Isolation and characterization of human esophageal microvascular endothelial cells: mechanisms of inflammatory activation

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Eosophagitis is one of the most common maladies in the Western world, occurring in up to 7% of individuals in the United States. The etiology of gastroesophageal reflux disease (GERD) is clearly linked to the prolonged contact of acidic gastric contents with the esophageal luminal surface. However, what remains largely undefined is why certain individuals with GERD experience mild “heartburn” symptoms with minimal histological inflammation, whereas other individuals develop complications including chronic inflammation, ulceration, stricture formation, metaplastic transformation into Barrett’s esophagus, as well as adenocarcinoma (33). Host esophageal defense factors that ultimately determine the degree of mucosal injury in response to esophageal acid exposure must be invoked. The investigation of mucosal defense in esophageal disease has been limited, with a majority of work focusing on damage to tight junctions and loss of epithelial integrity in response to acid (30, 31). Attempts to characterize the molecular and cellular basis for esophageal inflammation have remained limited, largely the result of the relative inaccessibility of in vitro esophageal cell populations for investigation.

Within the histological descriptions characterizing GERD, there are classic features of gastrointestinal inflammation. Selective recruitment of circulating eosinophils are frequently identified in mild-moderate esophagitis, and in ulcerative esophagitis, the most severe manifestation of GERD, infiltration of polymorphonuclear leukocytes from the microcirculation into the esophageal tissue, can be detected in >80% of endoscopic specimens (18). Given our current understanding of endothelial-leukocyte interaction in the local recruitment of select immunocyte populations, the accumulation of immune cells in the esophagus points to a central and active role for the microvascular endothelium in GERD. Other clinical and histological features of GERD include local microvascular hemorrhage, edema, and ulcers, all manifestations of inflammatory processes in the gastrointestinal tract. Thus if we consider GERD an inflammatory process, defining the cellular and molecular mechanisms in...
MATERIALS AND METHODS

 Patients. Normal esophageal full-thickness specimens were obtained from discarded cadaveric tissue from transplant donors as well as normal margins from patients undergoing esophagectomy. Normal colonic and ileal resection specimens were used from patients undergoing scheduled surgical bowel resection. The use of human tissues for isolation of EC was approved by the Institutional Review Board of The Medical College of Wisconsin.

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Assessment of CAM surface expression. Surface expression of CAM was performed by using radioactive immunoprecipitation (RIA) as described earlier (4, 38). EC monolayers were assessed unstimulated or after activation. Mouse monoclonal antibodies (5 μg/ml) recognizing human E-selectin, ICAM-1, and VCAM-1 (R&D Systems) were used for CAM detection. After incubation with primary antibodies at 4°C, monolayers were rinsed and incubated with goat anti-mouse biotinylated Fab’ fragment (Jackson ImmunoResearch Laboratories, West Grove, PA). Bound secondary antibody was detected with 125I-labeled streptavidin (80 μCi/ml; Amersham Pharmacia Biotech, Arlington Heights, IL). EC monolayers were lysed with 1.0% (vol/vol) Triton X-100 in PBS (pH 7.4; BioWhittaker), and radioactivity was quantified in a gamma counter. Data from triplicate wells were expressed as the mean of 125I-labeled streptavidin bound [counts per min (cpm)/well]. Control experiments using a nonspecific monoclonal antibody (mouse IgG; Sigma) were performed in parallel at equal concentrations and incubation conditions.

Static and dynamic leukocyte adhesion assays. Static U-937 adhesion to HEMEC was measured as described previously (4, 38). After 24 h of activation, EC monolayers were trypsinized and resuspended in growth medium. After incubation with primary antibodies at 4°C for 1 h, nonadherent cells were removed and plates were gently washed 3 times with PBS. Monolayers were stained by using a modified Wright’s stain (DiffQuik; Baxter Scientific), and adherent leukocytes were counted in 10 random high-power fields (magnification, ×20) by using an ocular grid. Adhesion was expressed as number of adherent leukocytes per square millimeter. In addition, EC-leukocyte interactions were assessed by using low shear stress flow adhesion assay as previously described (27, 38). EC monolayers were analyzed directly or after stimulation with a combination of TNF-α (100 U/ml) and IL-1β (1 ng/ml) in a low-shear-stress flow chamber. U-937 cells or neutrophils (1 × 10⁶ per ml of growth medium) were flowing over EC monolayers at 1 dyn/cm², and adhesion was recorded by using a charge-coupled device camera attached to an inverted tissue culture microscope for 5 min. Data were analyzed by counting the number of adherent leukocytes (rolling plus static adhesion) over 10 random high-power fields by using a grid, and adhesion was expressed as the number of adherent leukocytes per high-power field (magnification, ×200).

Flow cytometry. Unstimulated and stimulated HEMEC were trypsinized and resuspended in growth medium. After two washes with ice-cold Hank’s balanced salt solution (pH 7.4; BioWhittaker), cells were resuspended in FACS buffer [PBS containing 2% (vol/vol) FBS, 1% (wt/vol) BSA, and 0.05% (wt/vol) NaN₃]. Staining was performed by using anti-VCAM-1 antibodies (R&D Systems). Cells were washed in FACS buffer and incubated with FITC-labeled goat-anti mouse IgG (Santa Cruz Biotechnology). After being washed, cells were resuspended in 1% (wt/vol) paraformaldehyde (Fisher Scientific, Pittsburgh, PA) in PBS and analyzed by using FACScan (Becton Dickinson, Mountain View, CA). Matched isotype IgG antibodies were included as negative controls. Results are expressed as arbitrary fluorescence units vs. unstimulated HEMEC.

RNA extraction and semiquantitative RT-PCR. iNOS gene expression was assessed in unstimulated and activated confluent cultures of HEMEC with or without pharmacological inhibitors. Endothelial cells were stimulated with a combination of 100 U/ml TNF-α (R&D Systems) and 1 μg/ml LPS (Sigma) or IL-1β (100 U/ml) + TNF-α (100 U/ml) for 6 h at 37°C. Total RNA was extracted by using RNAzol B (Teltest, Friendswood, TX) and quantitated by optical density. Total RNA (1 μg) was reverse transcribed by using SuperScript II RT (GIBCO-BRL, Grand Island, NY) in a total reaction volume of 20 μl. Reverse-transcription product (1 μl; cDNA) was PCR amplified by using AmpliTag DNA polymerase (Perkin Elmer, Norwalk, CT) and 0.5 μl each of 10 μM iNOS forward and reverse primers. In the iNOS reaction, β-actin primers were included in the reaction as an internal control for the efficiency of the RT and the amount of RNA used in the RT-PCR. The 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 primer sequences and PCR product sizes were as follows: iNOS, 5’-TCT TGG TCA AAG CTG TCG TC-3’ (forward) and 5’-CAT TGC CAA ACC TAC TGC TC-3’ (reverse), 237 bp; and β-actin (25 cycles) 5’-CCA GAG CAA GAG AGG CAT CC-3’ (forward) and 5’-AGG TTA ATT TGC CAA AGC AGC AG-3’ (reverse), 436 bp, respectively. PCR products were run on 1% agarose gels, stained with 0.5 μg/ml of ethidium bromide, visualized under UV light, and photographed. MAdCAM-1 gene expression was assessed in activated HEMEC [TNF-α, (10 ng/ml); IL-1β, (100 ng/ml)] for 5 h at 37°C, and then 18 h. Total RNA was extracted and reverse transcribed as described above. Briefly, 1 μl of cDNA product was used per PCR reaction in a total volume of 40 μl containing 1.5 mM MgCl₂, 0.2 mM dNTP mix, 0.5 mM primer 5’-CCC CTG TCA AAG CAA CAA ATT AGC-3’ (forward) and 5’-AGG TTT ATT TGC CAA AGC AGC AG-3’ (reverse) (352 bp), and 1 unit of Taq polymerase. Cycle conditions were 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min (32 cycles). PCR products were visualized and described as above. Control reactions were run by using β-actin primers for 25 cycles as described above.

SDS-PAGE and Western blot analysis. Gel electrophoresis and Western blot analysis was performed as described earlier (38, 39). In brief, HEMEC unstimulated or after stimulation were lysed in lysin buffer (in mM: 50 Tris-HCl, pH 7.4, 2 EDTA, 2 EGTA, 75 NaCl, 25 NaF, 25 β-glycerophosphate, 1 Na₃VO₃, 1 Na₂MoO₄, 1 PMSF, and 1% Nonidet P-40 and 5 μg/ml each of leupeptin, aprotinin, and pepstatin) on ice. An equal volume of SDS treatment buffer (125 mM Tris-HCl, pH 6.8, 4% (wt/vol) SDS, 20% (vol/vol) glycerol, 10% (vol/vol) 2-mercaptoethanol) was added to the cell lysate, and samples were boiled. Equal amounts of protein were separated by SDS-PAGE and transferred to nitrocellulose membranes (38, 39). The membranes were blocked and incubated with specific MAPK antibodies (p44/p42 MAPK, p38 MAPK, and JNK; phosphorylated and nonphosphorylated, respectively (Cell Signaling, New England Biolabs, Beverly, MA) or phospho-tyrosine antibodies (Upstate Biotechnology). Immunodetection of bound primary antibody was performed by horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence (Amersham Pharmacia Biotech, Arlington Heights, IL).

For MAdCAM-1 immunoprecipitation, cells were lysed in buffer containing [in mM: 10 Tris-HCl (pH 7.6), 2 EDTA, 100 NaCl, and 1 sodium vanadate, with 1% Triton X-100 and protease inhibitors]. After 60 min of incubation on ice, cell debris was removed by centrifugation (10,000 g, 4°C, 10 min), and supernatants were collected. Total protein (160 μg) was incubated with 0.5 μg of MAdCAM-1 monoclonal antibody (Bender MedSystems, Vienna, Austria) for 1 h at 4°C, then 20 μl of protein G-agarose (Santa Cruz Biotechnology) was added and incubated at 4°C with gentle rotation overnight. After centrifugation at 1,000 g for 5 min, pellets were washed with PBS 4 times. Then pellets were resuspended in 30 μl of SDS treatment buffer, boiled for 5 min, and analyzed as
described above by using MadCAM-1 monoclonal antibody (0.5 μg/ml).

**JNK activity assay.** JNK activity was assayed by using a nonradioactive JNK activity assay kit (Cell Signaling). Briefly, 250 μg of cell lysates were incubated with 20 μl of resuspended c-Jun fusion protein beads overnight at 4°C with gentle agitation. After being washed, the pellet was incubated with the supplied kinase buffer supplemented with 100 μM ATP for 30 min at 30°C. The reaction was then terminated by the addition of SDS treatment buffer, and samples were analyzed by Western blot analysis by using phospho-c-Jun antibody (Ser63).

**Nuclear protein extraction and gel electromobility shift assay.** Nuclear protein extracts were prepared from HEMEC as described previously (38, 41). In brief, nuclear extracts were incubated with 2 μg/μl poly(dI-dC) and 32P-end-labeled double-stranded synthetic deoxyoligonucleotide probe (for NF-κB: 5'-GCC CGG GGA GGA TTC CTG GGC CCC-3'), and the labeled DNA was purified on push columns (Bio-Rad, Hercules, CA). Protein-DNA complexes were then resolved in polyacrylamide gels for 2 h at room temperature in Tris-acetate buffer (pH 7.5). Dried gels were exposed to X-ray film to detect NF-κB nuclear translocation overnight at −70°C.

**Immunolocalization of NF-κB p65 subunit.** HEMEC were cultured on glass coverslips and stimulated as indicated in RESULTS for 3 h at 37°C. Cells were fixed and permeabilized with ice-cold methanol and blocked with 5% BSA (wt/vol) in PBS for 1 h. The p65 was immunolocalized by using anti-p65 antibody followed by incubation with a biotinylated secondary antibody (both from Santa Cruz Biotechnology) and fluorescein isothiocyanate-streptavidin (Pierce). Slides were mounted and visualized by using a fluorescence microscope (Olympus BX-40).

**Histological analysis.** Full-thickness esophageal tissue was fixed in 4% (wt/vol) paraformaldehyde in PBS, saturated in 20% (wt/vol) sucrose in PBS, and embedded in optimum cutting temperature compound (Sakura, Osaka, Japan). Frozen sections (6 μm) were prepared and stained by using a cell and tissue staining kit (R&D Systems) and mouse monoclonal anti-human antibodies (VCAM-1, ICAM-1, E-selectin; R&D Systems) or mouse IgG as a negative control, respectively. After immunodetection using diaminobenzidine, sections were counterstained in Mayer’s hematoxylin and mounted. Positive staining is visible as a dark-brown precipitate. In parallel experiments, esophageal full-thickness tissue was paraffin embedded and stained with hematoxylin-eosin according to standard protocols. Factor VIII-associated antigen antibody (Sigma) staining was used to confirm microvessels in tissue sections.

**Analysis of data.** Statistical analysis was performed by using StatView 4.51 and SuperANOVA software for the Macintosh (Abacus Concepts, Berkeley, CA). When single comparisons were made, Student’s t-tests were used, applying paired or unpaired analysis as appropriate. When multiple comparisons between groups were performed, one-way or two-way ANOVA was used as appropriate followed by the Student-Newman-Keuls test. P ≤ 0.05 was considered statistically significant.

**RESULTS**

**Morphological and phenotypic characterization of HEMEC.** The human esophagus is a highly vascularized structure, with a rich microvascular bed present within the submucosal stroma beneath the muscularis mucosa. Figure 1, A and B demonstrates the esophageal microvessels used to generate the HEMEC iso-
lates. There were a total of six individual HEMEC cultures generated during the study period used in the following experiments.

HEMEC cultures were routinely expanded for 2–3 mo reaching passage 16, and an individual culture was maintained as long as passage 18. HEMEC stored in liquid nitrogen [10% dimethylsulfoxide (vol/vol) in FBS] were recultured without loss of function or viability. As assessed by phase contrast microscopy, confluent HEMEC monolayers isolated from histologically normal esophageal mucosa assume a characteristic “cobblestone” appearance morphologically distinct from that of gastrointestinal fibroblasts or smooth muscle cells. Interestingly, HEMEC displayed a slightly different morphology compared with HIMEC in which, in addition to the classic cobblestone appearance, a “spindle cell” morphology frequently emerged from clusters of EC (Fig. 1C). EC possess receptors for modified serum lipoproteins and readily uptake these substances in vivo and in vitro (28, 36, 51). The ability of HEMEC to internalize acetylated human LDL was assessed in vitro by using Dil-ac-LDL. After 4 h, a marked accumulation of Dil-ac-LDL was observed within HEMEC (Fig. 1D) but not in parallel cultures of human esophageal fibroblasts or gastrointestinal smooth muscle cells (not shown). Having demonstrated that HEMEC may demonstrate a spindle cell morphology, their ability to readily uptake Dil-ac-LDL proves that a mesenchymal or fibroblast-like morphology can be a normal feature of cultured esophageal microvascular EC. To further define the endothelial origin of HEMEC, additional experiments were per-

Fig. 1. Continued D: characterization of HEMEC. Endothelial characterization of HEMEC monolayers was performed by using fluorescence and immunofluorescence microscopy. HEMEC Dil-ac-LDL uptake was demonstrated as speckled cytoplasmic fluorescence. This is a characteristic feature of endothelial cells, which confirms that cells with both a "cobblestone" as well as a "spindle" morphology are endothelial in origin. Original magnification, ×200. Immunofluorescence detection of claudin-1, ICAM-2, occludin, platelet endothelial cell adhesion molecule-1 (PECAM-1), ZO-1, ZO-2, and vascular endothelial (VE)-cadherin demonstrated surface expression on HEMEC, which clustered at the cell-cell junctions (original magnification, ×200). Immunofluorescence detection of von Willebrand factor showed intracellular cytoplasmic speckled pattern, which again confirms the endothelial origin of HEMEC (original magnification, ×200).
was potently induced by TNF-α/LPS (Fig. 2). Compared with HIMEC, stimulated HEMEC constitutively expressed significantly less IL-8 as assessed for multiple time points (6, 12, 24 h) in culture.

**Modulated expression of MadCAM-1 in HEMEC.** We then assessed the ability of HEMEC to express MadCAM-1, an adhesion molecule critical for leukocyte trafficking and immune homeostasis in human intestine (43). Unstimulated HEMEC did not express detectable amounts of MadCAM-1 mRNA using specific primers in a semiquantitative RT-PCR analysis. However, marked upregulation of MadCAM-1 transcripts was detectable after stimulation with TNF-α and IL-1β (100 U/ml each), two prototypical proinflammatory cytokines. Stimulation with IL-5, a cytokine associated with early esophagitis (26), and IL-6, an acute-phase proinflammatory cytokine, did not result in detectable MadCAM-1 transcripts (Fig. 3A, top). Amplification of β-actin transcripts served as an internal control (Fig. 3A, bottom). Western blot analysis of immunoprecipitated MadCAM-1 protein demonstrates a low-level constitutive expression of MadCAM-1 in unstimulated HEMEC, and there was a marked upregulation of MadCAM-1 on stimulation with TNF-α/IL-1β (Fig. 3B). HEMEC stimulation with IL-6 did not lead to enhanced MadCAM-1 expression compared with unstimulated HEMEC. Finally, stimulation with gram-negative LPS also strongly enhanced the protein expression of MadCAM-1 in HEMEC (Fig. 3B).

**Differential adhesion molecule expression in HEMEC and HIMEC.** EC express CAM on their cell surface, a physiological phenomenon tightly regulated by cytokines and inflammatory activators (17, 23, 48, 49). We investigated the regulated expression of CAM in HEMEC in response to dual inflammatory stimulation with TNF-α/LPS. This potent, proinflammatory activation by cytokine and LPS is known to induce cellular activation and enhanced CAM expression in HIMEC (5). We assayed the modulated expression of ICAM-1, VCAM-1, and E-selectin by RIA in HEMEC and HIMEC, unstimulated or after TNF-α/LPS or IL-1β activation for 6 h. TNF-α/LPS stimulation demonstrating the expression of all three CAMs were markedly and significantly enhanced (Fig. 4A), with highest con-

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**Fig. 2.** Differential secretion of IL-8 by HEMEC. ELISA analysis of HEMEC cell culture supernatants demonstrate lower IL-8 secretion on proinflammatory stimulation with TNF-α (100 U/ml)/LPS (1 μg/ml) compared with human isolated microvascular EC (HIMEC). Each condition was assessed in triplicate, and data are expressed as mean chemokine concentration ± SE (pg/ml). *P < 0.05.

**Fig. 3.** Mucosal addressin cell adhesion molecule (CAM)-1 (MadCAM-1) expression in HEMEC. A: semiquantitative RT-PCR demonstrates the level of MadCAM-1 mRNA expression in HEMEC with various proinflammatory stimuli (IL-1β, IL-6, and TNF-α (100 U/ml of each)), IL-5 (15 ng/ml), and LPS (1 μg/ml) after 18-h stimulation. Unstimulated HEMEC did not express detectable amounts of MadCAM-1 mRNA, but there was a marked upregulation of MadCAM-1 transcripts after TNF-α and IL-1β stimulation. IL-5 and IL-6 activation did not result in detectable MadCAM-1 transcripts (A, top). β-actin served as an internal control (A, bottom). B: Western blot analysis of MadCAM-1 immunoprecipitate revealed upregulation of MadCAM-1 protein in response to TNF-α, IL-1β, and LPS stimulation. IL-6 did not increase MadCAM-1 expression above levels detected in unstimulated HEMEC. Image is representative of 3 independent experiments.
Fig. 4. A and B: differential expression of CAMs in gastrointestinal microvascular endothelial cells. Detection of CAM surface expression in HEMEC and HIMEC by using monoclonal antibodies, biotinylated secondary antibodies, and 125I-labeled streptavidin radioimmunoassay. A: HEMEC and HIMEC both expressed constitutive ICAM-1, but not VCAM-1 and E-selectin. TNF-α/LPS activation markedly upregulated surface expression of all 3 CAM in both HEMEC and HIMEC. HEMEC consistently expressed higher levels of VCAM-1 after activation with TNF-α/LPS when compared head to head with HIMEC. Representative data from a single paired experiment are shown; similar results were observed in 3 separate paired experiments. Data are expressed as means ± SD of triplicate wells. *P < 0.05.

B: IL-1β induces differential expression of CAM in HEMEC and HIMEC. The proinflammatory cytokine IL-1β caused equivalent increases in ICAM-1 expression in HEMEC and HIMEC. However, IL-1β stimulation resulted in lower levels of E-selectin expression in HEMEC compared with HIMEC. Representative data from a single paired experiment are shown; similar results were observed in 3 separate experiments. Data are expressed as means ± SD of triplicate wells. *P < 0.05.

C and D: esophageal microvascular expression of CAMs ICAM-1, VCAM-1, and E-selectin in vivo. Immunohistochemical localization of CAMs in full-thickness, normal human esophageal frozen sections. Monoclonal antibodies against ICAM-1 (C), E-selectin (D), VCAM-1 (E), and positive isotype mouse IgG control (F) were used to detect CAM expression in endothelial submucosal microvessels. Factor VIII-associated antigen staining in paired sections was used for identification of endothelial cells (data not shown). Antibody staining is visible as a dark brown precipitate. There was positive staining for ICAM-1 and VCAM-1, and to a lesser extent E-selectin, in normal esophageal microvessels (arrowheads, Fig. C–F; original magnification, ×400). Additional experiments were performed on esophageal sections that were not counterstained to demonstrate the specificity of immunostaining for VCAM-1 compared with isotype control (Fig. G and H). Representative images from triplicate experiments are shown.
selectin or VCAM-1 in response to IL-1. VCAM-1 was felt to be clinically relevant, because this CAM has been demonstrated to play a critical role in the recruitment of eosinophils, which are frequently identified in esophagitis and GERD (18).

HIMEC demonstrated low-level expression of E-selectin or VCAM-1 in response to IL-1β, unlike HEMEC, which greatly increase expression of all three of these CAMs in response to this cytokine (Fig. 4). The lack of VCAM-1 induction by IL-1β in HEMEC is similar to previous findings in dermal microvascular EC, in which IL-1β failed to induce VCAM-1 after in vivo injection into the skin of human volunteers (16). Additional experiments were carried out with IL-4 and IL-5, cytokines implicated in the etiopathogenesis of esophagitis and GERD. When administered either alone or in combination, these cytokines did not alter CAM expression above baseline. When IL-4 and IL-5 were used in combination with TNF-α/LPS, there was no further increase in CAM expression in HEMEC (data not shown).

We then analyzed the expression of the CAMs ICAM-1, VCAM-1, and E-selectin in intact microvessels from esophageal tissues (Fig. 4, C–H). With the use of frozen sections of freshly resected esophageal tissues, we detected all three of these CAMs in the esophageal microvessels, confirming that the expression of these molecules in HEMEC is relevant to the in vivo situation.

Leukocyte adhesion and functional assessment of NO generation. Cultured EC exposed to cytokines will dramatically increase their capacity to bind leukocytes after in vitro activation (3, 6). HEMEC were incubated for 24 h with and without activators (TNF-α/LPS or TNF-α/IL-1β) and then cocultured with U-937 cells, a monocyte-like cell line, for 1 h in a static adhesion assay. Under unstimulated conditions, only low numbers of U-937 cells adhered to HEMEC (Fig. 5, A–E). After stimulation with TNF-α/LPS, there was a marked increase in U-937 cell binding (Fig. 5C). In these experiments, we also evaluated whether a selective iNOS inhibitor, L-NIL, would alter HEMEC adhesion for leukocytes. We have previously shown that NO produced by the iNOS pathway plays a critical downregulatory role in HEMEC activation and leukocyte adhesion (5). Interestingly, pretreatment of HEMEC with 20 μM L-NIL, a dose selective for iNOS inhibition, significantly increased baseline adhesion of U-937 cells, a finding not present in HIMEC (5). This suggests that HEMEC exhibit constitutive iNOS expression, which differs from HIMEC in which L-NIL exerted no effect on leukocyte adhesion in unstimulated HIMECs. When we assessed the effect of TNF-α/LPS activation with selective iNOS inhibition by using L-NIL, there was a similar significant increase in leukocyte binding, suggesting a regulatory role of iNOS in activated HEMEC similar to HIMEC. These data are summarized in Fig. 5E, in which adherent leukocytes are expressed per millimeter squared of HEMEC surface, and iNOS inhibition resulted in significant increases in leukocyte adhesion in all conditions tested (P < 0.05).

In addition to evaluating the activation of HEMEC in response to classic proinflammatory cytokines and LPS, we performed experiments to determine the effect of cytokines specifically implicated in esophagitis and GERD, such as IL-4 and IL-5. These cytokines either alone or in combination failed to induce alterations in U-937 adhesion to HEMEC above baseline in both static and flow adhesion assays (data not shown).

iNOS expression in HEMEC. To assess the role of iNOS, a major downregulatory modulator in the inflammatory activation of human microvascular ECs, mRNA and protein levels of iNOS were determined in HEMEC stimulated with TNF-α/LPS by semiquantitative RT-PCR (Fig. 6A) and Western blot analysis (Fig. 6B) by using human iNOS-specific antibodies. It is of interest that both approaches revealed constitutive low-level expression of iNOS in unstimulated HEMEC, a physiological feature not present in unstimulated HIMEC (5). After TNF-α/LPS stimulation for 6 h, there was a marked increase in the expression of iNOS in HEMEC (Fig. 6, A and B). This increase was blocked by pretreatment of HEMEC with curcumin (Fig. 6, A and B) and the specific NF-κB inhibitors Bay 11 and SN-50 (Fig. 6A). These data suggest a regulatory role for iNOS in unstimulated as well as TNF-α/IL-1β- and TNF-α/LPS-activated HEMEC and support the functional data demonstrated in Fig. 5.

MAPK signaling in HEMEC activation. We then sought to characterize the signaling mechanisms involved in TNF-α/LPS activation of HEMEC. Phosphorylation/dephosphorylation of protein kinases plays a key role in the regulation of cell activation, and we have shown that TNF-α/LPS stimulation of HEMEC led to tyrosine phosphorylation of several proteins identified by immunoblotting of cell lysates by using an anti-phosphotyrosine antibody (Fig. 7A). These tyrosine phosphorylated proteins included proteins of molecular mass 42, 57, 76, 85, 116, and 130 kDa. We sought to determine the identity of these phosphoproteins and whether physiological activation in response to acidic pH would also induce activation of these signaling cascades.

Previously, we (38) demonstrated that TNF-α/LPS activation of HEMEC resulted in activation in serine/threonine MAPK, including p42/44 MAPK (ERK), p38 MAPK, and JNK, which are known to play a central role in the inflammatory activation of EC. In this study, we show phosphorylation of all three MAPK members in response to TNF-α/LPS-activated HEMEC (Fig. 7B). To study physiological stimuli linked to esophageal inflammation and GERD, we performed experiments examining the effect of IL-4 and IL-5 on MAPK activation in HEMEC. Our results indicate that IL-4 and IL-5 neither alone nor in combination have any effect on MAPK activation in HEMEC (data not shown). These data suggest that alternative pathways
are involved in HEMEC activation, and we focused investigation on VCAM-1 expression and acid pH-induced activation of HEMEC.

JNK activation by TNF-α/LPS and acid exposure in HEMEC. As revealed by a recent investigation, acid exposure significantly enhances phosphorylation of MAP kinases in esophageal epithelium, thus potentially contributing to carcinogenesis in Barrett’s esophagus (47). To determine whether acid exposure leads to activation of these signaling pathways in HEMEC, cells were exposed for multiple time points with acidified growth medium (pH 4.5), and immunodetection of phosphorylated MAP kinases was subsequently performed in EC lysates. Although normally not exposed to acidic pH, esophageal ECs might become activated by acid contact, presuming a disturbed mucosal squamous epithelial barrier, as seen in erosive reflux esophagitis and peptic esophageal ulceration. When HEMEC were exposed to acidic pH, there was a significant and rapid activation of JNK and to a lesser extent p42/44 MAPK (Fig. 7, C and E) and no p38 MAPK phosphorylation and activation (data not shown). Of interest, the phosphorylation of p42/44 MAPK was less rapid and less pronounced compared with JNK phosphorylation. Although an increase in phospho-p42/44 MAPK was detected at 10 and 15 min after acid exposure, there was no increase in kinase activity when functional assessment of this MAPK protein was performed (Fig. 7E). The increase in phospho-ERK may represent autophosphorylation of this kinase, which will not result in increased functional activity. These experiments suggest that exposure of esophageal microvascular ECs to acidic pH will result in selective activation of JNK.
Kinase activity of JNK in HEMEC. A kinase activity assay of TNF-α/LPS and acid-activated HEMEC demonstrated an increased JNK activity (Fig. 7D). Pharmacological pretreatment before HEMEC activation revealed that the antioxidant curcumin and NF-κB inhibitor Bay 11 abolished JNK activity (Fig. 7D). Wortmannin, a phosphatidylinositol 3-kinase inhibitor, partially inhibited the activation of JNK in HEMEC, suggesting a potential contribution of this pathway in HEMEC.

Dose-dependent inhibition of VCAM-1 expression by curcumin. Inflammatory activation of HEMEC with cytokines and LPS resulted in a rapid phosphorylation of JNK completely inhibited by curcumin and Bay 11. We then focused our attention on the link between JNK activation and the expression of VCAM-1 in HEMEC. Increasing dosages of curcumin pretreatment demonstrated a dose-dependent inhibition of VCAM-1 expression in response to activation with TNF-α/LPS by using both cell surface RIA (Fig. 8A) as well as flow cytometry (Fig. 8B). To correlate the inhibitory effect of curcumin on JNK activation and CAM expression, we performed experiments by using this compound on HEMEC in the low-shear-stress flow adhesion assay. At 20 μM concentration, curcumin completely inhibited the enhanced adhesion of both U-937 cells as well as freshly isolated human peripheral blood neutrophils to TNF-α/LPS-activated HEMEC (Fig. 8C). PD-98059, the selective p42/44 MAPK inhibitor, and SB-203580, the specific inhibitor of p38 MAPK, failed to inhibit VCAM-1 expression in HEMEC, again demonstrating the importance of the JNK pathway in the expression of this CAM (data not shown).

NF-κB activation in HEMEC. Activation of EC in response to cytokines may ultimately result in activation of the transcription factor NF-κB and gene transcription including the expression of CAMs (E-selectin, ICAM-1, VCAM-1) as well as iNOS. To further assess whether the transcription factor NF-κB was involved in TNF-α/LPS and acid activation of HEMEC, NF-κB electrophoretic mobility shift assays were performed. Activation of NF-κB from HEMEC nuclear extracts was detected after 3 h of TNF-α/LPS or pH 4.5 stimulation that was blocked by curcumin (Fig. 9). Antibody against p65 subunit of NF-κB resulted in supershift, confirming NF-κB activation. This result was further supported by immunofluorescence staining of nuclear localization of p65 (Rel A), a member of the NF-κB protein family. In unstimulated HEMEC, p65 was detected in the cytoplasm of the cells (Fig. 10A). After stimulation with TNF-α/LPS for 3 h, p65 was translocated into the nucleus (Fig. 10B). These results clearly indicate a mechanistic role for NF-κB in the response to inflammatory stimuli in HEMEC.

DISCUSSION

The present study is the first systematic investigation of microvascular endothelium from the human esophagus, with a specific focus on the molecular and cellular mechanisms that underlie inflammatory activation of these cells. There were six notable findings that emerged from this work, specifically: 1) this was
Fig. 7. Western blot analysis of HEMEC activation. A: Western blot analysis of HEMEC lysate by using anti-phophotyrosine antibody reveals multiple proteins undergoing tyrosine phosphorylation after TNF-α/LPS stimulation. Tyrosine-phosphorylated proteins are at 42, 57, 76, 85, 116, and 130 kDa. Image is a representation of 3 independent experiments. B: Western blot analysis of unstimulated and TNF-α/LPS-stimulated HEMEC lysates utilizing specific phosphoantibodies against p42/44 MAPK, p38 MAPK, and JNK demonstrate the phosphorylation of all 3 MAPK family members. Antibodies against nonphosphorylated proteins were used to confirm equal protein loading. C: Western blot analysis demonstrates the transient phosphorylation of JNK after TNF-α/LPS activation at 5 min as well as acidic pH exposure (pH 4.5) at 2 min in HEMEC. Phosphorylated and nonphosphorylated JNK antibodies are utilized to detect phosphorylated and total JNK. D: increased JNK kinase activity in TNF-α/LPS-stimulated HEMEC immunoprecipitates detected by specific phospho-c-Jun (Ser63) antibody that was completely inhibited by curcumin and Bay 11 and partially inhibited by Wortmannin. E: exposure of HEMEC to acidic pH 4.5 resulted in the phosphorylation of p42/44 MAPK (ERK), which did not correlate with kinase activity for ERK. All depicted images are representations of 3 independent experiments.
the first description of HEMEC isolation and culture; 2) characterization of HEMEC revealed that they are a microvascular endothelial population that are phenotypically and functionally distinct from HIMEC; 3) HEMEC express MAdCAM-1; 4) HEMEC and intact human esophageal microvessels express the CAMs ICAM-1, VCAM-1, and E-selectin; 5) HEMEC express unique patterns of the iNOS compared with HIMEC; and 6) cytokine and LPS activation of HEMEC involves NF-κB and MAPKs, and exposure to acidic pH will result in selective activation of JNK.

There has been limited investigation of the microvasculature and EC function in the pathophysiology of esophageal inflammation and GERD. Sbarbati et al. (45) directly addressed the involvement of the microvasculature in esophagitis by using ultrastructural analysis with transmission electron microscopy. In 30 patients with documented esophagitis but no Barrett’s metaplasia, they found signs of microangiopathy in all individuals ranging from interrupted to duplicated or thickened basal laminae in the esophageal microvessels. In areas of Barrett’s columnar metaplasia, the microvasculature showed an ectatic lumen and a thin basal lamina without duplications or interruptions. They concluded that microangiopathy is associated with epithelial damage in GERD.

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 (15, 24). Microvascular endothelial activation and adhesion of circulating immune cells is an early and rate-limiting step in the initiation and maintenance of the inflammatory re-

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**Fig. 8.** Effect of curcumin on HEMEC VCAM-1 expression and leukocyte binding. A: radioimmunoassay of HEMEC pretreated with curcumin followed by TNF-α/LPS activation demonstrate inhibition of VCAM-1 expression in a dose-dependent manner. Monolayers of HEMEC were pretreated with curcumin (1–20 μM) before activation with TNF-α/LPS for 6 h. Significant inhibition of VCAM-1 expression was detected with 10–20 μM curcumin pretreatment. B: similar inhibitory effect of curcumin was seen by using flow cytometry for detection of HEMEC VCAM-1 expression. Representative data from 3 separate experiments are shown. Data are expressed as means ± SD from triplicate wells. *P < 0.05. C: curcumin pretreatment resulted in inhibition of increased leukocyte adhesion by the TNF-α/LPS-activated HEMEC. With the use of a low-shear-stress flow adhesion assay, HEMEC monolayers were assessed unstimulated or after activation with TNF-α/LPS, before the interaction with U-937 monocytes under physiological flow conditions. HEMEC bind U-937 through activated expression of VCAM-1 and E-selectin. Representative data from a total of 3 separate experiments are shown. Ten microscopic high-power fields (HPF) were counted per experiment. Data are expressed as means ± SE of leukocytes/HPF; *P < 0.05.

**Fig. 9.** NF-κB activation in HEMEC. Gel electromobility shift analysis shows that TNF-α/LPS and exposure to acidic pH 4.5 resulted in activation of NF-κB in HEMEC that was supershifted with anti-p65 antibody (arrow). Activation of NF-κB was completely inhibited by pretreatment of HEMEC with curcumin. Image is a representation of 3 independent experiments.
spontaneous. We focused our investigation on the contribution of HEMEC to esophageal inflammation and leukocyte recruitment.

Leukocyte recruitment into the esophagus during GERD is the direct result of endothelial activation. The selective recruitment of eosinophils in esophagitis, particularly pediatric esophagitis, has been explored by numerous investigators. Early reports by Brown (9) and Winter (51) found that eosinophil recruitment into the esophageal mucosa was a characteristic feature of esophagitis, in adults as well as children. In their analysis, eosinophil infiltration of the epithelium was found in 52% of patients and was the only histological feature of esophagitis in 23% of confirmed cases. The state of activation of esophageal eosinophils was evaluated by Justinich (20) by using an ultrastructural electron microscopic analysis. In 12 patients with esophagitis, activated eosinophils were identified crossing the microvascular endothelium and migrating into the mucosa, a finding not present in normal controls. Previous investigation has suggested that specific cytokines and chemokines will mediate the leukocyte trafficking associated with esophagitis, specifically IL-4, IL-5, and eotaxin. IL-4, a cytokine associated with the progression of esophagitis into metaplastic transformation (Barrett’s esophagus) (11), and IL-5, a key cytokine implicated in the pathogenesis of human and animal models of esophagitis, as well as eosinophil trafficking (26), failed to exert a direct effect on HEMEC during the early time periods we studied. Likewise, investigation of chemokine expression patterns from HEMEC did not demonstrate production of eotaxin, the CC chemokine implicated in the selective recruitment of circulating eosinophils. These data suggest that local eosinophil recruitment in esophagitis and GERD may result from eotaxin production from stromal cells in the esophagus that do not include the microvascular endothelium.

HEMEC did demonstrate unique patterns of leukocyte recruitment and CAM expression in response to cytokine and bacterial activators. High levels of VCAM-1 were demonstrated in HEMEC compared with HIMEC. Likewise, tissue expression of VCAM-1 in esophageal microvessels was demonstrated. The EC adhesion molecules VCAM-1, ICAM-1 (1), and P-selectin but not E-selectin (40) mediate eosinophil tethering and binding. Previous reports have suggested that endothelial expression of VCAM-1 interacting with its cognate ligand, VLA-4 (α4β1-integrin) expressed on eosinophils, will mediate trafficking of this leukocyte population (44), which may be attenuated by small-molecule α4β1-antagonists (22). These data suggest that VCAM-1 expression by HEMEC may play a significant role in selective leukocyte recruitment into the esophagus.

Lymphocyte trafficking is mediated by interactions between homing receptors on the lymphocyte and vascular addressins expressed on specific endothelium that are controlled by a variety of inflammatory mediators. Lymphocyte homing to mucosal surfaces including Peyer’s patches and the lamina propria of the intestine is critically dependent on a single-chain 60 kDa glycoprotein adhesion molecule, the mucosal vascular addressin or MAdCAM-1 (2). MAdCAM-1 is expressed on ECs within the mesenteric lymph nodes, the lamina propria of both the small and large intestine, and the mammary gland during lactation. During chronic gut inflammation in IL-10 knockout mice there is a 10- to 11-fold increase in MAdCAM-1 expression (21). Increased MAdCAM-1 has been detected in the colonic lamina propria microvessels in mice with hapten-induced colitis, colitis secondary to IL-2 deficiency, and severe combined immunodeficient (SCID) mice reconstituted with CD4+CD45RB+ T cells. Antibodies directed against either MAdCAM-1 or its leukocyte ligand α4β7 have blocked the development of chronic inflammation in the SCID RB45 reconstitution model of colitis, demonstrating the central role of this leukocyte addressin in the development of an inflammatory bowel disease (IBD) lesion. In human IBD (both Crohn’s disease and ulcerative colitis), MAdCAM-1 expression has been shown to be dramatically increased compared with control tissues (8). This important role for MAdCAM-1/α4β7-integrin interaction in chronic gut
inflammation has led to a successful pilot trial of anti-a4 antibody therapy in IBD patients. Because of the central role of MAdCAM-1 in normal intestinal immune homeostasis, as well as chronic gut inflammation in IBD, we sought to determine whether this molecule would also be expressed in the upper gut and the esophageal microvasculature. HEMEC expressed MAdCAM-1 at the mRNA and protein levels that increased in response to inflammatory activation, and this is the first report of esophageal localization of MAdCAM-1. This suggests the possibility of similar mechanisms of endothelial-leukocyte interaction occurring in severe refractory esophagitis and as seen in IBD.

Our laboratory has previously characterized an important regulatory role for iNOS-derived NO in the regulation of inflammatory activation of HIMEC (38). Similarly, HEMEC also expressed iNOS that would downregulate leukocyte binding. However, distinctly different from HIMEC, HEMEC expressed constitutive iNOS that decreased basal leukocyte adhesion to this endothelial population. The physiological implication of this finding suggests that esophageal microvessels will recruit lower numbers of leukocytes, which is true in routine histological evaluation of the esophagus compared with the small and large bowel.

Esophagitis and GERD are presently believed to result from the prolonged contact of acidic gastric reflux with the esophageal mucosal lining. Investigation of the cellular and molecular mechanisms that follow acid exposure have centered around reparative and proliferative effects after acid, because the most dreaded complication of GERD is metaplastic transformation into Barrett’s esophagus, a precursor lesion to adenocarcinoma of the esophagus. Acidic pH has been demonstrated to promote cell growth in explants of Barrett’s tissue maintained in organ culture. In these experiments, acidic pH resulted in an increase in proliferation as measured by tritiated thymidine uptake and expression of cyclooxygenase-2, an antiapoptotic protein (12, 13, 32). In more recent experiments focused on identifying intracellular signaling mechanisms that would undergo activation in response to acid, Souza and colleagues (47) characterized the activation of MAPK pathways in response to acid in SEG-1 cells, a Barrett’s adenocarcinoma-derived esophageal epithelial cell line. In these experiments, transient exposure of transformed epithelial cells to acidic pH resulted in cell proliferation, a decrease in apoptosis, which corresponded with activation of p38 MAPK, ERK, and a delayed activation of JNK. Increased proliferation was abolished by inhibition of p38 MAPK or JNK, whereas inhibition of apoptosis was linked to ERK activity. Souza et al. (47) correlated these in vitro studies, transient exposure of Barrett’s esophagus patients, taken before and after 3 min of esophageal acid perfusion. These in vitro analyses demonstrated a significant functional increase in p38 MAPK activity and a trend in the increase in JNK activity. Our studies focused on the effect of acidic pH on the activation of primary esophageal EC, and also characterized MAPK family member activation (JNK) as well as NF-kB. Because HEMEC are a primary cell line derived from adult differentiated tissues, they were extremely sensitive to acid stimulation. Sustained acid treatment (>6 h) that is necessary to define CAM expression and enhanced leukocyte binding would result in significant cell death. Because of this outcome, we were unable to carry out experiments for CAM expression and leukocyte binding in response to prolonged acid exposure in HEMEC. However, short-term acid exposure, performed in the characterization of signal transduction cascades did not result in cell toxicity. Our studies suggest that the MAPK cascades will also be involved in the inflammatory activation of nonimmune cell populations in the esophagus, leading to chronic inflammation and leukocyte infiltration that accompanies esophagitis and GERD.

In summary, we have characterized HEMEC at the morphological, phenotypic, and functional levels. MAdCAM-1 as well as unique patterns of CAM expression were demonstrated in HEMEC. The NO biology of HEMEC appears unique, because constitutive iNOS is expressed in this EC population. HEMEC undergo activation in response to cytokines and LPS as well as acidic pH exposure. Future use of HEMEC will help to define unique patterns of leukocyte recruitment and mechanisms of inflammation in the human esophagus.

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DISCLOSURES

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