Cyclosporine A Enhances Leukocyte Binding by Human Intestinal Microvascular Endothelial Cells through Inhibition of p38 MAPK and iNOS

PARADOXICAL PROINFLAMMATORY EFFECT ON THE MICROVASCULAR ENDOTHELIUM

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The calcineurin inhibitor cyclosporine A (CsA) modulates leukocyte cytokine production but may also effect nonimmune cells, including microvascular endothelial cells, which regulate the inflammatory process through leukocyte recruitment. We hypothesized that CsA would promote a proinflammatory phenotype in human intestinal microvascular endothelial cells (HIMEC), by inhibiting inducible nitric-oxide synthase (iNOS, NOS2)-derived NO, normally an important mechanism in limiting endothelial activation and leukocyte adhesion. Primary cultures of HIMEC were used to assess CsA effects on endothelial activation, leukocyte interaction, and the expression of iNOS as well as cell adhesion molecules. CsA significantly increased leukocyte binding to activated HIMEC, but paradoxically decreased endothelial expression of cell adhesion molecules (E-selectin, intercellular adhesion molecule 1, and vascular cell adhesion molecule-1). In contrast, CsA completely inhibited the expression of iNOS in tumor necrosis factor-α/lipopolysaccharide-activated HIMEC. CsA blocked p38 MAPK phosphorylation in activated HIMEC, a key pathway in iNOS expression, but failed to inhibit NFkB activation. These studies demonstrate that CsA exerts a proinflammatory effect on HIMEC by blocking iNOS expression. CsA exerts a proinflammatory effect on the microvascular endothelium, and this drug-induced endothelial dysfunction may help explain its lack of efficacy in the long-term treatment of chronically active inflammatory bowel disease.

The calcineurin inhibitor cyclosporine A (CsA) is a potent immunosuppressive agent that has formed the pharmacologic cornerstone of solid organ transplantation. CsA prevents the activation of lymphokine genes essential for T cell proliferation by disrupting calcium-dependent signal transduction pathways in leukocytes. Although pharmacologic studies of CsA have focused primarily on T cell responses, there is emerging evidence that this agent may exert potent effects on blood vessels, where it disturbs production of nitric oxide (NO) ultimately promoting arterial hypertension, inducing long-term vascular dysfunction, and contributing to obliterative vasculopathy in chronic transplant rejection (1–4).

Because of the powerful immunosuppressive effect of CsA, and its success in the prevention of transplant rejection, this agent underwent extensive clinical evaluation for the treatment of chronic inflammatory disorders, including inflammatory bowel disease (IBD; Crohn’s disease and ulcerative colitis). Initial studies focused on short courses of high-dose intravenous CsA therapy, and have demonstrated impressive success for the treatment of fulminant colitis and the healing of refractory Crohn’s disease fistulas. However, multiple attempts to convert high-dose CsA to an oral maintenance strategy, akin to the success in transplant immunosuppression, have paradoxically failed to demonstrate efficacy (5–11). The precise cellular and molecular mechanisms that underlie this lack of efficacy in the long term treatment of chronic intestinal inflammation have remained undefined, and may result from the emerging profile of adverse effects of CsA on nonimmune cell populations, including the vascular endothelium.

Endothelial cells lining the microvasculature are now appreciated to play a central “gatekeeper” role in inflammation through their ability to recruit circulating immune cells into tissues (12, 13). Microvascular endothelial activation and adhesion of circulating immune cells is an early and rate-limiting step in the initiation and maintenance of the inflammatory response. Advances in vascular biology have defined an important regulatory role for NO within the blood vessel during

oxido synthase: VCAM-1, vascular cell adhesion molecule-1; ICAM-1, intercellular adhesion molecule-1; MAPK, mitogen-activated protein kinases; SAPK, stress-activated protein kinase; JNK, c-Jun NH₂-terminal kinase; ERK, extracellular signal-regulated kinase; TNF-α, tumor necrosis factor α; LPS, lipopolysaccharide; NFkB, nuclear factor κB; IBD, inflammatory bowel disease; DCF-DA, dichlorodihydrofluorescein diacetate; L-NMMA, Nω-nitro-L-arginine; L-NIL, Nω-nitro-L-lysine; PEG-SOD, polyethylene glycol-conjugated superoxide dismutase; ROS, reactive oxygen species; PBS, phosphate-buffered saline; CAM, cell adhesion molecule; iNOS, inducible nitric-oxide synthase.
inflammation (14), where endothelial-derived NO maintains vascular homeostasis through its ability to down-regulate endothelial cell activation and leukocyte binding (15–19). The effect of CsA on microvascular endothelial cells, specifically their ability to regulate leukocyte adhesion during inflammatory activation, is currently undefined.

The signaling pathways that mediate the inflammatory activation of nonimmune cells, including endothelial cells, are presently under intense investigation. The mitogen-activated protein kinase (MAPK) pathway is a conserved family of eukaryotic signal transduction molecules known to play a major role in the activation of multiple cell types in response to inflammatory stimuli (20–23). The MAPK superfamily is comprised of the extracellular signal-regulated protein kinase (ERK1/2, p42/44 MAPK), stress-activated protein kinase (SAPK; c-Jun NH2-terminal kinase (JNK)), and p38 MAPK. MAPKs are known to play a key role in the activation of endothelial cells, leading to an inflammatory phenotype characterized by increased expression of cell adhesion molecules, and enhanced leukocyte binding (24, 25). Moreover, expression of multiple proinflammatory genes in endothelial cells is regulated by the transcription factor nuclear factor κB (NF-κB) (26, 27). The impact of CsA on signal transduction pathways in nonimmune cell types is distinct from its well characterized immunosuppressive effect on immune cells (28). However, the effect of CsA on tissue-specific microvascular endothelial activation has not been defined.

We characterized the effect of CsA on the activation of human intestinal microvascular endothelial cells (HIMEC), the microvascular population that mediates leukocyte recruitment during chronic intestinal inflammation in IBD. We hypothesized that CsA would exert a deleterious, "proinflammatory" effect on HIMEC, through selective effects on intracellular signaling cascades during inflammatory activation, leading to a loss of NO production and enhanced leukocyte adhesion. To test this hypothesis, we utilized primary cultures of HIMEC to directly assess the effect of CsA on endothelial-leukocyte interaction. We focused experiments on the effect of CsA on two critical intracellular mechanisms that regulate the inflammatory activation of HIMEC, specifically the expression of iNOS and cell adhesion molecules, as well as the signal transduction pathways that underlie inflammatory activation of these cells. The results of this investigation demonstrate that CsA inhibits the production of iNOS-derived NO in activated HIMEC, normally a key down-regulatory mechanism for limiting endothelial cell activation induced by tumor necrosis factor-α (TNF-α) and lipopolysaccharide (LPS). Furthermore, CsA differentially inhibited signal transduction pathways during HIMEC activation, blocking activation of p38 MAPK, a major mechanism for iNOS gene transcription (29), ultimately resulting in a paradoxical, proinflammatory effect of this normally powerful immunosuppressive drug.

MATERIALS AND METHODS

Patients—Macroscopically normal mucosal specimens for HIMEC isolation were obtained from patients undergoing scheduled bowel resection. The use of discarded human tissues was approved by the Institutional Review Board of the Medical College of Wisconsin.

Isolation and Culture of Intestinal Microvascular Endothelial Cells—HIMEC isolation was adapted from dermal microvascular endothelium (30, 31). In brief, the surgical specimen was rinsed and full-thickness samples of intestinal tissue were obtained. Mucosal strips were dissected, washed, digested in type II collagenase solution (Worthington) and were then subjected to mechanical compression to express clusters of microvascular endothelial cells, which were plated onto fibronectin-coated tissue culture dishes, and grown in HIMEC medium (MCDB 131 medium (Sigma) supplemented with 20% fetal bovine serum and endothelial cell growth supplement (Upstate Biochemical, Lake Placid, NY)). After 7–10 days, microvascular endothelial cell clusters were physically isolated, and a pure culture was obtained. Endothelial cultures were verified by modified lipoprotein uptake (Dil-ac-LDL, Biomedical Technology, Inc., Stoughton, MA) and expression of factor VIII-associated antigen (32). All HIMEC experiments were carried out using cells obtained between passages 8 and 14. HIMEC isolates from resected normal intestinal tissue from six patients were utilized for this study.

U937 Monocyte-like Leukocytes—U937 cells, a human monocyte-like cell line, originally derived from a histiocytic lymphoma, were obtained from American Type Culture Collection (Manassas, VA) and maintained in culture with RPMI 1640 medium and 5% fetal bovine serum. Cells were cultured over three passages per week and were used between passages 5 and 10, once re-established in culture.

Pharmacologic Modulation of HIMEC—CsA (Alexis, San Diego, CA) was added to the endothelial cultures for specified time periods at a concentration of 1–10 μM. Additional immunomodulators were tested including FK506 (1 μM; Calbiochem, San Diego, CA) and rapamycin (1 μM; Calbiochem). The contribution of signal transduction pathways to HIMEC activation and gene expression were defined using specific inhibitors, including: p38 MAPK inhibitor SB203580 (10 μg/ml; Calbiochem); p42/44 MAPK inhibitor PD98059, which inhibits MEK (7 μM; Calbiochem); the tyrosine kinase inhibitors genistein (1 μg/ml; Sigma) and herbimycin (1 μM; Calbiochem); the antioxidant curcumin (10 μM; Sigma); and the inhibitor of NF-κB activation Bay 11 (5 μM; Biomol Research Laboratories, Plymouth Meeting, PA).

Pharmacologic Modulation of Nitric Oxide and Superoxide Production—Nω-Monomethyl-l-arginine (l-NMMA, Sigma) (1 mM) or N-iminoethyll-lysine (l-NIL, Alexis Biochemicals) (1 mM) were added to endothelial monolayers at the time of induction as competitive inhibitors of nitric-oxide synthase, respectively. In addition, l-NIL was utilized at a dosage of 20 μM, which preferentially inhibits iNOS function (33). Polyethylene glycol-conjugated superoxide dismutase (PEG-SOD, Sigma) (100 units/ml) was applied to HIMEC monolayers to preferentially increase degradation or inhibit production of intracellular superoxide anions during the 24-h activation with cytokines (TNF-α, 100 units/ml; R&D Systems, Minneapolis, MN) and LPS (1 mg/ml, Escherichia coli 0111:B4, Sigma). PEG-SOD was applied to the HIMEC monolayers for 2 h prior to and during the 24-h activation period.

Assays of Endothelial Activation Using Leukocyte Adhesion—Assays of U937 adhesion to HIMEC were measured as described previously (31). In brief, endothelial cells were seeded onto fibronectin-coated 24-well tissue culture plates (Corning Glass) at 0.5 × 105 cells/well using HIMEC medium and grown to confluence over 48–72 h. Unless otherwise noted in the figure legends, endothelial cells were stimulated directly or following stimulation with a combination of TNF-α (100 units/ml) and LPS (1 mg/ml). After 24 h of activation, HIMEC monolayers were rinsed and U937 cells (106/ml) were co-cultured on endothelial monolayers and allowed to adhere at 37°C in a 5% CO2 incubator. Following a 1-h incubation, nonadherent cells were removed, and residual cells were rinsed 3 times with Dulbecco’s phosphate-buffered saline (PBS) (Invitrogen) followed by gentle shaking of the tissue culture plate. Monolayers were then fixed and stained in modified Wright’s stain (DiffQuick, McGraw, IL), and adherent leukocytes were counted in 10 random high power fields (×20) using an ocular grid. Adhesion was expressed as number of adherent leukocytes/mm2.

In addition to the static adhesion assay described above, an endothelial-leukocyte low shear stress flow adhesion assay was used to assess HIMEC activation and function. The endothelial flow chamber was based on the design of McIntyre and co-workers (34), which allows a flow of leukocytes at 1 dyne/cm² over the endothelial monolayer. Endothelial cells were plated onto fibronectin-coated 35-mm tissue culture dishes (Corning Glass) at 1 × 105 cells/dish using HIMEC medium and grown to confluence over 48–72 h. Endothelial cells were analyzed directly or following stimulation with a combination of TNF-α (100 units/ml) and LPS (1 μg/ml). After 24 h, monolayers were rinsed and assayed in a low shear stress flow chamber (34, 25). U937 cells (1 × 106 ml of HIMEC medium) flowing at a rate of 1 dyne/cm² were co-cultured over the endothelial monolayers and allowed to adhere at 37°C, and adhesion was recorded using a CCD camera attached to an inverted tissue culture microscope for 5 min. Data were analyzed by counting the number of adherent leukocytes on 10 random high power fields (×20) using an ocular grid and adhesion was expressed as number of adherent leukocytes/mm².

Reverse Transcriptase-PCR for iNOS—iNOS gene expression was assessed in unstimulated and activated confluent cultures of HIMEC, with or without CsA and other pharmacologic inhibitors. Endothelial cells were stimulated with a combination of 100 units/ml TNF-α (R&D Systems) and 1 μg/ml LPS (Sigma) for 6 h at 37°C. Total RNA was
extracted using RNaZol B (Tel-Test, Friendswood, TX) and quantitated by optical density. 2 μg of RNA was reverse transcribed using SuperScript II RT (Invitrogen) in a total reaction volume of 20 μl. 1 μl of reverse transcription product (cDNA) was PCR amplified with AmpliTaq DNA polymerase (PerkinElmer Life Sciences) and 0.5 μl each of 10 μM forward and 10 μM reverse primers were included in the reaction as an internal control for the efficiency of the reverse transcriptase and the amount of RNA used in the reverse transcriptase-PCR. Each PCR cycle consisted of a denaturation step (94 °C, 1 min), an annealing step (60 °C, 1 min), and an elongation step (72 °C, 1.5 min) with a total of 35 cycles, followed by an additional extension step (72 °C, 7 min). The amplification primers for human iNOS were synthesized based on published sequences (36, 37). The primer sequences (F = forward, r = reverse primers) and PCR product sizes were as follows: iNOS: 5′-TCTTGTGTCAAAAGCTGTCGCT-3′ (F) and 5′-CATTGGCCAACATCTGGTGTCG-3′ (R). 237 bp PCR products were run on 1% agarose gels and stained with 0.5 μg/ml ethidium bromide, visualized under UV light, and photographed.

Measurement of NO Production—HIMEC (2 × 10⁶) were cultured overnight in 60-mm fibronectin-coated tissue culture dishes (Corning Glass). After rinsing the monolayers, medium was replaced with 2 ml of MCD 131 supplemented with 2% fetal bovine serum and supernatants were collected after 48 h. Total NO₂⁻/NO₃⁻ production was assessed by reduction to NO and measurement of chemiluminescence using a Sievers 280a Chemiluminescence Analyzers Inc. (Boulder, CO). Medium collected with 0 μM and 2 μg/ml of Elf-1 fusion protein for 30 min at 30 °C. The reaction was then terminated by addition of SDS treatment buffer and samples were analyzed by SDS-PAGE and Western blotting using phospho-Elf-1 antibody (Ser395). Western blots were visualized by ECL.

Kinase Assay of MAPK Activity—Assays of kinase activity were carried out using nonradioactive kinase assay kits (New England Biolabs) according to the instruction manuals as follows: p42/44 MAP kinase activity assay: 200 μg of total cellular protein from HIMEC lysates were incubated with 15 μl of resuspended, immobilized phospho-p42/44 MAPK (Thr202/Tyr204) monoclonal antibody overnight at 4 °C with gentle agitation. After washing, the pellet was resuspended in 50 μl of kinase buffer with 200 μM ATP and 2 μg of ATF-2 fusion protein as a substrate for 30 min at 30 °C. These reactions were terminated by addition of SDS treatment buffer and analyzed by SDS-PAGE and Western blotting using phospho-ATF-2 (Thr76) antibody.

Nuclear Protein Extraction and Gel Electromobility Shift Assay—Nuclear extracts were prepared as described (41). Briefly, HIMEC were lyzed in buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM dithiothreitol, and 10 μg/ml of each aprotinin and leupeptin). Cell homogenates were centrifuged (14,000 × g, 1 min, 4 °C) and the pellet was resuspended in buffer A containing 0.1% (v/v) Nonidet P-40. After centrifugation (14,000 × g, 10 min, 4 °C), 90 μl of buffer B (200 mM NaCl, 0.5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml of each aprotinin and leupeptin) were added to the supernatant (cytosolic fraction). The pellet was suspended in 60 μl of buffer C (20 mM HEPES, pH 7.9, 25% (v/v) glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, and 10 μg/ml of each aprotinin and leupeptin). Samples were incubated on ice for 10 min. After centrifugation (14,000 × g, 10 min, 4 °C), 90 μl of buffer D was added to the supernatant (nuclear fraction). Protein concentrations were determined as described above. Nuclear extracts were incubated with 2 μg/ml poly(dI-dC) and [γ-32P]ATP and 2 μg/ml of oligonucleotide probe. DNA-protein complexes were resolved on 10% polyacrylamide gels for 90 min at 2000 V constant voltage. Gels were dried and exposed to x-ray film for 18 h at -80 °C.

Expression and Assays of MAPK Activities—HIMEC were seeded at 2.5 × 10⁴ cells per well and grown for 48–72 h in individual coated wells (Corning Glass). Cells were then washed with PBS (3 times) before centrifugation (14,000 × g, 10 min, 4 °C). 50 μl of each of 100 ng/ml of TGF-β1 and 10 ng/ml of TGF-β2 were added to plates and incubated for 4 h. Cell lysates were analyzed by SDS-PAGE and transferred to nitrocellulose membranes (40). The membranes were blocked and incubated with specific antibodies (phosphorylated, nonphosphorylated MAPK (Cell Signaling, New England Biolabs, Beverly, MA), and iNOS (Santa Cruz Biotechnology, Santa Cruz, CA)), as described. Detection was by secondary antibody coupled to horseradish peroxidase and ECL™ (Amersham Biosciences).

Immunoprecipitation—Cell homogenate was prepared in lysis buffer (50 mM Tris, pH 7.5, 1 mM EDTA, 1% Triton X-100 in 60-mm fibronectin-coated tissue culture dishes (Corning Glass) until confluence was reached. Endothelial monolayers were assessed unstimulated, or following 24 h activation. HIMEC were seeded at 1 × 10⁶ cells per well and grown for 48–72 h in individual coated wells (Corning Glass) until confluence was reached. Endothelial monolayers were assessed unstimulated or following 24 h stimulation with TNF-α (100 units/ml/LPS (1 μg/ml), in the presence or absence of CsA (1–10 μg/ml) 30 min prior to visualization. cells were loaded with 5 mM 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA) (Acros Organics/Fisher), a nontoxic compound that freely diffuses into cells. It is deacylated by cellular esterases to the membrane impermeable, nonfluorescent derivative 2′,7′-dichlorodihydrofluorescein, which in the presence of intracellular reactive oxygen species and peroxides, is oxidized rapidly to the highly fluorescent 2′,7′-dichlorofluorescein. Coverslips of viable HIMEC loaded with DCF-DA (10 μL) were incubated in 0.1% (v/v) Triton X-100 in PBS, and blocked with 5% bovine serum albumin (BSA) (Sigma) for 30 min at 30 °C. After blocking, the coverslips were rinsed with medium, inverted, and visualized on a fluorescence microscope (absorption 504 nm; emission 529 nm). DCF-DA were rinsed with medium, inverted, and visualized on a fluorescence microscope (absorption 504 nm; emission 529 nm). DCF-DA were incubated with DMSO (0.1% (v/v) 25 min at 4 °C with gentle agitation. After the supernatants were recovered by centrifugation, the supernatants were discarded and the complexes were washed with PBS (3 times) before further analysis.
RESULTS

CsA Increases Activated HIMEC-Leukocyte Adhesion—Primary cultures of HIMEC interacting with U937 monocyte-like cells were used to assess the effect of CsA on endothelial activation, and to test the hypothesis that CsA would cause a proinflammatory effect with enhanced leukocyte binding in the

TNF-α/LPS-activated endothelial cells. Monolayers of HIMEC can be used to assess endothelial function in vitro, including their ability to undergo activation and binding of leukocytes through specific interaction of cell adhesion molecules. U937 cells were used as a target leukocyte population for these assays, as this established monocyte-like cell line is known to

FIG. 1. A, modified Wright's stain of unstimulated HIMEC monolayer after 1 h co-culture with U937 monocyte-like cells. Low power (×40), brightfield microscopic view; firmly adherent U937 nuclei stain dark purple, whereas HIMEC nuclei stain light purple. HIMEC were seeded at 5 × 10⁵ cells per well and 48 h later were exposed to 10⁵ U937 cells. B, HIMEC monolayer from the same patient, pretreated with 10 μM CsA for 24 h prior to co-culture with U937 cells. There is no increase in leukocyte adhesion to the endothelial monolayer. C, HIMEC monolayer, stimulated with 100 units/ml TNF-α and 1 μg/ml LPS for 24 h prior to co-culture with U937 cells. Ten-fold increase in leukocyte binding to the HIMEC monolayer following inflammatory activation is notable. D, HIMEC monolayer pretreated with CsA prior to stimulation with TNF-α/LPS (as above) for 24 h prior to the 1-h U937 co-culture. Furthermore, a significant increase in leukocyte binding by HIMEC is noted, above the increase caused by TNF-α/LPS activation demonstrated in panel C. Note that the increase in leukocyte adhesion following CsA activation only occurs following activation with TNF-α/LPS, and is not seen in the unstimulated monolayers exposed to this agent. Representative images of three experiments on HIMEC cultures are shown. E, static adhesion of U937 monocyte-like cells to HIMEC monolayers in the absence and presence of CsA. Adhesion assays were performed on unstimulated monolayers, and endothelial monolayers were pretreated with CsA prior to stimulation with TNF-α/LPS for 24 h prior to the 1-h U937 co-culture. CsA did not affect binding of the unstimulated HIMEC, but caused a significant, 2-fold increase in leukocyte binding in the TNF-α/LPS-activated HIMEC. n = 3 total experiments, each performed with a distinct HIMEC cell line derived from three different patients, performed in duplicate; an asterisk denotes a significant difference between cells with and without CsA (p < 0.05); data are expressed as mean ± S.E. F, low shear stress flow adhesion of U937 monocyte-like cells to HIMEC in the absence and presence of CsA. Adhesion assays were performed on unstimulated monolayers, and endothelial monolayers preactivated for 24 h with 100 units/ml TNF-α + 1 μg/ml LPS prior to U937 co-culture. The low shear stress flow adhesion assay measures the binding of leukocytes to the endothelial surface under physiologic shear conditions of 1 dyne per cm². n = 3 total experiments, each performed with a distinct HIMEC cell line derived from three different patients, performed in duplicate; an asterisk denotes a significant difference between the cells treated and not treated with CsA (p < 0.05); data are expressed as mean ± S.E.
express the specific cell adhesion molecules VLA-4 and sialyl Lewis X, which mediate binding to their endothelial ligands VCAM-1 and E-selectin, expressed on activated HIMEC. HIMEC exhibit a tightly regulated pattern of U937 monocyte binding that readily increased with overnight TNF-α/LPS activation using an endothelial-leukocyte adhesion assay (Fig. 1, A and C). Fig. 1A demonstrates that unstimulated HIMEC bound low levels of U937, and pretreatment of unstimulated HIMEC with CsA did not affect leukocyte binding (Fig. 1B). In marked contrast, CsA pretreatment of the TNF-α/LPS-activated HIMEC demonstrated a dramatic enhancement in U937 binding (Fig. 1D). Quantification of this enhanced leukocyte binding by CsA-treated HIMEC demonstrated a significant increase (Fig. 1E). To confirm that this striking phenomenon of CsA-induced leukocyte hyperadhesion in activated HIMEC was not an artifact of the static adhesion assay, additional experiments were conducted in a low shear stress flow adhesion chamber, which models the physiologic interaction of leukocytes flowing over an endothelial monolayer. Using this assay, CsA similarly exerted no effect on the binding of leukocytes to the unstimulated HIMEC monolayers (Fig. 1F). However, CsA pretreatment of HIMEC activated for 24 h with TNF-α/LPS demonstrated a significant (p ≤ 0.05), 2-fold increase in U937 binding.

We then examined the potential mechanisms underlying the proinflammatory effect of CsA on activated HIMEC and leukocyte hyperadhesion. Endothelial activation with cytokines and/or LPS results in increased cell adhesion molecule (CAM) expression, including the molecules ICAM-1, VCAM-1, and E-selectin. Activation of HIMEC has previously been demonstrated to result in increased surface expression of these three adhesion molecules, leading to enhanced interaction with leukocyte ligands and up-regulation in leukocyte binding to the endothelium. Previous experiments in our laboratory have demonstrated that U937 adhesion to HIMEC is dependent on endothelial expression of E-selectin and VCAM-1. The effect of CsA on CAM expression in unstimulated and TNF-α/LPS-activated HIMEC was assessed using monoclonal antibodies and a whole cell radioimmunoassay as described under “Materials and Methods.” Fig. 2 demonstrates that resting HIMEC expressed low levels of ICAM-1, and undetectable levels of E-selectin and VCAM-1. Following activation with TNF-α/LPS, there was a dramatic increase in the cell surface of all three adhesion molecules. When HIMEC were pretreated with CsA, and then activated with TNF-α/LPS, there was a slight, but significant (p ≤ 0.05) decrease in the level of expression of all three CAMs. These data were surprising, given the significant, 2-fold increase in leukocyte binding, induced by CsA pretreatment in activated HIMEC. These findings suggested that mechanisms other than modulation of CAM surface expression mediate the enhanced leukocyte binding and proinflammatory effect of CsA on activated HIMEC.

CsA Inhibits Nitric Oxide Production in Activated HIMEC—A major mechanism regulating endothelial activation and homeostasis involves enhanced nitric oxide production from increased expression of the inducible nitric-oxide synthase (iNOS, NOS2). We have demonstrated that iNOS-derived NO down-regulates activation following cytokine (TNF-α, IL-1β) and LPS activation of HIMEC (33). To investigate the effect of CsA on HIMEC NO production and its function, experiments were performed using static adhesion assays with and without CsA in the presence and absence of the nonselective NOS inhibitor L-NMMA. L-NMMA functions as an inactive substrate for the multiple NOS enzyme isoforms, and at 1 mM concentration prevents NO production from in vitro endothelial monolayers. When L-NMMA was applied to HIMEC, there was a significant (p ≤ 0.05), greater than 2-fold increase in U937 adhesion, compared with TNF-α/LPS-activated HIMEC alone. HIMEC pretreated with CsA then activated with TNF-α/LPS demonstrated enhanced leukocyte binding similar to the effect of nonselective NOS inhibition with L-NMMA. When L-NMMA and CsA were simultaneously applied to HIMEC prior to activation, there was no additive effect (33) (Fig. 3A). Likewise, use of the iNOS-specific inhibitor t-NIL in conjunction with CsA showed effects similar to L-NMMA, with no further increase in leukocyte binding (Fig. 3A, right bars). This suggested that iNOS-derived NO was the major source of NO production in activated HIMEC.

Because the effect of CsA on activated HIMEC was identical to that of the NOS inhibitor L-NMMA, which is known to block NO production, we conducted experiments to examine directly the effect of CsA on NO production by activated HIMEC monolayers. We have previously established that HIMEC constitutively produce low levels of NO, which increase 2–3-fold following activation. Detection of very low levels of NO can be performed using chemiluminescence (33), where the NO metabolites nitrite, nitrate, and nitrosothiols are measured, reflecting the original levels of NO production. Pretreatment of HIMEC monolayers with CsA prior to activation with TNF-α/LPS for 48 h demonstrated a complete inhibition of increased NO production (Fig. 3B). These data strongly suggested that CsA would block enhanced NO production in activated HIMEC. Because iNOS plays a central role in the generation of enhanced NO in activated HIMEC, our next investigation focused on the effect of CsA on HIMEC activation and iNOS gene expression.

TNF-α/LPS Activation of MAPK Family Members in HIMEC—Our initial findings demonstrated that CsA exerted a significant effect on HIMEC during TNF-α/LPS-induced activation, but not on the unstimulated endothelial cells. To further characterize the effect of CsA on HIMEC activation, we focused our investigation on the MAPK signaling pathways, which have been implicated in TNF-α/LPS induction of multi-
ple cells types, including endothelial cells. We initially defined the signaling cascades that mediated TNF-α/LPS activation in HIMEC, using Western blotting of immunoprecipitated cell lysates with specific phosphoantibodies against all three of the MAPK family members (Fig. 4, A–C). Using phospho-specific antibodies for p42/44 MAPK (ERK1/2), p38 MAPK, and JNK/SAPK, respectively, all three of these signal transduction molecules revealed rapid phosphorylation in HIMEC following TNF-α/LPS activation. Time course experiments demonstrated that p38 MAPK underwent a rapid and transient activation, which peaked at 15 min, whereas p42/44 MAPK activation following TNF-α/LPS stimulation peaked at 30 min (Fig. 4, D and E).

The Effect of CsA on HIMEC Expression of iNOS—iNOS expression following inflammatory stimulation plays an integral role in the regulation of HIMEC activation. Effect of CsA on the expression of iNOS mRNA was determined using reverse transcriptase-PCR. TNF-α/LPS activation of HIMEC resulted in a readily detectable signal for the iNOS gene product (237 bp) and CsA pretreatment inhibited expression of iNOS (Fig. 5). Experiments were initiated to evaluate the role of specific MAPK in the expression of iNOS following TNF-α/LPS stimulation. The p38 MAPK inhibitor SB203580 completely inhibited the expression of iNOS mRNA in activated HIMEC, similar to the effect of CsA. The p42/44 MAPK inhibitor PD98059 partially inhibited HIMEC iNOS expression following TNF-α/LPS stimulation.

CsA Differentially Inhibits Activation of MAPK Family Members in HIMEC—These initial experiments focusing on signal transduction pathways underlying the expression of iNOS in HIMEC suggested that p38 MAPK was playing the dominant role in TNF-α/LPS-mediated expression, and the inhibitory effect of CsA was similar to the specific p38 MAPK inhibitor SB203580. Western blot analysis of immunoprecipitated cell lysate demonstrated that the inhibitory effect of CsA on expression of iNOS was similar to the p38 MAPK inhibitor SB203580, whereas inhibition of ERK (p42/44 MAPK) with PD98059 failed to inhibit iNOS expression in TNF-α/LPS-activated HIMEC (Fig. 6A). To confirm that CsA was inhibiting activation of p38 MAPK in TNF-α/LPS-stimulated HIMEC, immobilized phospho-p38 MAPK antibody was used to immunoprecipitate p38 MAPK in unstimulated and pretreated TNF-α/LPS-stimulated HIMEC. Then in vitro kinase assay was performed using ATF-2 as a substrate and phospho-ATF-2 antibody (Thr^71) to detect ATF-2 phosphorylation. TNF-α/LPS readily induced p38 MAPK in HIMEC, resulting in subsequent activation of ATF-2, and this activation was abolished by both CsA and the specific p38 MAPK inhibitor SB203580 (Fig. 6B). p42/44 MAPK activation was partially inhibited by CsA (Fig. 6C). Activation of HIMEC with TNF-α/LPS phosphorylated JNK/SAPK, which was inhibited by genistein, curcumin, and herbimycin. However, CsA exerted no effect on JNK/SAPK phosphorylation, and this pathway was not further investigated (Fig. 6D).

Effect of CsA on NFκB Activation in HIMEC—NFκB plays a key role in cytokine and LPS activation of endothelial cells (26). Therefore, we investigated the potential effect of CsA on NFκB activation in HIMEC. TNF-α/LPS stimulation readily activated NFκB in HIMEC, as demonstrated by gel shift analysis (Fig. 7A) as well as nuclear translocation of the p65

![Graph](http://www.jbc.org)
subunit of NF-κB detected by immunofluorescence microscopy (not shown). CsA pretreatment of HIMEC failed to inhibit activation of NF-κB, unlike Bay 11 and PD98059, which both blocked NFκB activation.

CsA Results in Increased HIMEC Oxidant Stress—Because CsA significantly increased activated HIMEC-leukocyte binding capacity independently of cell adhesion molecule surface density, we further characterized the mechanisms of CsA-induced leukocyte adhesion. Studies have demonstrated that endothelial activation results in a rapid increase in the generation of superoxide anions and intracellular oxidant stress (16, 42). We hypothesized that the loss of iNOS-derived NO in the CsA-treated HIMEC would similarly lead to enhanced activation of these cells through an oxidant-mediated mechanism. To assess this possibility, DCF fluorescence was used to assess the presence of reactive oxygen species in HIMEC undergoing activation with and without CsA. DCF-DA is an intravital dye that complexes with intracellular reactive oxygen species, and can be visualized by fluorescence excitation. There was low-level oxidant stress detected in unstimulated HIMEC (Fig. 8A). When HIMEC were stimulated with TNF-α/LPS, oxidant stress was rapidly induced (data not shown), but after 24 h, levels were only slightly above baseline (Fig. 8B). However, when HIMEC were treated with CsA at the time of TNF-α/LPS activation, there was a dramatic increase in reactive oxygen species detected at 24 h (Fig. 8C).

Superoxide Scavenger Reverses the Proinflammatory Effect of CsA on Activated HIMEC—Because both CsA and NOS inhibition blocked production of NO and increased intracellular oxyradical stress, experiments were performed to determine whether this mechanism contributed to the enhanced HIMEC-leukocyte binding capacity. To determine whether CsA enhanced leukocyte binding was the result of increased superoxide anion levels in the TNF-α/LPS-activated HIMEC, we employed a pharmacologic inhibition of superoxide anion with the scavenger PEG-SOD. In these functional experiments using the endothelial-leukocyte adhesion assay, concurrent treatment of HIMEC with PEG-SOD completely reversed the enhanced leukocyte binding induced by CsA (Fig. 9). These data imply that excess superoxide generated in the activated endothelium treated with CsA plays a critical role in the enhanced leukocyte binding.

FIG. 4. MAPK activation in HIMEC following stimulation with TNF-α/LPS. Immunoprecipitates of unstimulated and TNF-α/LPS-stimulated HIMEC lysates were analyzed by Western blotting utilizing specific phosphoantibodies against p42/44 MAPK, p38 MAPK, and JNK. A, TNF-α/LPS-induced phosphorylation of p42/44 MAPK (ERK1/2); B, phosphorylated p38MAPK was readily detected following TNF-α/LPS activation in HIMEC; and C, similarly, p54 and p46 MAPK (JNK, SAPK) were phosphorylated by TNF-α/LPS in HIMEC. Nonphosphorylated antibodies were used to detect the total amount of these kinases to confirm equal loading between lanes. D, a time course of p38 MAPK activation in HIMEC following TNF-α/LPS stimulation revealed a rapid and transient increase in phosphorylated p38 MAPK that peaked 15 min following stimulation. E, phosphorylation of p42/44 MAPK peaked at 30 min following TNF-α/LPS stimulation. Representative images from three separate experiments are shown.

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Effect of FK506 and Rapamycin on HIMEC-Leukocyte Interaction

Additional calcineurin inhibitors and pharmacologic agents have emerged in transplant immunosuppression with the potent ability to block rejection, and have undergone preliminary testing in trials of patients with chronic inflammation and IBD. FK506 is known to inhibit calcineurin, Na⁺/H⁺ ATPase, FK506-binding protein activity, and proliferation of lymphocytes. Rapamycin is a macrolide antibiotic with potent antifungal and immunosuppressive qualities. It forms a stable complex with the FK-binding protein (FKBP-12) with a high affinity to the mammalian target of rapamycin. This interaction causes dephosphorylation and inhibition of p70S6 kinase, activated cells pretreated with CsA, SB203580, or PD98059 (p < 0.05).

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ultimately inhibiting cell cycle re-entry and expansion of lymphocyte populations, leading to its immunosuppressive effect in transplantation. Both FK506 and rapamycin have been implicated in causing endothelial cell dysfunction, similar to the effect of CsA (43). We performed experiments to determine whether the proinflammatory effect of CsA on the microvascular endothelium was unique, or would be seen in the other most frequently used potent transplantation immunosuppressive compounds. Flow leukocyte-adhesion assays were performed on HIMEC pretreated with CsA, FK506, and rapamycin. Both FK506 and rapamycin induced a pattern of enhanced U937 adhesion, which was similar to CsA in HIMEC activated with TNF-α/LPS (Fig. 10). This preliminary investigation suggests that endothelial dysfunction and enhanced inflammatory activation is a common feature shared by all three of these major immunosuppressive compounds. The mechanisms underlying the enhanced leukocyte binding in the FK506 and rapamycin-treated endothelial cells were not specifically defined.

**DISCUSSION**

We have demonstrated that the immunosuppressive agent CsA affects gene expression patterns in human intestinal microvascular endothelial cells following inflammatory activation. The CsA effect was mediated through differential inhibition of signaling pathways, ultimately resulting in a paradoxical proinflammatory phenotype characterized by enhanced leukocyte binding. As expected, stimulation of HIMEC with TNF-α and bacterial lipopolysaccharide resulted in activation of the MAPK cascades and NFκB, and enhanced expression of the proinflammatory cell adhesion molecules ICAM-1, VCAM-1, and E-selectin. Activated HIMEC demonstrated an established enhanced adhesive interaction with leukocytes,
which represents an important early control step in the inflammatory response. In addition to the increased expression of these proinflammatory cell adhesion molecules, activation with TNF-α/LPS also induced the expression of iNOS and increased NO production, a key mechanism that ultimately mediates the down-regulation of HIMEC activation. Although treatment of HIMEC with CsA caused a modest decrease in the expression of proinflammatory molecules (CAM), it exerted a potent, and complete inhibition of the anti-inflammatory enzyme iNOS in these microvascular endothelial cells. The net effect of CsA on HIMEC activation was a loss of NO production and a significant, proinflammatory increase in leukocyte binding, an unexpected, paradoxical effect for this drug, which is normally used clinically to inhibit inflammatory responses. Although CsA has been previously demonstrated to exert an inhibitory effect on the expression of iNOS in other cell types, including vascular smooth muscle cells and mesangial cells, this is the first report of its direct effect on the microvascular endothelium, a nonimmune cell population that plays a critical regulatory role in inflammatory responses. The specific inhibitory effect of CsA on iNOS expression in nonimmune cells has not been completely defined, which prompted our investigation of the intracellular signaling mechanisms involved in HIMEC activation and the effect of CsA on these activation pathways.

Perhaps the most important aspect of this investigation centers on the characterization of differential inhibition of MAPK signaling pathways by CsA. CsA selectively inhibited p38 MAPK during the activation of intestinal microvascular endothelial cells, which ultimately promoted a proinflammatory phenotype in these cells. An equally important aspect of this investigation was the characterization of both proinflammatory and anti-inflammatory gene expression profiles simultaneously induced by cytokine and LPS activation of microvascular endothelial cells. TNF-α and LPS readily activated MAPK family members as well as NFκB in HIMEC. MAPK and NFκB activation resulted in proinflammatory expression of CAM, and a simultaneous expression of iNOS, a key regulatory mechanism for limiting activation in HIMEC. Although CsA affected multiple gene expression patterns, it exerted its most powerful effect on the expression of the regulatory mechanism (i.e. iNOS expression) through a potent inhibition of p38 MAPK activation.

CsA is highly effective as an immunomodulator, but has demonstrated mixed results in the treatment of long term chronic inflammation, particularly inflammatory bowel disease. Much of the investigation regarding the therapeutic efficacy of CsA has focused on the effect of this compound on lymphocytes, specifically T cells and the generation of cytokines, with most research focusing on interleukin 2 (28). Our investigation of the pharmacologic activity of CsA on nonimmune cell populations suggests that this compound also exerts potent effects on microvascular endothelial populations, which play key regulatory roles in the inflammatory process. Our work suggests that CsA exerts multiple effects on the blood vessel wall, the net result of which appears to be an enhancement of inflammation through increased leukocyte binding activity.

Selective inhibition of signal transduction pathways is a rapidly emerging strategy in molecular medicine. Design of compounds, which will effect specific cellular activation pathways underlying disease mechanism, while limiting adverse reactions, is the ultimate goal of drug design. Inhibition of JNK and p38 MAPK has already been demonstrated to show clinical efficacy in short term trials of severely ill patients with Crohn’s disease (44). Likewise, the short term use of intravenous CsA has shown efficacy in the treatment of patients with both ulcerative colitis and Crohn’s disease (7–8), whereas long term use has failed to demonstrate clinical improvement.

Although experiments examining the role of the vasculature in animal models of intestinal inflammation are essential, there are important distinctions in the nitric oxide biology of rodents, which may not allow for a direct extrapolation of findings regarding our understanding of human microvascular function during intestinal inflammation (45). Experiments in human intestinal inflammation must rely on in vitro approaches, and the use of tissue-specific microvascular endothelial cell cultures represents an essential strategy for gaining insight into molecular and cellular mechanisms affected by pharmacologic strategies. Our investigation of HIMEC activation and the proinflammatory effect of CsA may offer a potential mechanism for the lack of efficacy of long term CsA therapy in IBD. We have demonstrated previously the lack of iNOS-derived NO in endothelial cells isolated from chronically damaged areas of IBD bowel in contrast to adjacent normal areas of intestine. This loss of iNOS-derived NO impairs the ability of microvascular endothelial cells to down-regulate inflammatory activation, and may thus contribute to chronic intestinal inflammation. iNOS-derived NO is preserved in uninvolved areas of gut in IBD patients, suggesting that an intact ability to produce NO in the microvasculature prevents worsening or extension of the disease process (46). Our present investigation suggests that CsA adversely affects NO production from the intestinal microvasculature, impairing vascular homeostasis, leading to enhanced leukocyte recruitment, and worsening uncontrolled, chronic intestinal inflammation. The effect of CsA on HIMEC activation may also offer mechanistic insights into the high prevalence of IBD in solid organ transplantation, where transplant recipients treated with long term calcineurin inhibitor immunosuppression (i.e. CsA and FK506) experience rates of clinical IBD 10 times greater than in the general population (47).

Defining the beneficial and deleterious effects of established pharmacologic agents represents an important goal in improving therapeutic approaches. Our present investigation may provide a mechanistic explanation regarding a proinflammatory effect of CsA on the organ-specific microvascular endothelium, and the failure of this compound in the long term treatment of chronic inflammatory bowel disease. If CsA blocks the generation of iNOS-derived NO in the activated intestinal microvasculature, then replacing endogenously produced NO with pharmacologic NO delivery may offer a long term strategy for improved immunomodulatory therapy using calcineurin inhibitors. In addition, we have shown that iNOS-derived NO functions as an antioxidant to quench superoxide anions generated during HIMEC activation. Endothelial delivery of antioxidant, in addition to coupling CsA to a pharmacologic NO donor compound, may restore the function of NO whose production was inhibited by CsA. Conjugation of NO donor compounds with pharmacologic agents has been initiated, as NO-mesalamine has already undergone preclinical investigation, and has demonstrated improved efficacy and safety in the treatment of animal models of IBD (48).

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