iNOS expression in human intestinal microvascular endothelial cells inhibits leukocyte adhesion

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Binion, David G., Sidong Fu, Kalathur S. Ramanujam, Yuh Cherng Chai, Raed A. Dweik, Judith A. Drazba, Justin G. Wade, Nicholas P. Ziets, Serpil C. Erzurum, and Keith T. Wilson. iNOS expression in human intestinal microvascular endothelial cells inhibits leukocyte adhesion. Am. J. Physiol. 275 (Gastrointest. Liver Physiol. 38): G592–G603, 1998.—Increased nitric oxide (NO) production by inducible nitric oxide synthase (iNOS) has been associated with intestinal inflammation, including human inflammatory bowel disease. However, NO can downregulate endothelial activation and leukocyte adhesion, critical steps in the inflammatory response. Using primary cultures of human intestinal microvascular endothelial cells (HIMEC), we determined the role of NO in the regulation of HIMEC activation and interaction with leukocytes. Both nonselective (Nω-monomethyl-L-arginine) and specific (N-iminoethyl-L-lysine) competitive inhibitors of iNOS significantly increased binding of leukocytes by HIMEC activated with cytokines and lipopolysaccharide. Increased adhesion was reversible with the NOS substrate L-arginine and was not observed in human umbilical vein endothelial cells (HUVEC). Activation of HIMEC significantly upregulated HIMEC iNOS expression and NO production. NOS inhibitors did not augment cell adhesion molecules or increased expression of the inflammatory response.

Recent advances in vascular biology have defined an important regulatory role for NO within the vascular wall (26). Endothelial cells are now known to possess multiple mechanisms for NO production via constitutive endothelial NOS (eNOS; NOS-3) and high-output iNOS after inflammatory activation (19, 22, 42). Endothelial cell-derived NO plays an integral role in vascular physiology, regulating both blood flow and vessel wall remodeling through direct effects on vascular smooth muscle cells (15). In addition to these effects on

INCREASED PRODUCTION of nitric oxide (NO) has been correlated with intestinal inflammation in human inflammatory bowel disease (IBD) and animal models of IBD (33, 46). This association has led to the hypothesis that overproduction of NO mediates tissue damage during intestinal inflammation. High-output production of NO from L-arginine is due to transcriptional activation (27, 53) and increased expression of the inducible form of the nitric oxide synthase (NOS) enzyme [inducible NOS (iNOS); NOS-2]. The proposed proinflammatory role for NO in intestinal inflammation has been further strengthened by the demonstration that inhibition of NOS with pharmacological agents can improve inflammation in experimental models of IBD (21, 32). However, specific mechanisms by which NO mediates inflammation are not known.

Endothelial cells lining the microvasculature are now known to play a critical “gatekeeper” role in the inflammatory process through their ability to recruit circulating immune cells into tissues and foci of inflammation (16, 36). Endothelial activation in response to cytokines and bacterial products results in cell adhesion molecule expression and chemokine production, which mediate increased binding and transmigration of leukocytes across the vascular wall (16). Thus endothelial activation and leukocyte interaction are thought to be critical regulatory steps in the initiation and maintenance of the inflammatory response.

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blood pressure and flow, endothelial cell-derived NO also maintains vascular homeostasis through its ability to downregulate endothelial cell activation and leukocyte binding (11, 35). In intravital microscopy studies of mesenteric venules in small animals, NOS inhibition resulted in significantly increased endothelial binding and transmigration of immune cells (2, 24, 25). These data suggest that NO exerts an anti-inflammatory effect by limiting leukocyte adhesion to the microvascular endothelium, a key step in the inflammatory process. A recent study using mice with targeted disruption of the iNOS gene has confirmed that NO can also exert anti-inflammatory effects in the intestinal mucosa (29). In direct contrast to previous studies in which pharmacological inhibition of NOS decreased inflammation in animal models of IBD, iNOS knockout mice developed more severe intestinal inflammation and increased perivascular leukocyte recruitment compared with wild-type animals when subjected to acetic acid colitis.

Because of the discrepancy between the potential pro- and anti-inflammatory effects of NO in various animal models of intestinal inflammation, we sought to directly determine the role of NO in human intestinal microvascular endothelial cell (HIMEC) activation and leukocyte interaction. Direct analysis of NO biology in HIMEC is now possible, as these cells have been recently isolated and established in tissue culture from resected human intestinal specimens (6). We hypothesized that NO produced by HIMEC would function in an anti-inflammatory capacity by downregulating endothelial activation and leukocyte interaction. We also hypothesized that after inflammatory activation, the physiologically relevant microvascular endothelial cells (HIMEC) would be capable of expressing iNOS and that this high-output pathway of NO production would underlie the regulation of leukocyte adhesion. To test these hypotheses, we utilized primary cultures of HIMEC generated from normal intestinal tissue to directly assess the effect of NO production on HIMEC-leukocyte interaction, the enzymatic mechanisms of endothelial NO production, and the cellular and molecular mechanisms that underlie NO modulation of HIMEC-leukocyte interaction. In addition, we performed parallel experiments using a well-characterized control endothelial population, human umbilical vein endothelial cells (HUVEC), to highlight the unique tissue and microvascular-specific properties of HIMEC. In this report, we demonstrate that in the human intestinal microvasculature, endothelial NO derived from iNOS plays a critical downregulatory role in the inflammatory process by decreasing endothelial activation and the avidity of endothelial cell adhesion molecules for their leukocyte ligands.

**MATERIALS AND METHODS**

Isolation and culture of mucosal microvascular endothelial cells. HIMEC isolation was performed using a technique adapted from dermal microvascular endothelium (6, 28). In brief, surgical specimens were rinsed, and full-thickness samples of intestinal tissue were obtained. Mucosal strips were dissected and washed to remove debris and contaminating bacteria, minced, and digested in a type II collagenase solution (Worthington Biochemical, Freehold, NJ). Mechanical compression was used to express clusters of microvascular endothelial cells, which were plated onto fibronectin-coated tissue culture dishes and grown in MCDB 131 medium (Sigma Chemical, St. Louis, MO) supplemented with 20% fetal bovine serum (FBS) and endogenous cell growth factor (ECGF; Boehringer Mannheim, Indianapolis, IN). After 7–10 days of culture, microvascular endothelial cell clusters were physically isolated, and a pure culture was obtained. Endothelial cultures were recognized by modified lipoprotein uptake (Dil-ac-LDL; Biomedical Technology, Stoughton, MA) and expression of factor VIII-associated antigen (1). All experiments were carried out on cultures between passages 8 and 10. HIMEC lines derived from normal margins of resected intestinal tissue from six patients were utilized for this study.

Human umbilical vein endothelial cells. Human umbilical vein endothelial cells (HUVEC) were isolated from placental samples of intestinal tissue were obtained. Mucosal strips were placed in PBS (0.25% xylene cyanole and loaded on a 1% agarose gel and stained bands were visualized under ultraviolet (UV) light and photographed.

Northern blot analysis for iNOS. Ten micrograms of total RNA from HIMEC were dissolved in denaturing buffer (50% deionized formamide, 6% formaldehyde, 10 mM sodium phosphate buffer, 0.5 mM EDTA, pH 7.4) and heated at 70°C for 10 min. The samples were mixed with 5 µl of loading buffer [50% glycerol, 1 mM EDTA (pH 8.0), 0.25% bromophenol blue, 0.25% xylene cyanol] and loaded on a 1% agarose gel containing 1.1 M formaldehyde. After completion of electrophoresis, total RNA was bound to Hybond-N (Amersham, Arling-
tion.

One millimolar Stain; Baxter Scientific, McGraw, IL), and adherent leukocytes by a positive pressure blotter (Stratagene, La Jolla, CA) and fixed by UV cross-linking. The prehybridization and hybridization solution consisted of 0.25 M NaH2PO4, 7% SDS, and 1 mM EDTA (pH 8.0). After 30-min prehybridization at 60°C, hybridizations were carried out overnight at 60°C with a 1.2-kb partial-length human iNOS cDNA probe [obtained from V. Laubach and P. Sherman, Burroughs Welcome, Durham, NC (44)] labeled with 32P-dCTP (Amersham) by a random primer method (rediPrime; Amersham). Filters were washed in 2× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0)-0.1% SDS at room temperature twice for 15 min each and once in 0.1× SSC-0.1% SDS at 60°C for 15 min. The filters were exposed to Kodak BioMax film (Kodak, Rochester, NY) at -80°C with intensifying screens. The concentration and loading of RNA in each lane was standardized by hybridization with a 1.1-kb cDNA probe for constitutively expressed glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Clonetech, Palo Alto, CA).

Measurement of NO production. HIMEC (1 × 10^5) cells were cultured overnight in 60-mm fibronectin-coated tissue culture dishes (Corning Costar, Cambridge, MA). After the monolayers had been rinsed, the medium was replaced with 2 ml of MCDB 131 supplemented with 2% FBS, and supernatants were collected after 48 h. Total NO production was assayed by chemical reduction of nitrite, nitrate, and nitrosodiethylenediamine by vanadium chloride and measurement of chemiluminescence using a Sievers 280 NO analyzer (Sievers, Boulder, CO). Medium incubated in a 5% CO2 incubator in the absence of cells, unstimulated cell-conditioned medium, and TNF-α (100 U/ml) + LPS (1 µg/ml)-activated cell-conditioned medium were assessed, and results were recorded as the concentration (µM) of NO production. NO levels were adjusted for protein concentration for HIMEC from each well as determined by the method of Bradford (8).

Endothelial leukocyte adhesion assay. Endothelial cells were seeded onto fibronectin-coated 24-well tissue culture plates (Corning Costar) at 0.5 × 10^6 cells/well, using HIMEC medium supplemented with EGF, and allowed to grow to confluence after 48–72 h. Endothelial cells were stimulated with a combination of TNF-α (100 U/ml; R&D Systems) and interleukin-1β (100 U/ml; R&D Systems), or TNF-α (100 U/ml) and IFN-γ (200 U/ml). After 48 h, total NO production was assessed by chemical reduction of nitrate, nitrite, and nitrosodiethylenediamine by vanadium chloride and measurement of chemiluminescence using a Sievers 280 NO analyzer (Sievers, Boulder, CO). Medium incubated in a 5% CO2 incubator in the absence of cells, unstimulated cell-conditioned medium, and TNF-α (100 U/ml) + LPS (1 µg/ml)-activated cell-conditioned medium were assessed, and results were recorded as the concentration (µM) of NO production. NO levels were adjusted for protein concentration for HIMEC from each well as determined by the method of Bradford (8).

Assessment of cell adhesion molecule surface expression. HIMEC were seeded at 2.5 × 10^5 cells/well and grown for 48–72 h in individual fibronectin-coated wells of a 48-well tissue culture cluster (Corning Costar) until confluence was reached. Endothelial monolayers were assessed unstimulated or after 12 h of activation. HIMEC were stimulated with TNF-α (100 U/ml) + LPS (1 µg/ml), either alone or with 1 mM l-NMMA. Mouse monoclonal antibodies recognizing human E-selectin, intercellular adhesion molecule-1 (ICAM-1), and VCAM-1 (Genzyme, Cambridge, MA) were used to inhibit HIMEC adhesion of U-937 monocytes. Anti-VCAM-1 monoclonal antibody (25 µg/ml) was applied to the activated HIMEC monolayer for 1 h before and during the coculture with U-937 monocytes. Control experiments using a nonspecific monoclonal antibody [mouse IgG1, Kappa (MOPC-31c); Sigma BioSciences] were performed in parallel under equal concentrations and incubation conditions.

Assessment of intracellular reactive oxygen species. HIMEC were stimulated with TNF-α (100 U/ml) + LPS (1 µg/ml) and L-NIL to preferentially increase degradation or inhibit production of intracellular superoxide anion during the 24-h activation period with cytokines and LPS. PEG-SOD and allopurinol were applied to the HIMEC monolayers for 2 h before and during the 24-h activation period.

Inhibition of leukocyte-endothelial cell binding with anti-cell adhesion molecule blocking antibodies. Monoclonal antibodies directed against the endothelial cell adhesion molecule vascular cell adhesion molecule-1 (VCAM-1; Genzyme, Cambridge, MA) were used to inhibit HIMEC adhesion of U-937 monocytes. Anti-VCAM-1 monoclonal antibody (25 µg/ml) was applied to the activated HIMEC monolayer for 1 h before and during the coculture with U-937 monocytes. Control experiments using a nonspecific monoclonal antibody [mouse IgG1, Kappa (MOPC-31c); Sigma BioSciences] were performed in parallel under equal concentrations and incubation conditions.
Olympus camera (PM20) with a fixed shutter speed of 16 s to allow for comparison between culture conditions.

Analysis of data. Statistical analyses were performed using Statview 4.5 and superANOVA software for the Macintosh. When single comparisons were made, 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, followed by the Student-Newman-Keuls test.

RESULTS

Inhibition of NO synthesis significantly increases HIMEC-leukocyte adhesion. Unstimulated HIMEC bound low levels of U-937 (Fig. 1A). After 24-h activation with TNF-α + LPS, HIMEC displayed a dramatic increase in leukocyte binding (Fig. 1B). When HIMEC were activated with TNF-α + LPS in the presence of the NOS inhibitor l-NMMA or the iNOS-specific inhibitor l-NIL, there was a further, marked increase in leukocyte adhesion (Fig. 1C). HIMEC-U-937 adhesion was quantified as described in MATERIALS AND METHODS. With NO inhibition with l-NMMA (Fig. 2) or l-NIL (Fig. 2B), there was a significant, two- to threefold increase in leukocyte binding following HIMEC activation with TNF-α + LPS or TNF-α + interleukin-1β (IL-1β), compared with activation alone. The enhancement of leukocyte binding by l-NMMA or l-NIL was specific to HIMEC and was not seen when HUVEC were treated with NOS inhibitors (Fig. 2C). In both HIMEC and HUVEC, l-NMMA produced a modest increase in basal adhesion, but this was not statistically significant. The effect of NOS inhibition on leukocyte binding was reversible by the addition of excess amounts of the NOS substrate, L-arginine, to both unstimulated and activated HIMEC (Table 1). The selective iNOS inhibitor l-NIL (20 µM) did not alter unstimulated HIMEC-leukocyte binding (data not shown).

HIMEC expression of iNOS and eNOS. Total RNA from unstimulated and TNF-α1 + LPS-activated HIMEC and HUVEC was analyzed for iNOS and eNOS gene expression by RT-PCR. Five HIMEC lines displayed absent-to-low levels of iNOS that increased after TNF-α1 + LPS treatment (Fig. 3A). iNOS gene expression in activated HIMEC was confirmed by Northern blot analysis (Fig. 3B). In marked contrast, HUVEC failed to display iNOS expression with activation. eNOS mRNA was expressed in all endothelial cultures, and levels were not modulated by cytokine and LPS activation (Fig. 3A).

HIMEC production of NO. To confirm that increased expression of iNOS resulted in production of NO, supernatants from HIMEC were assayed for release of NO by chemiluminescence. As shown in Fig. 3C, there was a 2.5-fold increase in NO production (P < 0.01) in the activated HIMEC.

Activated HIMEC cell adhesion molecule surface expression is not increased during NOS inhibition. Analysis of cell adhesion molecule surface expression (Fig. 4A) revealed that resting HIMEC had low levels of ICAM-1 and undetectable levels of E-selectin and VCAM-1. After activation with TNF-α + LPS, there was a dramatic increase in all three adhesion molecules tested (Fig. 4A). When HIMEC were activated in the presence of the NOS inhibitor l-NMMA, there was essentially no change in the level of cell adhesion molecule expression. This result was in marked con-
The contrast to the significant increase in leukocyte adhesion with L-NMMA treatment shown in Fig. 2A.

Previous experiments in our laboratory and published data have demonstrated that U-937 adhesion to endothelial cells is mediated primarily by endothelial expression of two cell adhesion molecules, E-selectin and VCAM-1 (12, 13). Because the increase in HIMEC-U-937 adhesion observed during NOS inhibition did not appear to be mediated by an increase in the density of these cell adhesion molecules, we hypothesized that an alteration in cell adhesion molecule binding affinity might underlie the increase in leukocyte adhesion. Since VCAM-1 is a primary regulator of U-937-endothelial adhesion, we assessed its role in modulating HIMEC-U-937 binding. When blocking antibodies directed against VCAM-1 were used in the HIMEC-U-937 binding assay, antibodies failed to significantly decrease activated HIMEC-leukocyte binding at 24 h (Fig. 4B). However, increased HIMEC-U-937 adhesion elicited by the NOS inhibitor L-NMMA was significantly decreased with anti-VCAM-1 antibody pretreatment. This suggests that, despite unchanged HIMEC expression of VCAM-1 (Fig. 4A), endothelial NO derived from iNOS also modulated leukocyte adhesion.

**Table 1. Effect of L-NMMA on baseline and activated adhesion and reversibility with l-arginine**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Adhesion (U-937/mm²)</th>
<th>% Control</th>
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</thead>
<tbody>
<tr>
<td>Baseline adhesion</td>
<td></td>
<td></td>
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<tr>
<td>Unstimulated</td>
<td>16.2 ± 3.2</td>
<td></td>
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<tr>
<td>L-NMMA</td>
<td>41.4 ± 11.5</td>
<td>230.3 ± 19.1*</td>
</tr>
<tr>
<td>L-NMMA + l-arginine</td>
<td>13.8 ± 5.0</td>
<td>102.3 ± 35.6f</td>
</tr>
<tr>
<td>Activated adhesion</td>
<td></td>
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<tr>
<td>LPS/TNF</td>
<td>902.1 ± 118.4</td>
<td></td>
</tr>
<tr>
<td>LPS/TNF + L-NMMA</td>
<td>1,617.8 ± 218.9</td>
<td>179.5 ± 7.8#</td>
</tr>
<tr>
<td>LPS/TNF + L-NMMA + l-arginine</td>
<td>953.0 ± 120.2</td>
<td>119.5 ± 26.96</td>
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After exposure of human intestinal microvascular endothelial cells (HIMEC) to the above treatments for 24 h, U-937 monocytes were added to HIMEC for 1 h, and adhesion was determined as described in MATERIALS AND METHODS. Concentrations were as follows: Nω-monomethyl-l-arginine (L-NMMA), 1 mM; l-arginine, 10 mM; lipopolysaccharide (LPS), 1 μg/ml; tumor necrosis factor-α (TNF-α), 100 U/ml; n = 4–6 separate experiments, performed in duplicate. *P < 0.05 vs. unstimulated control; †P < 0.05 vs. L-NMMA by two-way ANOVA and Student-Newman-Keuls test; ‡P < 0.01 vs. LPS/TNF control; ††P < 0.01 vs. l-NMMA by two-way ANOVA and Student-Newman-Keuls test.

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**Fig. 2. Adhesion of U-937 monocyte-like cells to HIMEC in absence and presence of a NOS inhibitor (1 mM L-NMMA) (A) or a specific inducible NOS (iNOS) inhibitor [20 µM 2-Methyl-1H-imidazole-5-carboxamidine (L-NIL)] (B). Adhesion assays were performed on unstimulated monolayers and endothelial monolayers were preactivated for 24 h with specific agents [100 U/ml TNF-α + 1 µg/ml LPS or 100 U/ml TNF-α + 100 U/ml interleukin-1β (IL-1β)] before U-937 coculture; n = 5 total experiments, each performed with a distinct HIMEC cell line derived from 5 different patients, performed in duplicate. *P < 0.05; **P < 0.001. Data are means ± SE. C: adhesion of U-937 monocyte-like cells to human umbilical vein endothelial cells (HUVEC) in absence and presence of a NOS inhibitor (1 mM L-NMMA). Adhesion assays were performed on unstimulated monolayers and on endothelial monolayers preactivated for 24 h with specific agents (as in A and B) before U-937 coculture; n = 3 total experiments, performed on 4 separate cell lines derived from 3 different placenta. Each experiment was performed in duplicate; L-NMMA treatment induced no significant difference in leukocyte adhesion to the activated HUVEC. A similar lack of effect with NOS inhibition was observed with the selective iNOS inhibitor L-NIL (data not shown).
through possible effects on cell adhesion molecule binding capacity.

NOS blockade results in increased HIMEC oxidant stress. Because increased HIMEC-leukocyte binding capacity with NOS inhibition was not mediated by alterations in cell adhesion molecule surface expression, we assessed an alternative potential mechanism of leukocyte hyperadhesion. Endothelial activation results in a rapid increase in intracellular superoxide anion generation and oxidant stress (35, 48). We hypothesized that NO production might play a critical regulatory role in the oxidant mechanism of HIMEC activation. To determine the role of NO production on this pathway, DCF-DA fluorescence was used to assess the presence of ROS in HIMEC. There was low-level oxidant stress detected in unstimulated HIMEC (Fig. 5A). 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. 5B). However, when HIMEC were treated with L-NMMA at the time of TNF-α + LPS activation, there was a dramatic increase in ROS detected at 24 h (Fig. 5C and D).

Excess intracellular superoxide underlies increased HIMEC-U-937 adhesion during NO blockade. Since NOS inhibition resulted in increased concentrations of oxygen radicals in HIMEC, we sought to determine whether increased superoxide levels occurring with NOS inhibition alter leukocyte adhesion. PEG-SOD increases degradation of intracellular superoxide in activated endothelial cells, including HIMEC (unpublished observations and Ref. 30). We therefore treated HIMEC with PEG-SOD for 2 h before and concurrently during the 24-h activation time period. As shown in Fig. 6A, PEG-SOD completely attenuated the increased leukocyte adhesion induced by NOS inhibition with L-NMMA in the TNF-α + LPS-activated endothelial cells. To confirm that superoxide generation was mediating the increase in leukocyte adhesion, allopurinol, a selective inhibitor of xanthine oxidase-derived superoxide anion, was applied to HIMEC before activation with and without L-NMMA (Fig. 6B). Inhibition of superoxide generation by allopurinol significantly decreased the L-NMMA-induced increase in HIMEC adhesion for leukocytes.

DISCUSSION

In the present study we have demonstrated a regulatory role for NO in human intestinal inflammation through its inhibitory effect on microvascular endothelial activation and leukocyte recruitment. Using pure human intestinal endothelial populations in vitro, we have demonstrated that HIMEC synthesize increased...
amounts of NO after stimulation with proinflammatory cytokines and LPS. The enzymatic mechanism of NO production in HIMEC is distinct, consisting of a high-output pathway of iNOS expression and activity after stimulation, in addition to constitutive eNOS, which did not change expression. In contrast, iNOS expression was not demonstrated in passaged HUVEC cell lines that were used in this study. Pharmacological inhibition of NOS in activated HIMEC resulted in a significant increase in leukocyte binding, whereas this effect did not occur in activated HUVEC. Finally, our data suggest that NO regulation of HIMEC-leukocyte interaction involves an antioxidant mechanism, in which NO functions intracellularly to deplete endothelial superoxide anion, and this reduction in oxidant stress may decrease HIMEC cell adhesion molecule affinity for leukocyte integrin ligands.

Although NOS inhibitors increased the ability of HIMEC to bind leukocytes, they had essentially no effect on HUVEC. We attribute this finding to fundamental differences in the expression of NOS isoenzymes in these two different endothelial cell types. HIMEC expressed iNOS after activation, as demonstrated by increased iNOS mRNA and NO levels, but expression of this enzyme could not be detected in our established HUVEC lines. The lack of iNOS expression in HUVEC was confirmed by functional studies showing an absence of hyperadhesion in activated HUVEC with either nonselective NOS inhibition (L-NMMA) or selective iNOS inhibition (L-NIL). This absence of iNOS expression is consistent with previous reports in passaged HUVEC (7, 23, 40); however, it is possible that primary cultures of HUVEC might be capable of expressing iNOS.

Our adhesion studies demonstrated the functional effect of iNOS expression in activated HIMEC. Although not statistically significant, baseline adhesion was modestly increased by the nonselective NOS inhibitor L-NMMA, indicating a possible involvement of eNOS in regulating endothelial-leukocyte adhesion in the resting microvasculature. However, after inflammatory activation, iNOS appears to play the dominant role in regulating endothelium-leukocyte interaction. Although it is possible that increased eNOS activity could contribute to the regulation of activated endothelium-leukocyte interaction, this possibility is made less likely by the following findings. The selective iNOS inhibitor, L-NIL, produced the same degree of hyperadhesion as the nonselective NOS inhibitor, L-NMMA, in the activated HIMEC. Furthermore, our activated HUVEC, which did not express iNOS, did not develop hyperadhesion with L-NMMA. The ability of HIMEC to express iNOS has important implications for the investigation of endothelium-leukocyte interaction, as it points to an in vivo role for NO in the regulation of leukocyte-vessel wall interaction in the intestinal microcirculation during states of activation and inflammation.

Although NO produced by iNOS in HIMEC played a significant functional role, the Griess reaction, a routine assay for the measurement of nitrite, a stable NO reaction product, did not detect changes in NO production from HIMEC (data not shown). However, chemiluminescence, an extremely sensitive assay for the detection of NO, was able to demonstrate a significant increase in NO production. We were unable to detect an effect of exogenous NO addition on unstimulated HIMEC-U-937 adhesion with the commonly used NO donor compound S-nitroso-N-acetyl-penicillamine
(Alexis Biochemicals) in concentrations ranging from 0.01 to 1 mM (data not shown). We attribute this lack of effect to the very low baseline HIMEC adhesion, which did not allow for assessment of further diminution in adhesion.

Because adhesion molecule expression is a primary pathway of increased adhesiveness of endothelial cells, we assessed the effect of NO on this expression. As anticipated, there was a marked induction of the adhesion molecules E-selectin, ICAM-1, and VCAM-1 after activation. Surprisingly, inhibition of NOS had no effect on stimulated adhesion molecule protein expression. The use of U-937 monocytes in our HIMEC-leukocyte adhesion assays offers the distinct advantage of a defined mechanism of endothelial interaction and binding (12, 13). U-937-HIMEC adhesion is mediated by endothelial expression of VCAM-1 and E-selectin, two molecules not present on resting HIMEC. However, the expression of these molecules was not altered with NOS inhibition, despite increased leukocyte adhesion.

To assess how unaltered densities of adhesion molecules could mediate differential leukocyte binding, we evaluated oxidant-mediated HIMEC activation and the effect of NO on this process.

Oxidant stress is known to play an integral role in the activation of multiple cell types, including endothelial cells (17, 18). Intracellular NO can regulate oxidant stress through its ability to react rapidly with radical oxygen species, including superoxide anion (17, 41). There is clear evidence for protective effects of NO on oxygen radical scavenging in endothelial cells and neurons (10, 52). The concern that the combination of NO with superoxide will result in the generation of peroxynitrite, an even more dangerous compound that has been implicated in the pathogenesis of intestinal inflammation, has also been the focus of intense research (17, 38, 39). However, a growing body of evidence indicates that this may not occur readily in biological systems, where the predominance of one radical species (either NO or \( O_2^- \)) precludes this development (31).

Recent data from our laboratory suggest that cytokine-induced HIMEC activation is also mediated via oxidant stress, specifically the generation of superoxide anion by xanthine oxidase (3). This is similar to the endothelial activation pathway that has been demonstrated in feline mesenteric venules (48). In the present study, DCF-DA fluorescence in HIMEC demonstrated that levels of oxygen radicals in activated cells were markedly increased with NOS inhibition. This suggests that when high-output NO is produced by iNOS expression in the intestinal microvasculature, it can act to reduce oxidative stress. Agents that reduced superoxide levels (PEG-SOD and allopurinol) decreased DCF-DA fluorescence and abolished the hyperadhesive-

Fig. 5. A: high-power (×400), fluorescence microscopic view of live, resting HIMEC monolayer treated with 5 µM 2',7'-dichlorofluorescein diacetate (DCF-DA) for 30 min to detect reactive oxygen species (ROS). Essentially no fluorescence is demonstrated. B: DCF-DA fluorescence for ROS in live HIMEC monolayer (derived from same patient as in A) stimulated with 100 U/ml TNF-α + 1 µg/ml LPS for 24 h. C: DCF-DA fluorescence for ROS in HIMEC monolayer stimulated with TNF-α + LPS (as above) in presence of NOS inhibition (1 mM L-NMMA) for 24 h. D: quantification of DCF-DA fluorescence in resting and activated HIMEC monolayers stimulated as shown, treated with and without 1 mM L-NMMA for 24 h. High-power fluorescence photomicrographs were digitized and analyzed using NIH Image v. 1.6 software as described in MATERIALS AND METHODS. Data shown are from a representative experiment; n = 6 experiments.
ness caused by NOS inhibition. This finding directly implicates the persistence of superoxide anion as a causative factor in the hyperadhesion of HIMEC when NO release is prevented. Thus we can infer that during the normal course of inflammatory activation in HIMEC, NO produced by iNOS results in decreased levels of superoxide anion and hence limits activation.

Another possible mechanism of NO regulation of oxidant stress is via inhibition of xanthine oxidase activity (14). Thus the hyperadhesion observed with NOS inhibitors could reflect the loss of NO-mediated downregulation of xanthine oxidase.

In addition to increasing adhesion molecule expression, oxidant stress may modulate the ability of these molecules to bind their ligands on leukocytes. Because U-937 binding is mediated through HIMEC expression of defined cell adhesion molecules, we studied the effects of NO on endothelial VCAM-1. During NOS inhibition, VCAM-1 mediated a significant part of the increased HIMEC-U-937 adhesion and played a lesser role in the activated HIMEC, which were not exposed to the NOS inhibitors and were thus able to produce NO. Our data suggest a potential regulatory role for NO in decreasing cell adhesion binding affinity. It should be noted, however, that our experiments relied on indirect evidence derived from antibody blockade of VCAM-1. It is also possible that modulation of levels of oxygen radicals may alter HIMEC adhesion molecule expression. For example, we have found that inhibition of superoxide production by allopurinol or enhancement of its degradation by PEG-SOD decreases VCAM-1 expression in HIMEC (unpublished observations). However, as discussed above, despite modulating oxygen radicals, alteration of NO levels did not affect adhesion molecule expression in our study.

It is also possible that NOS inhibitors may alter very late antigen-4, the leukocyte ligand for VCAM-1, either directly or via the secretion of soluble factors, such as chemokines, from HIMEC. This explanation is possible, as we have demonstrated decreased HIMEC production of the chemokines MCP-1 and IL-8 in previous studies (4).

Significant heterogeneity in the NO biology of microvascular and large vessel endothelial cell populations was an additional finding that resulted from this investigation. HIMEC, a microvascular endothelial population derived from a differentiated adult tissue, retained the capacity to synthesize iNOS in long-term culture, and this translated into unique functional responses compared with HUVEC, a routinely used large vessel endothelium. In our experiments, although passaged HUVEC cultures were used at earlier passages than HIMEC, the HUVEC cultures had no capacity to synthesize iNOS and were unaffected by NOS inhibition in functional assays of leukocyte binding. Our results also suggest that attempts to define specific microvascular processes in the intestinal inflammatory response may require the use of local cell populations whose phenotype and functional capacity may vary greatly from large vessel endothelium and standard endothelial cell models. Finally, the availability of HIMEC for in vitro functional assays was essential in defining the regulatory role of iNOS and NO in intestinal endothelial-leukocyte interaction. Defining these mechanisms would have been difficult using fixed intestinal tissue sections, as an alteration in cell adhesion molecule binding capacity and not a gross change in cell adhesion molecule density mediated these altered re-

Fig. 6. A: adhesion of U-937 monocyte-like cells to HIMEC pretreated with polyethylene glycol-conjugated superoxide dismutase (PEG-SOD) in absence and presence of a NOS inhibitor (L-NMMA). Adhesion assays were performed on endothelial monolayers pretreated for 24 h with specific agents before U-937 coculture. PEG-SOD was applied concurrently during the 24-h activation period. *P < 0.05; n = 3 total experiments, performed in duplicate. Data are means ± SE. B: adhesion of U-937 monocyte-like cells to HIMEC pretreated with allopurinol in absence and presence of a NOS inhibitor (L-NMMA). Adhesion assays were performed on unstimulated monolayers and endothelial monolayers preactivated for 24 h with specific agents before U-937 coculture. Allopurinol was applied concurrently during the 24-h activation period. **P < 0.05; n = 3 total experiments performed on 3 separate cell lines in duplicate; data are means ± SE.
responses. To confirm that this was not a phenomenon of primary cells, we found identical responses in immortalized HIMEC cell lines (data not shown) (5).

Many studies have shown that both immune and nonimmune cell types play a role in intestinal inflammation. The role of endothelial cells in regulating the acute inflammatory process is now appreciated. In general, increased expression of iNOS in intestinal inflammation has been localized mainly to intestinal epithelial cells and immune cells, such as macrophages. There is immunohistochemical evidence suggesting that iNOS can be detected in the inflamed mucosal microvascular endothelium, although expression is lower than is typically detected in the lamina propria mononuclear cell population, as well as the epithelium (50, 51). Other investigators have had difficulty identifying iNOS expression in the vasculature, and this has been proposed to result from enzyme expression in endothelial cells below the limit of immunohistochemical detection (9). Furthermore, McCafferty et al. (29) demonstrated markedly increased neutrophil infiltration in colitic iNOS knockout mice, suggesting that NO normally blunts neutrophil-microvascular adhesion and transmigration during inflammation. The concept that NO-derived free radicals play a role in downregulating oxidant stress in microvascular endothelial activation and leukocyte recruitment has also been demonstrated in iNOS knockout mice subjected to endotoxemia (20). Our analysis of pure HIMEC populations in vitro has demonstrated that iNOS is readily induced and is functional as a regulatory molecule in this cell population.

An earlier study demonstrated that NO can function as an effector molecule, which is critically important in host defense against tumor cells and microbes and appears to mediate tissue damage during inflammation (53). However, the exacerbation of experimental colitis in iNOS knockout mice supports a protective role for NO (37). Our data in HIMEC support a potentially protective role for NO in intestinal inflammation. The seemingly opposing roles for NO, as proinflammatory effector molecule and anti-inflammatory regulatory molecule, are not mutually exclusive in the intestinal mucosa during inflammation and may depend on the temporal context. Increased NO production in endothelial cells during inflammatory stimulation may function as an endogenous antioxidant, which ultimately turns off activation (26).

The overall role of NO in downregulating oxidant stress in microvascular endothelial activation and leukocyte recruitment may underlie pathological processes, including IBD. We have demonstrated that HIMEC isolated from the chronically inflamed mucosa of IBD patients have consistently demonstrated a significant, two- to threefold increase in leukocyte binding after inflammatory activation with cytokines and LPS (6). Similarly, alteration in the balance of NO and oxidant stress may provide a mechanism for enhanced leukocyte binding by HIMEC isolated from chronically inflamed mucosa and may prove to be a fruitful area of investigation.

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REFERENCES


