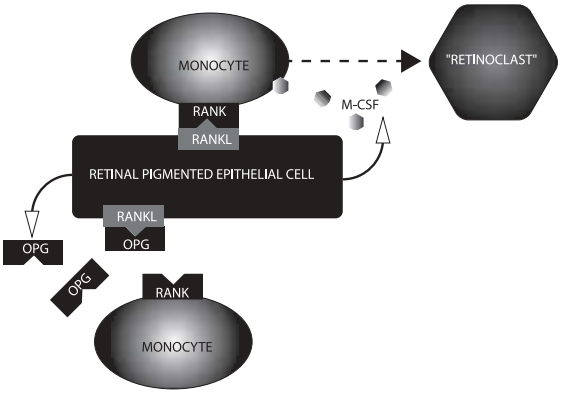

Figure 1

Hypothesized cell-cell interaction between monocyte cells or their precursors with RANKL, M-CSF-, and OPG-expressing retinal cell (RPE cell in this example) leading to transition of the monocyte to a "retinoclast" that participates in laying down and organizing fibrous or fibrovascular tissue in retinal diseases. RANKL expressed by the RPE cell interacts directly with RANK receptor on the monocyte cell. Soluble M-CSF (produced by the RPE cell) interaction with cognate receptor on the monocytes would also be necessary if the bone paradigm were followed. OPG is soluble receptor for the RANKL hypothetically produced by RPE cells to downregulate the response. Thus, the lower monocyte is blocked from RANK-RANKL-mediated interaction with the RPE cell.

Michigan Eye-Bank and Transplantation Center or from portions of eyes that have been enucleated for choroidal melanoma. These eyes do not have any evidence of AMD, on gross examination.

I believe that inflammation may be involved both early and late in the pathogenesis of AMD. The early contact between a monocyte and RPE cell may precipitate a cascade of events, including apoptosis. We have reported the production of pro-inflammatory chemokines, such as interleukin-8 (IL-8) and monocyte chemotactic factor-1 (MCP-1) by RPE cells, resulting from direct contact between monocytes and RPE cells.[1] These pro-inflammatory chemokines may further promote migration of additional leukocytes into the region of monocyte-RPE cell contact. In addition, during loss of RPE cells in age-related macular degeneration pigment and other cell components may be released, and in turn, may stimulate further low-grade inflammation. Obviously, this is not a fulminant inflammatory response, but rather a low-grade, smoldering inflammation in which monocytes, if they come into contact with an RPE cell, may promote destruction of the RPE cell. We have other work, currently ongoing, looking at the spread of intercellular signals between RPE cells, beyond the immediate point of RPE-monocyte contact. There may be mechanisms by which limited RPE cell contact with inflammatory cells may, in fact, lead to the larger geographic areas of RPE atrophy seen clinically in AMD.

In reference to the role of inflammation in hereditary retinal degenerations, I believe low-grade inflammatory responses may similarly play a role in the RPE loss associated with these diseases. Although, I have not specifically investigated this association, there is clinical evidence of low grade inflammation as evidenced by the vascular sheathing, mild vitreous cell, and occasional vascular exudation that may be seen in retinitis pigmentosa.

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