Modulation of Permeability and Adhesion Molecule Expression by Human Choroidal Endothelial Cells

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Purpose. The therapeutic potential of TA, an anti-inflammatory glucocorticoid, for the treatment of exudative retinopathy has been examined in several independent clinical studies. The modulation of permeability and adhesion molecule expression of an epithelial cell line has been described in vitro, with the use of cytokines and triamcinolone acetonide (TA). In the current study, the influence of proinflammatory cytokines and TA on permeability and adhesion molecule expression in human choroidal endothelial cells (CECs) was investigated.

Methods. Human CEC isolates treated with IFNγ, TNFα, and TA were evaluated by flow cytometry and immunocytochemistry for expression of intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, and major histocompatibility complex (MHC)-I and -II. The effects of IFNγ, TNFα, and TA on paracellular permeability of CEC monolayers were assessed in transendothelial cell resistance (TER) assays.

Results. Both IFNγ and TNFα significantly upregulated expression of ICAM1 and MHC-I on CECs. Expression of VCAM1 was induced after stimulation with both IFNγ and TNFα, whereas expression of MHC-II was induced only by stimulation with IFNγ. Cytokine-induced expression of ICAM1, MHC-I, and MHC-II antigen by CECs was significantly downregulated by TA. IFNγ stimulation also increased permeability of CEC monolayers, whereas subsequent TA treatment decreased permeability of CEC monolayers.

Conclusions. Human CEC isolates provide a useful in vitro model to study choroidal neovascular membrane characteristics and their potential response to pro- and anti-inflammatory agents. In addition, the results indicate that TA has the capacity to reduce adhesion molecule expression and permeability of choroidal vessels in vitro, confirming its potential as a therapeutic agent for treatment of exudative macular degeneration. (*Invest Ophthalmol Vis Sci.* 2002;43:3125–3130)

Intravitreal administration of glucocorticoids has been shown to be effective in reducing the incidence of experimentally induced neovascularization in rabbits,[1,2] monkeys,[3] pigs,[4] and rats.[5] The therapeutic potential of triamcinolone acetonide (TA) for the treatment of exudative age-related macular degeneration (AMD)[6,7] and cystoid macular edema in uveitis[8,9] has been examined in several independent clinical pilot studies. Subretinal vessels are derived from the choroidal vasculature, and new vessels penetrate the retinal pigment epithelium (RPE), compromising the integrity of the blood–retinal barrier (BRB). Glucocorticoids are known to display differential capacities to mediate anti-angiogenic, anti-inflammatory, and permeability effects, although the mode of action of TA on human choroidal endothelial cells (CECs) has not been completely defined.

Immunoglobulin superfamily (IgSF) molecules, including intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, and major histocompatibility complex (MHC)-I and -II, are key indicators of vascular endothelial cell activation. ICAM1 is constitutively expressed on CEC and RPE cell surfaces and is a critical component of cell–cell interaction during inflammatory responses, mediating leukocyte adhesion and extravasation.[10,11] Expression of adhesion molecules, including ICAM1, has been described in association with inflammatory cells in excised subretinal disciform lesions.[12] Furthermore, soluble factors released by reactive microglia may enhance expression of ICAM1 on vascular endothelial cells.[13] It has also been shown that microglial activation is involved in the pathogenesis of AMD[14] and that TA affects microglial morphology and quantitative expression of MHC-II in exudative AMD.[15]

Human CECs have been differentially isolated and purified by clonal elimination of contaminating cells.[16] In addition, a method for the isolation of human fetal CECs using CD31-coated beads (Dynabeads; Dynal, Oslo, Norway) has been reported.[17] In the current study, we used a novel method for the isolation of adult human CECs—*Ulex europaeus I* (UEAI) lectin–coated beads (Dynabeads). In an earlier study, we showed that TA has the capacity to modulate the expression of ICAM1 by and the permeability of a human epithelial cell line.[18] In the present study, we used human CEC primary isolates to investigate the effects of TA on the permeability of vascular endothelial cells and the expression of a range of IgSF molecules after stimulation with cytokines.

Materials and Methods

Human Choroidal Endothelial Cell Isolates

Human eyes of five donors were obtained from the Lions New South Wales Eye Bank, consistent with the Declaration of Helsinki, and were used as the source of primary CECs. Specimen ages (in years) and postmortem delay (pm; in hours) were as follows: 20 (pm 12), 24 (pm 11), 50 (pm 18.5), 54 (pm 16), and 61 (pm 9). After the vitreous was removed, the choroidal segment was separated from the neural retina and retinal pigment epithelium (RPE), cut into small pieces, washed with cold Hanks' balanced salt solution (HBSS) and 0.5 mg/mL penicillin-streptomycin (ThermoTrace Pty., Ltd., Noble Park, Australia) three times, and cut into 1- to 2-mm pieces. The pieces were incubated in an enzyme mixture containing 500 μg/mL collagenase 1A (Sigma, Australia Pty., Ltd., Sydney, Australia) and 1.2 U/mL Dispase II (Roche Diagnostics, Australia Pty., Ltd., Sydney, Australia) for 45 minutes at

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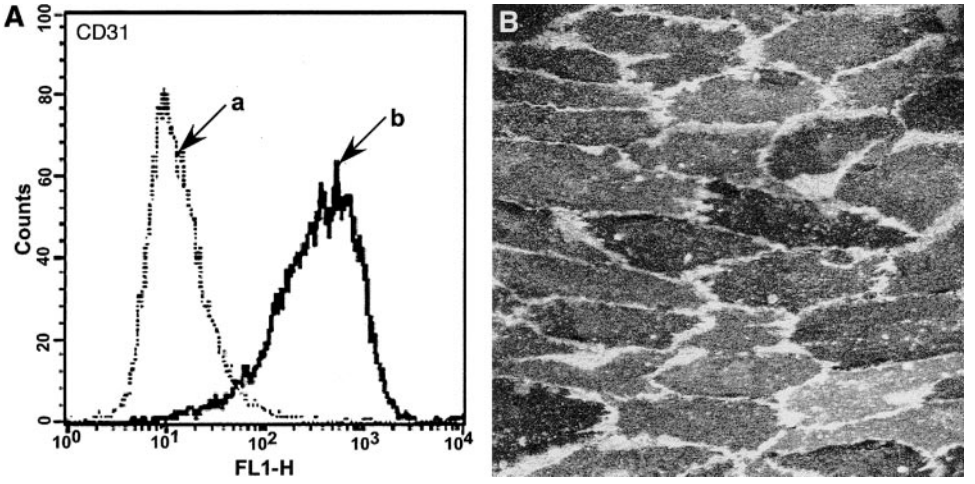

FIGURE 1. Expression of CD31 was used to determine the purity of primary isolated CECs. (**A**) Flow cytometry (FCM) histogram showing CECs labeled with mouse IgG_1 isotype control (a) and mouse anti-human CD31 antibody (b). (**B**) CEC monolayer immunolabeled with CD31 and visualized by streptavidin-fluorescein. Final magnification, ×1250.

37°C with constant agitation. Then, 400 μg/mL DNase I (Roche Diagnostics Pty., Ltd.) was added for a further 15 minutes. The choroidal digests were double filtered through 70- and 44-μm meshes, and the enzymes were neutralized by Iscoves modified Dulbecco's medium (IMDM) and 10% fetal bovine serum (FBS; ThermoTrace Pty., Ltd.).

The cells from individual donors were centrifuged at 400*g* at 4°C, and resuspended in 80 μL HBSS and 5% FBS. The cell suspension was then incubated with 12 μL *Ulex europaeus I* (UEAI) lectin (Sigma, Australia Pty., Ltd.)–coated beads (Dynbeads; Dynal) for 15 minutes at room temperature (RT). After incubation, the bead–endothelial cell complexes were washed five times by resuspending in HBSS and 5%FBS, mixed by gentle agitation for 1 minute, and separated in a magnetic particle concentrator. The bead–endothelial cell complexes were resuspended in growth medium (IMDM supplemented with 20% pooled human heated inactivated serum, obtained from authors and their colleagues; 100 μg/mL endothelial cell growth supplement [Collaborative Research Inc., Bedford, MA]; 20 μL/mL bovine retinal extract, 7 U/mL heparin [ThermoTrace Pty., Ltd.], 0.2 μg/mL insulin, and 0.5 mg/mL penicillin-streptomycin). The resuspension was then placed in a 60-mm tissue culture dish that had been coated with 0.1% gelatin and 1 μg/mL fibronectin (ThermoTrace Pty., Ltd.). After overnight incubation in a humidified atmosphere of 5% CO_2 and air at 37°C, the debris and dead cells were washed off with IMDM, and fresh growth medium was added. The CECs were passaged with 0.05% trypsin and 0.02% EDTA in HBSS after 7 to 10 days of primary culture, when large confluent areas of cells were visible.

Cultures were routinely assessed by flow cytometry (FCM) or immunocytochemistry and confirmed to be endothelial cells by positive labeling with CD31 (Fig. 1). Cells of passages 2 to 3 were used in all experiments.

Antibodies

The following primary antibodies were used: monoclonal mouse immunoglobulin G_1 (IgG_1) anti-ICAM1 (anti-CD54, 1:50 dilution), anti-VCAM1 (anti-CD106, 1:50 dilution), anti-E-selectin (anti-CD62E, 1:50 dilution) and anti-P-selectin (anti-CD62P, 1:50 dilution), all from BD Biosciences (Sydney, Australia); mouse IgG_1 anti-platelet–endothelial cell adhesion molecule (PECAM)-1 (anti-CD31, 1:50 dilution), anti-HLA-DR (anti-MHC-II, 1:50 dilution), and mouse IgG_{2a} anti-HLA-ABC (anti-MHC-I, 1:50 dilution), and mouse IgG_1 isotype control (1:50 dilution), all from Dako, Australia Pty., Ltd., (Botany, Australia). Sheep anti-mouse immunoglobulin $F(ab')_2$ fraction fluorescein isothiocyanate–conjugated (FITC, dilution 1:25, Amrad Biotech Pty., Ltd., Melbourne, Australia) was used as the secondary antibody for FCM. Biotinylated sheep anti-mouse Ig (dilution 1:100; Amersham Pharmacia Biotech Pty., Ltd., Sydney, Australia) was used as the secondary antibody for immunocytochemistry. All antibodies were titrated for FCM or immunolabeling before experimental use, and the minimum concentration for saturation labeling was chosen.

Reagents

IFNγ and TNFα (Sigma, Australia Pty., Ltd.) were dissolved in medium according to the manufacturer's instructions. TA (Sigma, Australia Pty., Ltd.) was dissolved in methanol (Selby-Biolab Scientific Pty., Ltd., Clayton, Australia) as a 10^{-2}-M stock solution. Optimal dose and time responses were established by FCM.

Flow Cytometry

CEC cells from five individual donors were seeded in 25-cm^2 flasks and cultured until confluent. The medium was removed, and the cells treated with medium alone (diluent control; methanol), 200 U/mL IFNγ for 48 hours, 200 U/mL TNFα for 48 hours, or IFNγ (or TNFα) for 4 hours with TA 5×10^{-6} M added for a further 44 hours.

FCM Labeling. After incubation, cultures were washed twice with HBSS and detached from the flasks with 0.05% trypsin and 0.02% EDTA for 2 minutes at 37°C. Cells (10^5) were pelleted by centrifugation at 463*g* for 5 minutes at 4°C and resuspended in 50 μL of primary antibody at 4°C. After a 1-hour incubation, cells were washed through 100 μL FBS by centrifugation (463*g*, 5 minutes, 4°C) and resuspended in 100 μL FITC-conjugated antibody for 45 minutes at 4°C. Cells were finally centrifuged through FBS and resuspended in 250 μL IMDM-FBS for FCM.

FCM Analysis. Fluorescence between 515 and 545 nm was measured by FCM (FACScan; BD Biosciences,) with an argon-ion laser set at an emission of 488 nm for excitation of FITC. Forward and side scatter measurements were within the same range for all populations, and 10^4 events were collected from each sample. Data analysis was performed with the accompanying software (CellQuest; BD Biosciences) and results presented either as histograms or bar graphs. Histograms show results from individual experiments and express the number of events versus $\log_{10}$ fluorescence intensity. Bar graphs show average normalized data ($n = 5$, from five donors) for peak channel fluorescence, which is a quantitative measure of the relative expression of the molecule on the cell surface.

Immunocytochemistry

In parallel with FCM experiments, CECs were seeded as described earlier, onto permeable membrane inserts (Transwell; Costar, Cambridge, MA), and immunolabeled using anti-ICAM1, anti-CD31, or the negative control (mouse IgG_1). After treatment, inserts were fixed in 2% paraformaldehyde at 4°C for 10 minutes, rinsed in PBS, and incubated at room temperature (RT) in 10% normal saline solution and 0.4% saponin and PBS for 20 minutes, before incubation with the primary

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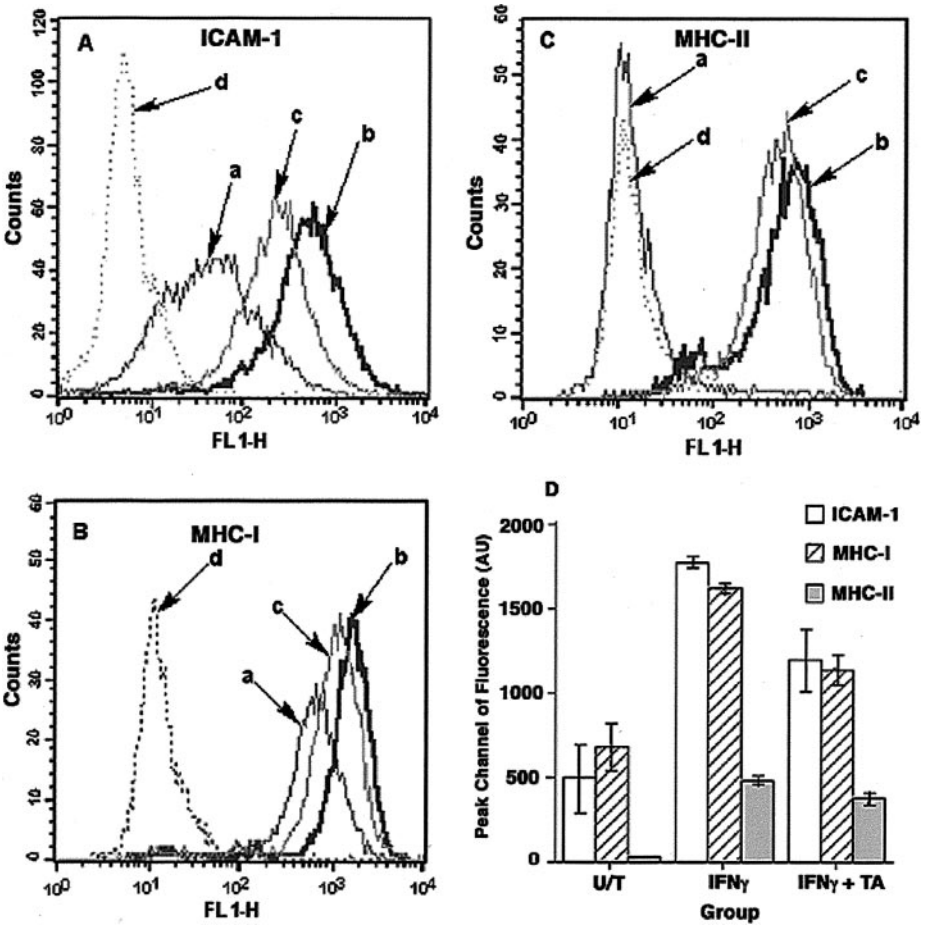

FIGURE 2. Representative FCM results showing expression on CECs of (**A**) ICAM1, (**B**) MHC-I, and (**C**) MHC-II. Stimulation with IFNγ for 48 hours significantly induced expression of ICAM1, MHC-I, and MHC-II, whereas addition of TA after 4 hours of stimulation with IFNγ significantly reduced expression of ICAM1, MHC-I, and MHC-II. The abscissa (FL 1-H) indicates $\log_{10}$ fluorescence intensity, and the ordinate indicates number of events. (a) Untreated CECs, (b) CECs stimulated with IFNγ (200 U/mL) for 48 hours, (c) CECs stimulated with IFNγ (200 U/mL) for 4 hours with TA (5×10^{-6} M) added for the remaining 44 hours, (d) CECs treated with isotype control mouse IgG_1. (**D**) Unstimulated CECs constitutively expressed ICAM1 and high levels of MHC-I but did not express MHC-II compared with the isotype control antibody. Stimulation with IFNγ (200 U/mL) for 48 hours significantly induced expression of ICAM1 (~3.5-fold), MHC-I (~3-fold), and MHC-II on CECs. A subsequent 44 hours of TA treatment (5×10^{-6} M) after a 4-hour exposure to IFNγ significantly reduced expression of ICAM1, MHC-I, and MHC-II on CECs. Data are the mean ± SEM of normalized data from five donors in five separate FCM experiments.

antibody at 4°C overnight. Inserts were then rinsed in PBS and incubated in biotinylated secondary antibody for 45 minutes. Bound antibody was detected with streptavidin-fluorescein and Cy3 (1:100 dilution; Zymed, San Francisco, CA) labeling. Inserts were mounted on glass slides in anti-fade glycerol (Dako Pty., Ltd.) and examined by confocal microscopy.

Transendothelial Resistance

Permeable membrane inserts (3-μm pore size, 6-mm diameter, area 28.3 mm^2; Transwell; Costar) were coated at RT with 35 μL of 0.1% gelatin overnight. The next day, wells were further coated with 70 μL laminin (Collaborative Research Inc.), collagen IV, and fibronectin for 2 hours (final concentrations: 1 μg laminin [50 μg/mL], 1 μg collagen IV [50 μg/mL], and 1.5 μg fibronectin [50 μg/mL]). After two washes in HBSS, the CECs (3.5×10^4/well) were plated onto coated permeable membrane inserts in a 150-μL volume of medium; 700 μL of medium was added to each well. The medium used in the transendothelial resistance (TER) experiments contained CEC growth medium and medium conditioned with human retinal mixed glia (1:1). The medium was changed every second day for the duration of the experiment. Electrical resistance was measured from day 2 with a resistance meter (ERS; Millipore, North Ryde, Australia), and the monolayers were treated once resistance was higher than 15 Ω/cm^2 (approximately 2–4 days). At that point, monolayers were either left untreated or were treated with TA (5×10^{-6} M) or IFNγ (150 U/mL) for 4 hours or with TA (5×10^{-6} M) after stimulation with IFNγ (150 U/mL).

The TERs of monolayers were calculated as the average resistance of the different groups minus the average resistance of the background control (medium and coated filter only) and then multiplied by the effective growing area (0.33 cm^2). Each data point represents the mean ± SEM of electrical resistance in an individual experiment (n = 4 permeable membranes). The experiments were repeated with CECs from three individual donors.

Statistical Analysis

Results were expressed as the mean ± SEM. Analysis of variance, followed by a multiple-comparison Bonferroni t-test, was used to analyze results. $P < 0.05$ was considered significant.

RESULTS

Phenotype of Unstimulated Human CECs

Constitutive expression of CD31 by human CECs was confirmed by FCM and immunocytochemistry (Fig. 1). FCM showed that CECs constitutively expressed high levels of ICAM1 (496 ± 225 arbitrary units [AU]) and MHC-I (685 ± 160 AU; Fig. 2). However, significant levels of MHC-II (Fig. 2), VCAM-1 (Fig. 3), E-selectin, and P-selectin were not detected on resting, unstimulated CECs.

TA and Cytokine Modulation of Expression of IgSF Molecules

Constitutive expression of ICAM1 was upregulated approximately 3.5-fold by IFNγ. TA, however, significantly downregulated the IFNγ-induced expression of ICAM1 after 44 hours of treatment ($P < 0.01$, Fig. 2A, histogram c). The CECs also constitutively expressed high levels of MHC-I, which was increased approximately twofold after stimulation with IFNγ (Fig. 2B, histogram b). TA significantly reduced upregulation of MHC-I ($P < 0.05$, Fig. 2B, histogram c). Expression of MHC-II

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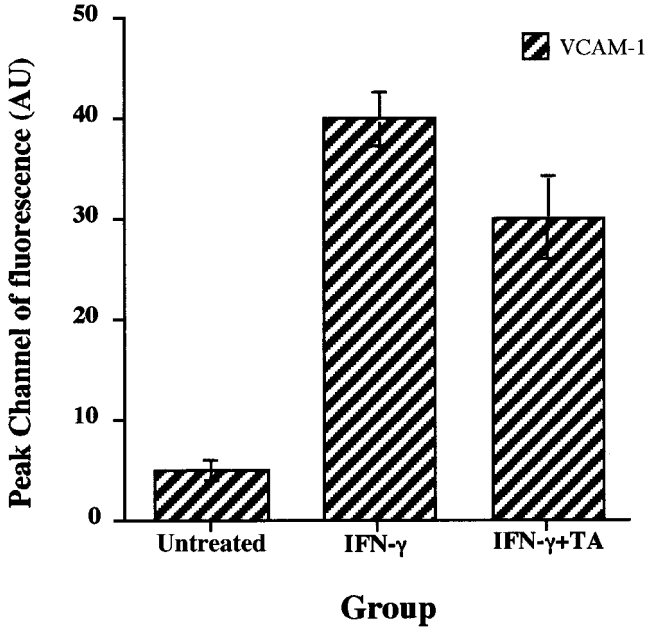

FIGURE 3. Resting CECs expressed insignificant levels of VCAM1. Stimulation with IFNγ for 48 hours induced VCAM-1 expression. Subsequent treatment with TA for 44 hours after 4 hours of initial exposure to IFNγ reduced expression of VCAM1 on CECs, although not significantly. Data are the mean ± SEM of normalized data from five separate experiments.

was not detected on resting unstimulated CECs; however, IFNγ induced expression of MHC-II (480 ± 13 AU), which was reduced by TA ($P > 0.05$, Fig. 2C, histograms b and c, respectively).

We also examined CECs for expression of VCAM1, which was almost undetectable on unstimulated CECs (Fig. 3). However, IFNγ induced low-level expression of VCAM1 (40 ± 3 AU), which was marginally but not significantly reduced by treatment with TA (Fig. 3). TA also reduced TNFα-induced expression of VCAM1 by approximately 35% (data not shown).

Stimulation with TNFα also upregulated expression of ICAM1 on CECs (approximately twofold; data not shown), although the level of upregulation was much less than that induced by IFNγ (approximately 3.5-fold; Fig. 2A, histogram b). TA significantly downregulated both IFNγ- and TNFα-induced expression of ICAM1 (IFNγ, Fig. 2A, histogram c; TNFα, data not shown). A similar profile of modulation was observed for expression of MHC-I (Fig. 2B, histogram b). No induction of MHC-II was apparent after stimulation with TNFα (data not shown). Stimulation with TNFα for 4 hours markedly induced E-selectin (fourfold) and moderately induced P-selectin, whereas IFNγ did not. TA had no effect on the expression of these molecules (data not shown).

Immunocytochemistry

Immunolabeling for ICAM1 was consistent with the FCM results. CECs grown on permeable membrane inserts showed cell membrane localization of ICAM1 (Fig. 4A), which was of greater intensity after 48 hours of stimulation with IFNγ (Fig. 4B). In unstimulated cultures, ICAM1-positive labeling was generally uniform, with occasional individual cells being more intensely immunoreactive. In the IFNγ-stimulated cultures (Fig. 4B), a patchy expression of ICAM1 occurred, perhaps due to clonal expansion of individual cells expressing high levels of ICAM1. A reduction in ICAM1 immunoreactivity was evident after treatment with TA (4 hours after stimulation, Fig. 4C). Staining with isotype control mouse IgG_1 indicated insignificant levels of nonspecific binding to CECs (Fig. 4D).

TA Modulation of TER on Resting and Activated Human CECs

A dose response to IFNγ was initially determined that showed treatment with IFNγ at 150 U/mL for 24 hours to be optimum for obtaining a differential effect on TER compared with untreated CEC monolayers (data not shown). CEC monolayers reached a stable TER (approximately 15–25 Ω/cm^2) 2 days after seeding onto permeable membranes. TA-treated monolayers had a significantly higher TER from day 1 through all time points after treatment (range from $P < 0.001$ to $P < 0.04$), except at day 4 ($P = 0.07$; Fig. 5A). Stimulation with 150 U/mL IFNγ markedly reduced resistance (~60%) from 1 day after treatment until the conclusion of the experiment (Fig. 5B). However, treatment with 5×10^{-6} M TA after stimulation with IFNγ modulated this change, with a significant increase in TER occurring at days 3 ($P < 0.01$), 5 ($P < 0.01$), and 6 ($P < 0.05$). Figure 5 illustrates results of typical experiments; similar results were obtained in three separate experiments.

DISCUSSION

In an earlier study, human CECs were differentially isolated, purified by clonal elimination of contaminating cells, and cul-

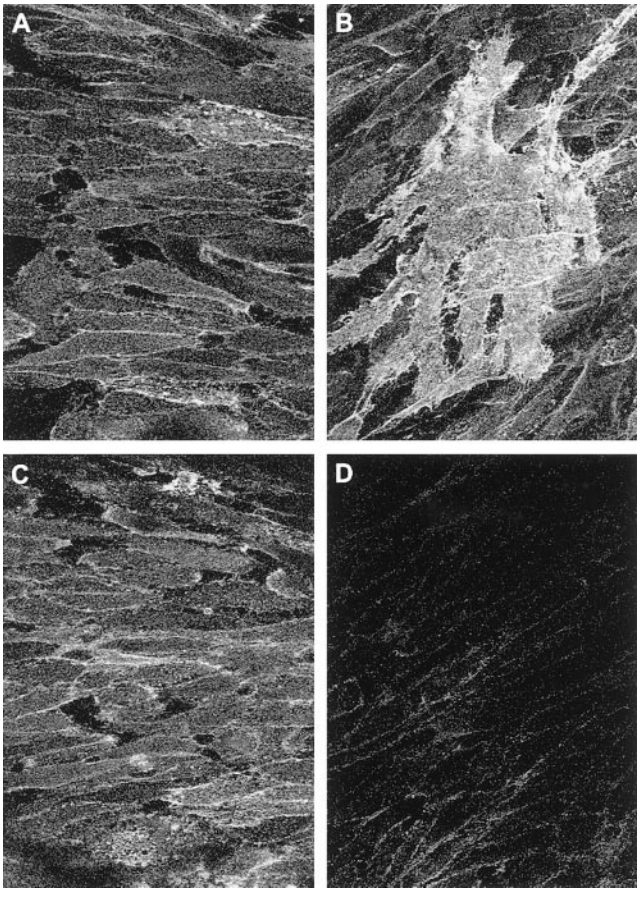

FIGURE 4. ICAM-1 immunostaining was consistent with FCM results. (**A**) Resting CEC monolayer showed cell membrane–localized ICAM1. (**B**) IFNγ-stimulated CEC monolayer displayed a greater intensity of ICAM1 staining on cell membranes. (**C**) Subsequent treatment of the IFNγ-stimulated CEC monolayer with TA reduced ICAM1 immunoreactivity. (**D**) Mouse IgG_1 isotype control staining of the CEC monolayer showed low-level background staining. Final magnification, ×950.

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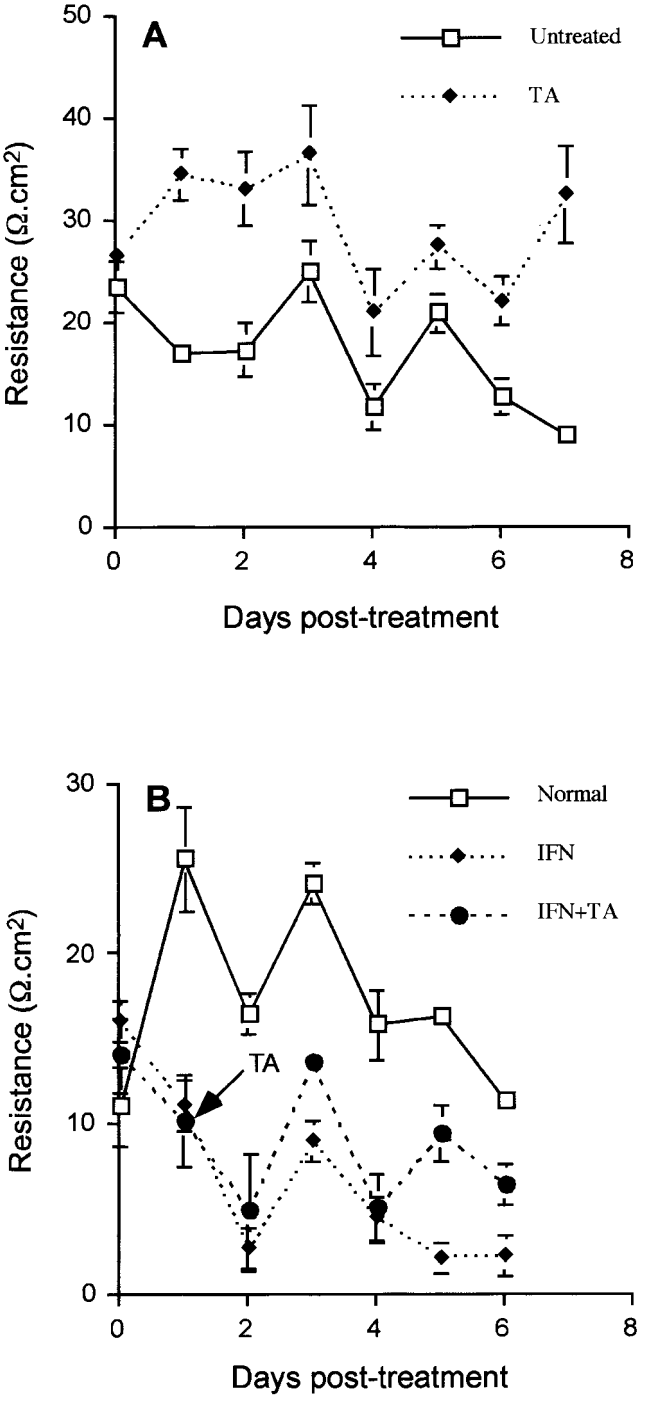

FIGURE 5. **(A)** Comparison of the TER of TA-treated CEC monolayers versus control CEC monolayers. TER was significantly higher in the TA-treated (5×10^{-6} M) monolayer from day 1 to the completion of the experiment, compared with the control monolayers. **(B)** IFNγ (150 U/mL) markedly decreased TER from day 1 after treatment to the conclusion of the experiment. Treatment with IFNγ followed by TA modulated the change, with a significant increase in TER at days 3, 5, and 6. Data are the mean TER ± SEM of four experiments ($n = 4$).

tured in a collagen gel.[16] In addition, a method for the isolation of bovine CECs involving use of lectin-coated beads (Dynabeads; Dynal) has been published.[19] More recently, a preliminary report described the use of CD31-coated beads to isolate human fetal CECs.[17] Primary human CEC isolates provide a pertinent in vitro model for studying choroidal neovascular membrane characteristics and their potential response to pro- and anti-inflammatory agents. The present results indicate that TA has the capacity to reduce expression of adhesion molecules and the permeability of human CECs in vitro, confirming its potential as a therapeutic agent for the treatment of exudative retinopathy.

Chronic inflammatory cells have been reported in AMD lesions[20–22] and surgically excised choroidal membranes,[23] and a variety of cell types are involved in subretinal neovascular lesions, including vascular endothelial cells and leukocytes.[24] It has been established that CECs are the primary source of exudation and neovascularization in exudative AMD[16]; however, it has been pointed out that in many cases of exudative AMD, there is a significant involvement of retinal vascular leakage.[25] Glucocorticoids, such as TA, influence the activity of various cell types (RPE, vascular endothelial cells, and leukocytes) involved in fibrovascular lesions and have shown anti-inflammatory, -exudative, and -angiogenic effects.[6–9,26] Glucocorticoid receptors are widely distributed in mammalian tissues and have been detected in human RPE cells[27] and bovine endothelial cells.[28] The rationale for the use of anti-inflammatory glucocorticoids for the treatment of exudative macular degeneration has been derived from observations of animal models and pathologic specimens that implicate immune processes in AMD. Evidence relating leukocytes and cytokines to the formation of new vessels in the choroid and the role of microglia in AMD[29,30] has been recently reviewed.[24]

The proinflammatory cytokines TNFα, IFNγ, and IL1β are major inducers of expression of ICAM1 in most cell types.[31] In a previous study, we demonstrated that TA ameliorates modulation of both permeability and expression of ICAM1 that is experimentally induced by treatment of the ECV304 epithelial cell line with phorbol myristate acetate (PMA), IFNγ, and/or TNFα, representing a model of epithelial and RPE cell permeability.[18] In the present study, using similar techniques, we investigated the influence of those cytokines and TA on human CEC primary isolates. Both IFNγ and TNFα significantly upregulated expression of ICAM1 and MHC-I on human CECs. This contrasts with our previous findings in ECV304 cells that indicated that expression of MHC-I was not significantly modulated by either cytokines or TA.[18]

TNFα is chemotactic for monocytes and fibroblasts, acting synergistically with IFNγ,[32] which has been shown to induce expression of MHC-II in human RPE cells.[33] In the present study we found that IFNγ, but not TNFα stimulation, induced expression of MHC-II on human CECs and that treatment with TA subsequently produced a small but consistent decrease in IFNγ-induced expression. It has been suggested that TNFα secreted by macrophages promotes choroidal neovascularization.[34] Histopathologic analyses of AMD-affected eyes has revealed downregulation of expression of MHC-II antigen on vascular elements associated with intravitreal administration of TA.[15] Collectively, the results of these studies reveal differential expression of IgSF in response to both pro- and anti-inflammatory agents by transformed epithelial and primary endothelial lineage cells.

Proinflammatory effects of TNFα on the blood–retinal barrier have also been demonstrated to include permeability changes involving microglia and Müller cells.[35] We suggested previously that modulation of epithelial resistance by TA in vitro is consistent with clinical observations, indicating that reduction of the permeability of the outer blood–retinal barrier and downregulation of inflammatory stimuli are significant effects of intravitreal TA in vivo.[18] Recent histopathologic analyses of human eyes showed diminished exudation associated with intravitreal administration of TA,[15] and in the present study TA produced a decrease in the permeability of resting human CECs. It appears that the clinical effects of TA in

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exudative AMD, reported in abstracts and peer-reviewed publications,[6,7] may involve downregulation of ICAM1, reduced choroidal leukostasis, and reduced paravascular permeability.[18]

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