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Research Article

The localization of circulating leukocytes within inflamed tissues occurs as the result of interactions with and migration across vascular endothelium, and is governed, in part, by the expression of adhesion molecules on both cell types. Recently, we have described a novel primary adhesion interaction between the structurally activated form of the adhesion molecule CD44 on lymphocytes and its major ligand hyaluronan on endothelial cells under physiologic laminar flow conditions, and have proposed that this interaction functions in an extravasation pathway for lymphocytes in vascular beds at sites of inflammation. While the regulation of activated CD44 on leukocytes has been characterized in depth, regulation of hyaluronate (HA) on endothelial cells has not been extensively studied. Here we demonstrate that the expression of HA on cultured endothelial cell lines and primary endothelial cultures is inducible by the proinflammatory cytokines TNFalpha and IL-1beta, as well as bacterial lipopolysaccharide. In addition, this inducibility appears strikingly restricted to endothelial cells derived from microvascular, but not large vessel, sources. The elevated HA levels thus induced result in increased CD44-dependent adhesive interactions in both nonstatic shear and laminar flow adhesion assays. Changes in mRNA levels for the described HA synthetic and degradative enzymes were not found, suggesting other more complex mechanisms of regulation. Together, these data add to the selectin and immunoglobulin gene families a new inducible [...]

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Proinflammatory Stimuli Regulate Endothelial Hyaluronan Expression and CD44/HA-dependent Primary Adhesion

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Abstract

The localization of circulating leukocytes within inflamed tissues occurs as the result of interactions with and migration across vascular endothelium, and is governed, in part, by the expression of adhesion molecules on both cell types. Recently, we have described a novel primary adhesion interaction between the structurally activated form of the adhesion molecule CD44 on lymphocytes and its major ligand hyaluronan on endothelial cells under physiologic laminar flow conditions, and have proposed that this interaction functions in an extravasation pathway for lymphocytes in vascular beds at sites of inflammation. While the regulation of activated CD44 on leukocytes has been characterized in depth, regulation of hyaluronate (HA) on endothelial cells has not been extensively studied. Here we demonstrate that the expression of HA on cultured endothelial cell lines and primary endothelial cultures is inducible by the proinflammatory cytokines TNF α and IL-1 β , as well as bacterial lipopolysaccharide. In addition, this inducibility appears strikingly restricted to endothelial cells derived from microvascular, but not large vessel, sources. The elevated HA levels thus induced result in increased CD44-dependent adhesive interactions in both nonstatic shear and laminar flow adhesion assays. Changes in mRNA levels for the described HA synthetic and degradative enzymes were not found, suggesting other more complex mechanisms of regulation. Together, these data add to the selectin and immunoglobulin gene families a new inducible endothelial adhesive molecule, hyaluronan, and help to further our understanding of the potential physiologic roles of the CD44/HA interaction; i.e., local cytokine production within inflamed vascular beds may enhance surface hyaluronan expression on endothelial cells, thereby creating local sites receptive to the CD44/HA interaction and thus extravasation of inflammatory cells. (*J. Clin. Invest.* 1998. 101:97–108.) Key words: CD44 • endothelial cell • cytokines • lymphocyte adhesion • hyaluronate

Introduction

The localization of emigrating cells at vascular beds within sites of inflammation depends on several discrete and successive events including cell tethering, rolling, activation, and arrest (1–3). After arrest, leukocytes migrate via endothelial junctions into the surrounding tissues. These carefully coordinated interactions between circulating leukocytes and endothelial cells have generally been viewed as being mediated primarily by two classes of ligand pairs: (a) selectins and their carbohydrate ligands, and (b) leukocyte integrins interacting with members of the immunoglobulin gene superfamily borne by endothelial cells.

We have recently described a novel interaction between T cells and endothelial cells which is similar under laminar flow conditions to that mediated by selectins and also has as its basis a distinct protein-carbohydrate ligand interaction, namely that between the cartilage link protein family member CD44 and its principal ligand, hyaluronate (HA)¹ (4). One well-known consequence of antigen stimulation on T cells is increased surface levels of CD44 (5–7). However, elevated levels of CD44 do not necessarily correlate with increased HA-binding, and thus the ability of CD44 to bind HA is not constitutive; rather, CD44 requires some form of structural alteration to engage this ligand (5, 8, 9). Although the mechanism of CD44 activation to bind HA remains to be completely elucidated, evidence has accumulated that T cell stimulation of normal lymphocytes in vitro or in vivo via signaling through the T cell receptor induces the activated form of CD44 and attendant primary adhesion on HA substrate (7, 10, 11). These observations have established the HA-binding form of CD44 as an early activation marker on T cells after T cell receptor stimulation and support a role for this interaction during the course of an immune response. Furthermore, there has been a suggested role for CD44 in human arthritis (12), a collagen-induced mouse model of arthritis (13), and in the progression of a delayed type hypersensitivity response (14). However, the basis for a relationship to these diseases has not been clarified. Based on our observations, we have postulated that CD44 on lymphocytes interacts with HA on endothelium and participates in the well-known preferential homing of activated lymphoblasts to tertiary sites of inflammation.

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1. *Abbreviations used in this paper:* ABEC, bovine aortic primary endothelial cells; BAEC, bovine pulmonary artery endothelial cells; bPG, bovine proteoglycan; DMEC, human dermal microvascular endothelial cells; EC, endothelial cell; Fl-HA, fluorescein conjugated hyaluronate; HA, hyaluronate; HAS, hyaluronate synthase; HUVEC, human umbilical vein endothelial cells; LN, lymph node; LN-PEC, primary murine peripheral lymph node endothelial culture; LPS, bacterial lipopolysaccharide; RT-PCR, reverse transcription polymerase chain reaction; SA-PE, phycoerythrin-labeled streptavidin.

The reciprocal ligand, HA, is a complex glycosaminoglycan that is a prominent component of the extracellular matrix, and has been suggested to play a key role in several biological processes including embryonic development (15), wound healing (16, 17), and tumor growth (18) by providing a provisional matrix for supporting cellular migration and adherence (19, 20). Moreover, studies have demonstrated that HA may function as a cellular signaling molecule under certain circumstances (21, 22). Our studies, in contrast, imply a potential role for HA on the luminal aspects of blood vessels. It has been reported that hyaluronan is found associated with vessels during development and in adult lung (23, 24), on luminal aspects within some venules of lymph nodes (25), and within dermal microvasculature in lesions of the chronic inflammatory skin disease psoriasis (26). A key implication of the CD44/HA activation and extravasation model we have proposed is that local regulation of HA on the luminal surfaces of blood vessels occurs during the course of an inflammatory response, and that this increased expression would lead to increased recruitment of leukocytes to the inflamed site.

In these studies, we have addressed whether proinflammatory stimuli, such as cytokines and the bacterial cell wall component bacterial lipopolysaccharide (LPS), regulate the cell surface expression of HA on endothelial cells, and whether this regulation alters adhesive interactions under nonstatic shear and laminar flow conditions. It is demonstrated that such stimuli have a marked influence on the level of HA expression on several endothelial cell lines as well as primary endothelial cultures, that this is sustained over a prolonged period, and that this increased expression results in increased adhesive interactions in *in vitro* assays. These results support the involvement of a new molecular class, glycosaminoglycans, as another defined type of cell surface molecule that can be upregulated by proinflammatory agents, and lend further credence to a role for the CD44/HA interaction in leukocyte extravasation at sites of inflammation.

Methods

Chemicals and reagents. Rooster comb hyaluronate was purchased from Sigma Chemical Co. (St. Louis, MO). Biotinylated bovine proteoglycan (bPG), extracted from bovine nasal cartilage (23), was kindly provided by C. Underhill (Georgetown University School of Medicine, Washington, DC). Rat anti-mouse CD44, KM81, which blocks HA binding (27), was obtained from the American Type Culture Collection (ATCC, Rockville, MD). Rat anti-mouse H-2 (clone M1/42) was obtained from K. Fischer-Lindahl (HHMI, UT Southwestern Medical Center, Dallas, TX). The following mouse cytokines

were used in these studies: recombinant TNF α (5×10^7 U/mg) and IL-1 β (2.2×10^7 U/mg) from Genzyme Corp. (Cambridge, MA), IFN γ (1.1×10^6 U/mg) from the Biological Response Modifiers Program (Frederick Cancer Research & Development Center, Frederick, MD), and recombinant IL-12 (1.5×10^7 U/mg) from PharMingen (San Diego, CA). Human recombinant cytokines TNF α (10^7 U/mg), IL-1 β (10^8 U/mg), and IFN γ (10^7 U/mg) were obtained from Genzyme Corp. Bacterial LPS and chondroitinase ABC from *Proteus vulgaris* were obtained from Sigma Chemical Co. Streptococcal hyaluronidase was purchased from ICN Biomedicals, Inc. (Irvine, CA).

Endothelial cell culture and stimulation. SVEC4-10 is an SV40 transformed murine lymph node endothelial cell line derived from lymph node stroma. The TME-3H3 endothelial line was similarly derived (28) and was kindly provided by A. Hamann (Department of Immunology, Medizinischen Klinik, Hamburg, Germany) and J. Lesley (Salk Institute, San Diego, CA). LEII and MPCE, murine lung capillary endothelial cell (EC) lines derived by A. Curtis (University of Glasgow, Scotland, UK), and bovine pulmonary artery and bovine aortic primary endothelial cultures (BAEC and ABEC), respectively, were kindly provided by P. Thorpe (UT Southwestern Medical Center). Primary cultures of dermal microvascular endothelium and human umbilical vein endothelial cells (HUVEC) were obtained from the Skin Center at Emory University (S.W. Caughman, Atlanta, GA). EC lines were maintained in RPMI 1640–high glucose, 15% FCS plus 1 mM pyruvate, 2 mM glutamine, and 50 μ M β -mercaptoethanol. Primary LN EC cultures were made by pooling cervical and axial nodes from three animals, as described (25, 29). Briefly, organs were minced, rinsed to remove lymphocytes, treated with collagenase for 30 min at 37°C, and plated on 35-mm culture dishes in supplemented IMDM (20% FCS). These cultures at confluence showed clear endothelial morphology of closely packed polygonal cells with overlapping cytoplasm. FACS[®] analysis indicated that > 95% of the population stained positive for endothelial specific markers MECA-32, CD31, and endoglin, and were negative for the macrophage marker MAC-1. After reaching initial confluence, primary lymph node endothelial cells were passaged and used directly or after one additional passage to fresh plates. HUVEC and human dermal microvascular endothelial cells (DMEC) were used upon reaching confluence or following their first or second passages. BAEC and ABEC were used at passage five. Endothelial cell lines were taken from frozen storage and used for three to a maximum of five passages.

For stimulation of EC, cells were passaged 24–48 h before addition of stimuli, when cultures were subconfluent. Cytokines or LPS were added to culture medium as follows: TNF α , IL-1 β , IFN γ , or IL-12 to 10 ng/ml final concentration, or LPS to 10 μ g/ml, except as noted in dose response experiments. Cells were maintained at 37°C in a 5% CO₂ atmosphere for indicated time periods. Control cultures without exogenous stimuli were incubated in parallel in all experiments. Cells were harvested for FACS analysis and RNA isolation by gentle pipetting after incubation with Versene (GIBCO BRL, Gaithersburg, MD) for 5 min at 37°C.

Fluorescence activated cell sorter (FACS) analysis. 5×10^5 cells

Table I.

Template	Forward primer	Reverse primer
HA synthase	5'-CCTAGCGTTGGTTACCATGAATC-3'	5'-CCCGACTCATACTTGGACA-3'
β -glucuronidase	5'-CCTACCACTTACATCGATGATATC-3'	5'-CCTCTAGGTGGTGCCGAAGTGAC-3'
Hyaluronidase	5'-GAATGTCCCAACTGAACGTTG-3'	5'-CTCAACCAGGTAGGCCTCC-3'
Hexase* α	5'-CTGCGTCGTCCTCGACGAGGCC-3'	5'-GTGTGCTCATGAAGTCATAGGTGCTG-3'
Hexase β	5'-CCGCGGCTGTTGTACATCTCCGC-3'	5'-CCACACTTCGACTACTGTGCC-3'
β -actin	5'-GTGGGCCGCTCTAGGCACCAA-3'	5'-CTCTTTGATGTCACGCACGATTCC-3'

* β -N-acetyl-D-hexosaminidase. Primer concentrations, RNA amounts, and PCR cycling conditions were titrated to establish optimal conditions for each primer set. Cycling conditions for each reaction are indicated in the legend to Fig. 7. Cycling conditions are indicated in figure legends. PCR reactions were performed semi-quantitatively and compared to β -actin PCR amplifications run in parallel.

were stained with bPG-biotin in 100 μ l PBS/2%FCS for 30 min and then washed with 500 μ l of PBS/2%FCS. Phycoerythrin-labeled streptavidin (SA-PE; Caltag Labs, San Francisco, CA) was added for an additional 30 min. For blocking of bPG staining, cells were pre-incubated with 100 μ l of soluble HA (500 μ g/ml) or treated with 200 μ l of hyaluronidase (20 U/ml) at 37°C for 30 min before addition of bPG. Cells were again washed and analyzed using a FACScan™ instrument and CellQuest™ software (Becton Dickinson, San Jose, CA).

Reverse transcription polymerase chain reaction (RT-PCR). Total RNA was isolated according to manufacturer's instructions using RNeasy (Qiagen, Crawfordsville, IN). RT-PCR amplification was performed as described (30). Please see Table I for primer sequences.

Adhesion assay under rotational and flow conditions. Nonstatic adhesion assays were performed similarly to those previously described (31, 32) in rotating 24-well tissue culture plates containing SVEC4-10 EC monolayers grown to confluence which had been incubated immediately previously for 4 h with medium, cytokine, or LPS. Plates were rotated at room temperature for 30 min, rinsed gently with RPMI 1640, and fixed with 1% glutaraldehyde. Determination of the number of bound cells was performed by counting microscopically. For each value, five wells were analyzed, counting five separate, randomly chosen fields (magnification of 200) of each well.

Physiological flow conditions were produced using a parallel plate flow chamber as previously described (4, 33). Briefly, flow occurs over a 35-mm tissue culture dish containing an adherent cell monolayer of EC. The culture dish and an opposing Plexiglas chamber are held 1.27 \times 10⁻² cm apart by a silicon gasket cut to form two flow chambers, each 0.6 cm wide. Experiments were carried out at a wall shear stress of 2.0 dyn/cm², unless otherwise indicated. After equilibration of flow with medium alone, BW5147 murine T cells (34) were resuspended at a concentration of 3 \times 10⁶ cells per ml in RPMI 1640 equilibrated to 37°C and pulled continuously across the flow chamber. For blocking studies, antibody or soluble HA was added at saturating concentrations to the cell suspension before flow. Interaction of lymphoid cells with the EC monolayer after equilibration of flow was monitored for 5–10 min with an inverted phase contrast micro-

scope connected to a video camera and recorder. Rolling cells were scored visually and data is reported as the average number of interacting cells/mm² per minute, based on an actual field of view of 0.6 mm \times 0.8 mm. Cell rolling velocities were obtained by determining the amount of time necessary for a given cell to move a calibrated distance. For average velocity and velocity distributions, 100 cells from two separate experiments for each experimental condition were followed.

For measurements of initial attachment, or tethering, cells were introduced into the flow chamber under incremental shear stresses after equilibration of medium under flow, as described (35). The number of cells attaching to the SVEC4-10 monolayer during the first 30 s at varying shear force was scored. Results are reported as the number of tethering cells per mm².

Results

HA expression is inducible on murine endothelial cell lines.

In order to determine whether HA can be regulated on EC, we analyzed the lymph node microvascular endothelial cell line SVEC4-10. We have shown previously that this line expresses constitutive basal surface HA and supports CD44-dependent rolling interactions (4). These were examined after 4-h treatment with TNF α . Results of bPG staining are shown in Fig. 1 A, where a marked increase in staining for the entire population above constitutive untreated expression levels can be seen after treatment. This increased staining was shown to be specific for HA by blocking with soluble HA and by treatment of EC with hyaluronidase before bPG staining, both of which diminish bPG staining to background levels (Fig. 1 A). In addition, using a control glycolytic enzyme, chondroitinase ABC, no change in bPG staining was observed (data not shown). Thus TNF α , a proinflammatory cytokine, can increase HA expression on an endothelial cell line.

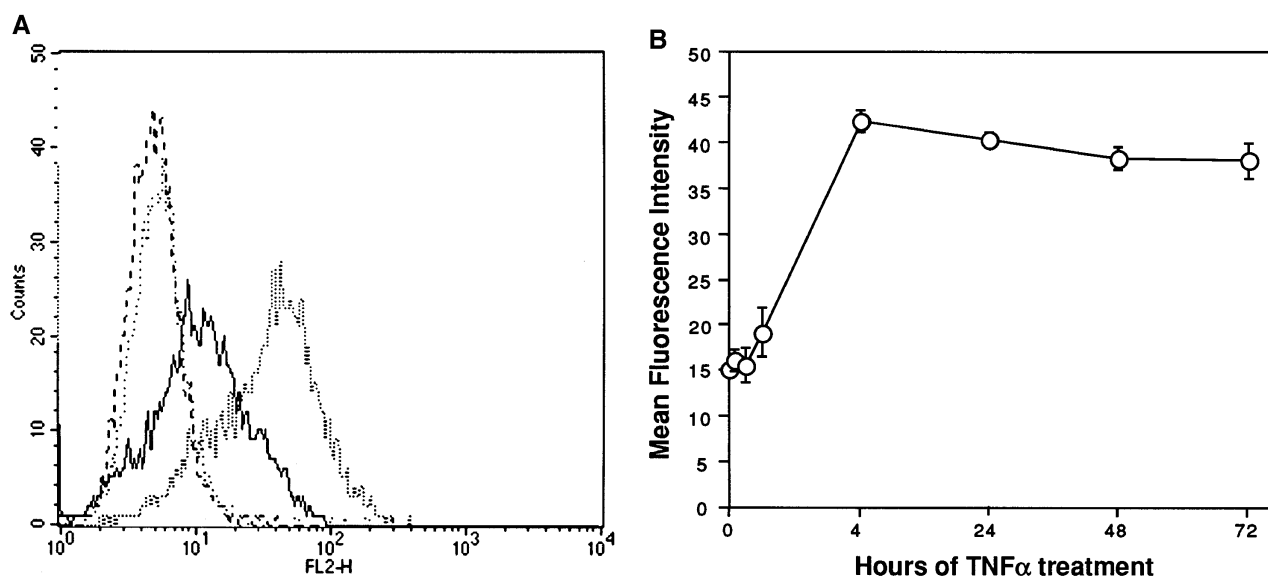


Figure 1. Induction of increased levels of cells surface HA on SVEC4-10 cells by treatment with TNF α . (A) EC were treated for 4 h with TNF α (10 ng/ml), stained with bPG-biotin/SA-PE, and analyzed by FACS for fluorescence. Untreated cells, which constitutively express HA, bind bPG (solid line), while TNF α treated cells show even greater levels of staining (stippled line). bPG staining is blocked to below the level of untreated EC by preincubation with either soluble HA or hyaluronidase (dotted and dashed lines, respectively). (B) Changes in levels of cell surface HA on SVEC4-10 cells in response to TNF α over time. Cells were treated and stained as in A and harvested at the indicated time points. Data are the means \pm SEM from three separate experiments.

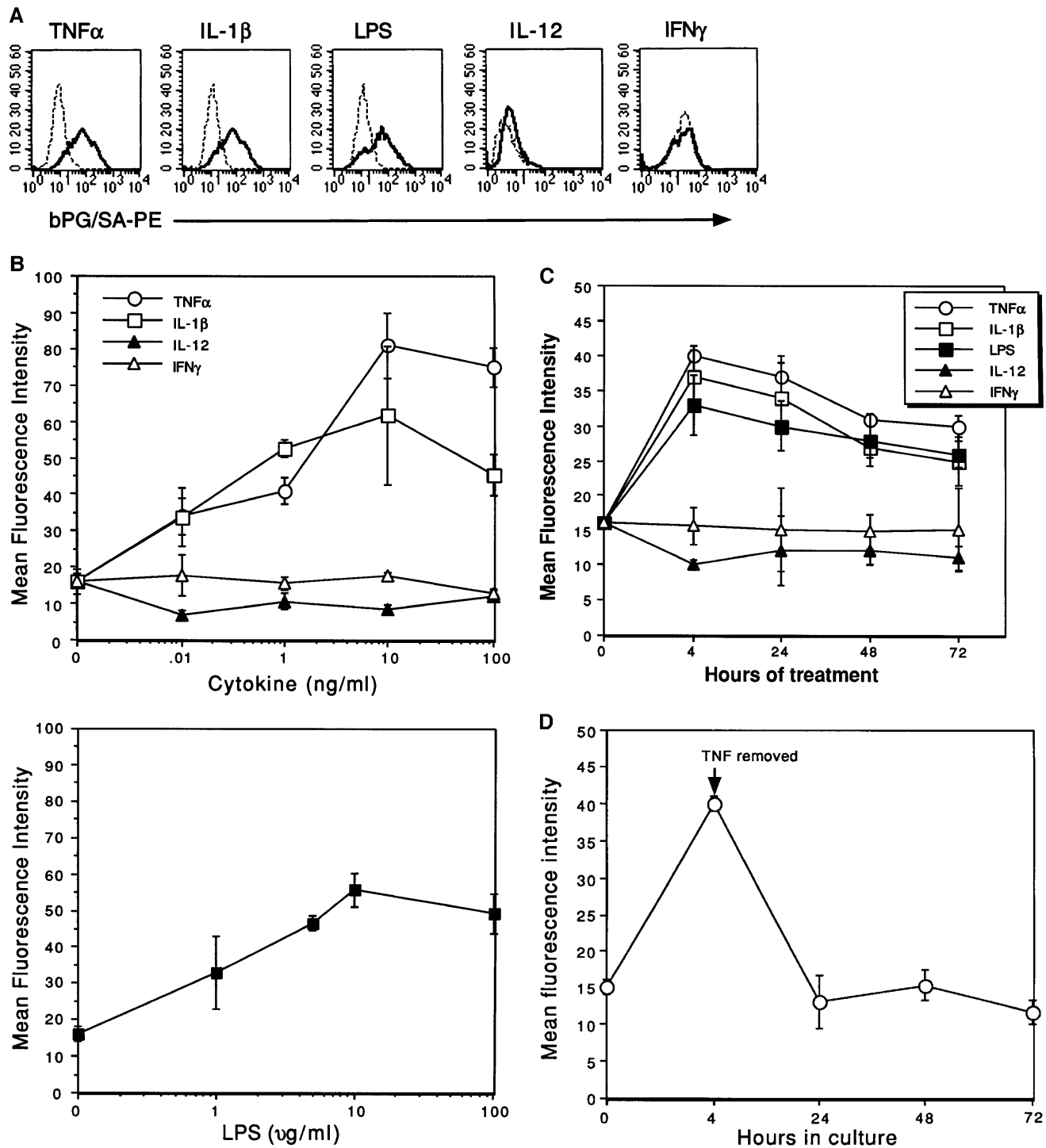


Figure 2. Characterization of HA levels on SVEC4-10 cells in response to cytokines or LPS. (A) SVEC4-10 cells were treated with 10 ng of TNF α , IL-1 β , IL-12, IFN γ , or 10 μ g of LPS. Cells were harvested after 4 h in culture and stained with bPG-biotin/SA-PE. Cells respond to TNF α , IL-1 β , and LPS treatment by expressing increased levels of HA, while IL-12 and IFN γ have no effect. Dotted lines, untreated cells; solid lines, treated cells. (B) Dose dependence of increased HA levels in response to cytokine or LPS treatment. Cells were treated with TNF α , IL-1 β , IFN γ , IL-12, or LPS at varying concentrations, as shown. IL-12 and IFN γ had no effect on SVEC4-10, even at the highest concentration, while TNF α , IL-1 β , and LPS each had a dose-dependent effect on levels of surface HA. (C) Changes in levels of cell surface HA on SVEC4-10 cells in response to cytokines or LPS over time. Cells were treated and stained as in Fig. 2 A and harvested at the indicated time points. Data are the means \pm SEM from three separate experiments. (D) Effect of cessation of TNF α treatment of EC on levels of HA as determined by bPG staining. TNF α was incubated with cells for 4 h and then removed (arrow). HA levels returned to baseline with 20 h of cytokine removal. Results are the mean \pm SEM from three experiments.

Various endothelial adhesion receptors are inducible over varying time intervals. Although expression at 4 h is suggestive of the more gradual expression seen with E-selectin induction, to gauge the rapidity and duration of the increased HA, expression levels at various time intervals after stimulation were examined. As shown in Fig. 1 B, the increase in the level of cell surface HA was not measurable until 1 h after incubation with TNF α , suggesting an absence of an intracellular pool rapidly mobilizable to the surface. The response peaked by \sim 4 h, and was still elevated at 72 h. Thus, elevated HA expression is induced after exposure to TNF α and peaks over a period of several hours. Furthermore, elevated levels are persistent over several days in the presence of cytokine. Since bPG staining was maximal after 4 h stimulation, this time point was used in subsequent experiments, unless otherwise indicated.

HA expression is inducible by other proinflammatory stimuli on SVEC4-10 cells. To determine whether the upregulation of HA expression occurs with additional stimuli, SVEC4-10 cells were tested with murine TNF α , IL-1 β , LPS, IFN γ , or IL-12. Fig. 2 A demonstrates that TNF α , IL-1 β , and LPS, common proinflammatory stimuli, all substantially increased expression of HA on SVEC4-10 cells, while IFN γ and IL-12 did not.

To further examine the range of concentrations of stimulant over which HA is inducible, SVEC4-10 cells were treated with TNF α , IL-1 β , IFN γ , IL-12, or LPS at varying concentrations. Results, shown in Fig. 2 B, indicate a dose-dependent relationship between bPG staining and cytokine concentration with TNF α , IL-1 β , or LPS. IFN γ and IL-12 treatments had no effect on HA expression even at the highest concentrations. We further assessed the kinetics of HA expression with these agents. The temporal pattern of increased bPG staining seen in response to TNF α was essentially recapitulated when SVEC4-10 cells were treated individually with TNF α , IL-1 β , or LPS. In each case, maximal increase was reached by 4 h and main-

tained to at least 72 h (Fig. 2 C). To further examine whether the prolonged elevation of HA expression required the continual presence of cytokine, SVEC4-10 cells were incubated with TNF α for 4 h, at which time the medium was exchanged and cells were further incubated without cytokine. As shown in Fig. 2 D, return of HA expression to the basal state occurred within 20 h of removing TNF α from the cultures. Thus, as with the induction of other adhesion receptor expression, the maintenance of elevated surface HA requires continual exposure to the stimulus.

HA expression is inducible on a subset of endothelial cell lines. To examine the scope of the upregulation of HA expression on a variety of endothelial lines with these stimuli, TME-3H3, derived from murine lymph node small vessel endothelium, and LEII and MPCE, independently derived murine lung capillary endothelial lines, were also tested with TNF α , IL-1 β , LPS, IFN γ , and IL-12. Fig. 3 demonstrates that, as with SVEC4-10 cells, TNF α , IL-1 β , and LPS all increased HA expression on TME-3 cells, while IFN γ and IL-12 did not. In contrast, none of the tested cytokines promoted changes in HA expression on either of the lung capillary-derived LEII or MPCE endothelial cell lines. Although from similar sources, it is notable that MPCE and LEII differ with respect to basal HA expression. The basis for this is unclear, but may reflect EC heterogeneity in vivo, clonal responsiveness to nonphysiologic stimuli in culture, or a combination thereof. These results together suggested that a subset of endothelial lines, in particular those derived from small venular endothelium, respond to a variety of cytokines with elevated HA expression.

HA expression is inducible on primary lymph node endothelial cultures. In order to extend the observations of regulatable expression of HA on EC to more physiologic counterparts, we tested a number of small and large vessel-derived primary endothelial cultures. We derived primary endothelial cell cultures from murine peripheral lymph nodes (LNPEC),

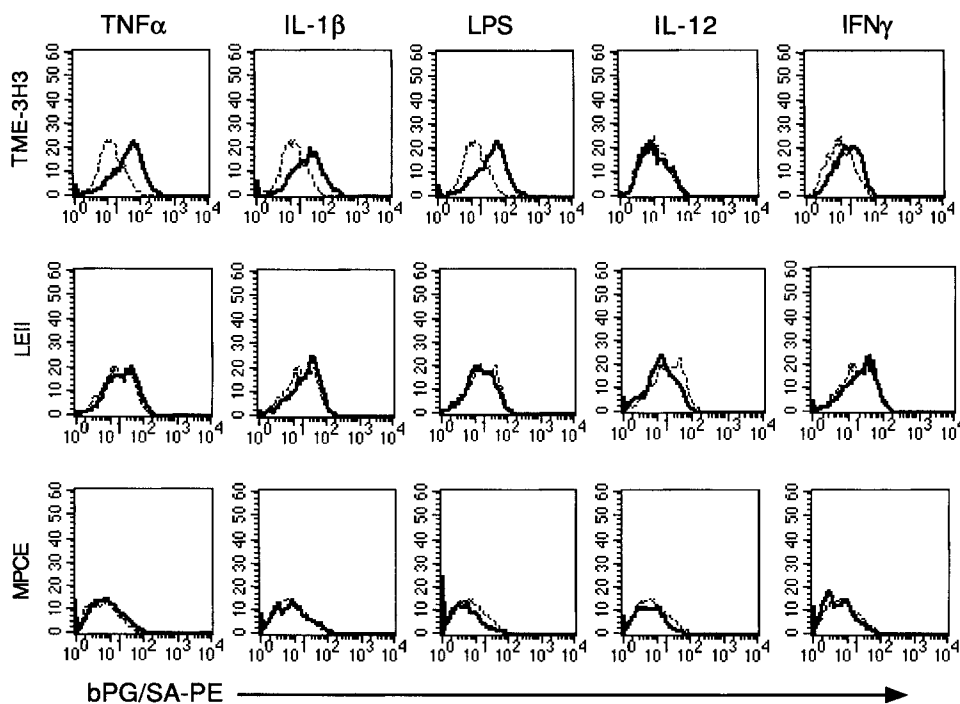


Figure 3. HA levels on endothelial cells in response to cytokines or LPS. TME-3H3, LEII, and MPCE cells were treated with TNF α , IL-1 β , IL-12, IFN γ , or LPS, as in Fig. 2 A. Cells were harvested after 4 h in culture and stained with bPG-biotin/SA-PE. TME-3H3 cells respond to TNF α , IL-1 β , and LPS treatment by expressing increased levels of HA, while LEII and MPCE cells are not affected. IL-12 and IFN γ had no effect on any of the EC lines. Dotted lines, untreated cells; solid lines, treated cells.

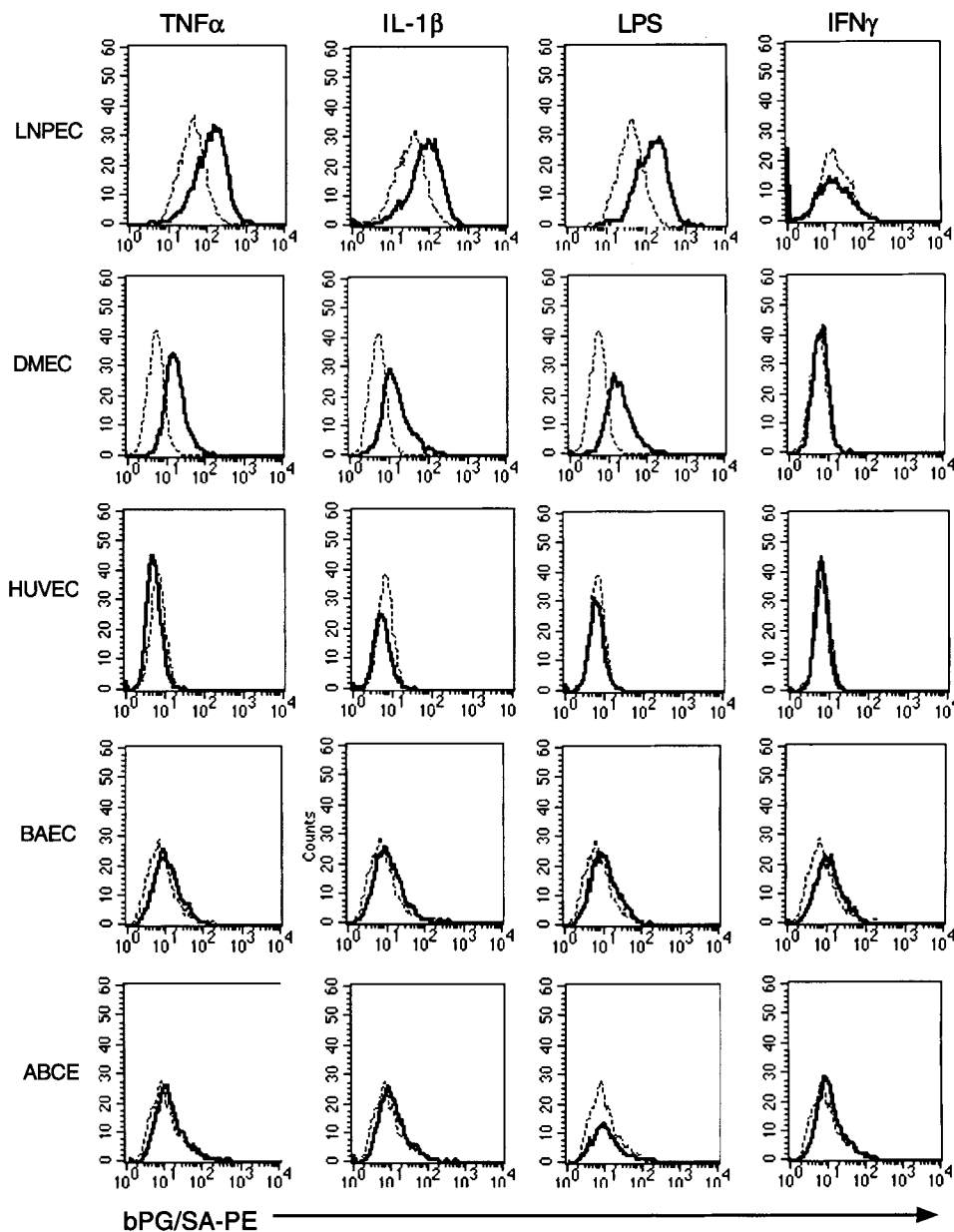


Figure 4. HA levels on primary lymph node endothelial cells in response to cytokines or LPS. LNPEC, DMEC, HUVEC, BAEC, and ABCE were treated, harvested, and stained as in Fig. 2 A. LNPEC and DMEC respond to TNF α , IL-1 β , and LPS treatment by expressing increased levels of HA, while IFN γ had no effect. HUVEC, BAEC, and ABCE did not increase surface HA expression in response to any of the stimuli.

described to have characteristics of high endothelial venular cells (29), and obtained DMEC, HUVEC, BAEC, and ABCE cultures. Cultures were stimulated with cytokines or LPS for 4 h as described for the EC lines above. As with the SVEC4-10 and TME-3 lines, TNF α , IL-1 β , and LPS, but not IFN γ induced the upregulation of HA expression on both microvascular cultures, LNPEC and DMEC. In contrast, the large vessel-derived HUVEC, BAEC, and ABCE (Fig. 4) were unaffected by these treatments. In addition, this regulation occurred over a similar time course to continuous cell lines (data not shown). Thus, in all cells tested, sources of microvascular primary endothelial but not large vessel endothelial cells respond to proinflammatory stimuli with increased surface HA expression.

Changes in surface HA levels result in increased adhesive interactions under nonstatic conditions. We have suggested that local changes in endothelial cell HA expression represent

a regulatory point that can result in adhesion and ultimately extravasation of activated or effector T cells. To determine if the increased level of HA found on SVEC4-10 cells in response to proinflammatory stimuli had a measurable impact on lymphocyte/EC interactions, treated cells were compared with control EC for their ability to sustain CD44 dependent primary adhesion under conditions of shear stress using two types of adhesion assays. In a nonstatic (rotation) adhesion assay, we used the T cell line BW5147, which we have shown to constitutively express the HA binding form of CD44 (4), and measured the number of BW5147 cells bound onto treated versus untreated SVEC4-10 monolayers (Fig. 5 A). Consistent with our previous results, BW5147 cells bound to some extent to SVEC4-10 monolayers even in the absence of cytokine treatment, reflecting the baseline level of HA expressed by these cells. After 4-h culture with TNF α , IL-1 β , or LPS, the change in level of surface HA was reflected in a three- to five-

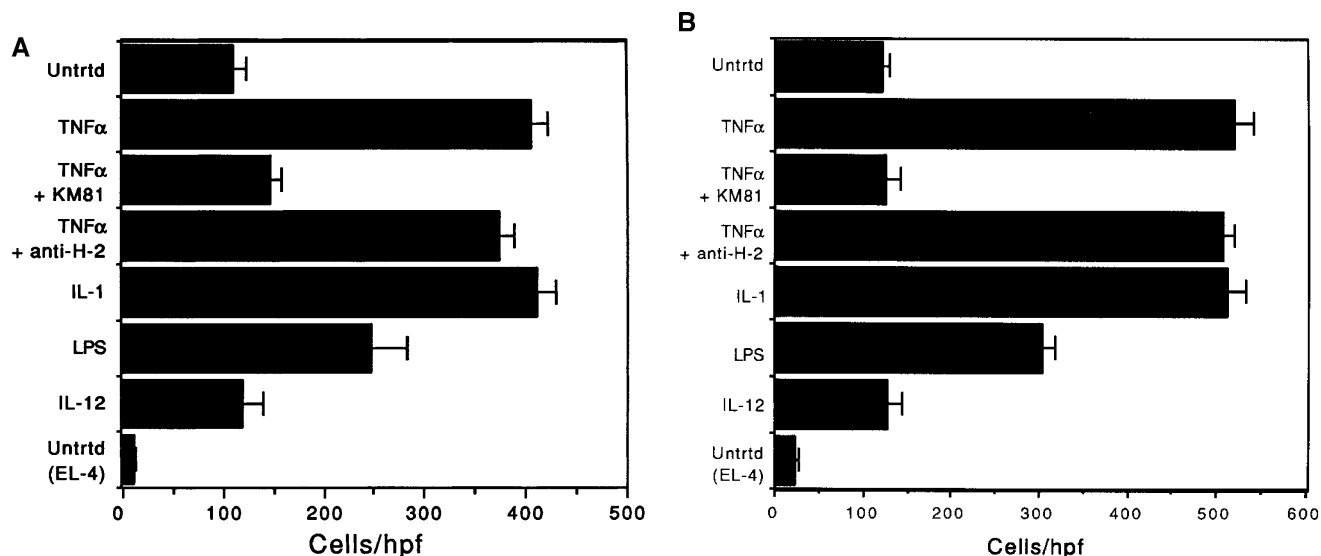


Figure 5. Effect of cytokine or LPS treatment on CD44 mediated adhesion under rotational shear force. (A) Adhesion of BW5147 cells to SVEC4-10 monolayers under shear with or without 4 h treatment with cytokine or LPS, as indicated. For blocking of adhesion, TNF α treated EC were incubated with medium containing either KM81 anti-CD44 or anti-H-2 mAb. Bound cells were counted microscopically in five randomly chosen fields of each well; five wells were counted for each condition. Background adhesion was determined using the CD44 negative cell line, EL4. (B) Adhesion of BW5147 cells to primary LN EC monolayers. Experiments were performed as described in A.

fold increase in binding of BW5147 cells. As with bPG staining, IL-12 treatment of EC did not increase the level of cell attachment. Inclusion of the HA-blocking anti-CD44 mAb, KM81, blocked $\sim 80\%$ of the increased binding of BW5147 cells to TNF α treated SVEC4-10 (Fig. 5 A), indicating the CD44 dependence of the interaction, while the isotype matched control anti-H-2 had no effect. Although the bulk of binding is clearly CD44 dependent, the residual binding after EC treatment may result from the induction on EC of additional adhesion molecules that interact with complementary ligands on BW5147 cells, giving rise to some CD44-independent adhesion. Primary lymph node EC also showed increased binding of BW5147 cells comparable to SVEC4-10 after TNF α , IL-1 β , or LPS treatment (Fig. 5 B) and binding was again inhibitable by KM81. Thus, under nonstatic conditions, interactions mediated by CD44 and HA are supported and regulatable by the degree of HA expression on EC.

Changes in surface HA levels result in increased primary adhesion interactions under conditions of laminar flow. We evaluated the functional consequences of elevated HA expression on interactions of EC with lymphoid cells under laminar flow in several ways. First, rates of tethering, representing the initial contact made with endothelium, of BW5147 cells on treated versus untreated EC were determined. Second, we assessed changes in the number of lymphoid cells engaging in rolling interactions on treated EC. Finally, we compared the velocity distributions and mean velocities of rolling cells on treated and untreated EC.

SVEC4-10 cells were used in a parallel plate adhesion assay after 24-h treatment with TNF α when both complete confluence and maximal HA expression of the monolayer was assured. To obtain a measure of the efficiency with which initial contact (tethering) occurred under conditions of flow, the level of tethering cells per mm 2 was determined and found to be significantly higher on TNF α treated EC at comparable shear

stresses than seen on untreated cells ($P < .005$, Fig. 6 A). Significantly, in the physiological ranges of 2.0 dyn/cm 2 and greater, rates were two- to threefold higher. Attachment of cells was always followed by rolling. Thus, increased expression of HA has a marked influence on the efficiency of initial cell contact under shear stress.

As shown in Fig. 6 B, the number of BW5147 lymphoid cells rolling after treatment of SVEC4-10 with TNF α was almost fourfold greater than that seen on untreated cells. Rolling was inhibitable both with blocking anti-CD44 antibody and with soluble HA, indicating the CD44/HA dependence of this interaction. An isotype matched control antibody had no effect on rolling interactions. The remaining small amount of non-CD44 mediated interaction is also much reduced compared with that seen in the less discriminating rotational assay. Thus, levels of endothelial expression of HA can have direct consequences for the efficiency of primary rolling interactions mediated by CD44.

The velocities and shear stress responses of the CD44-dependent interactions we have previously described between lymphoid cells and endothelial lines (4) are in the same range as those observed between the selectin family of adhesion molecules and their endothelial carbohydrate ligands (33, 36–40). Therefore, it was of interest to determine whether increased HA expression levels were additionally reflected in altered rolling velocities of BW cells as a result of more avid primary adhesion interactions. The average velocity of BW cells rolling on TNF α treated SVEC4-10 cells was 7 μ m/s, a 67% decrease in velocity relative to BW rolling on untreated SVEC4-10 (21 μ m/s). A representative distribution of velocities within a population of 100 cells was determined and is shown in Fig. 6 C, where a dramatic downward shift in the range of velocities is readily apparent. On untreated SVEC4-10, only 23% of the BW5147 cells roll at velocities ≤ 10 μ m/s, compared with 81% of cells rolling at ≤ 10 μ m/s on TNF α treated EC. Although

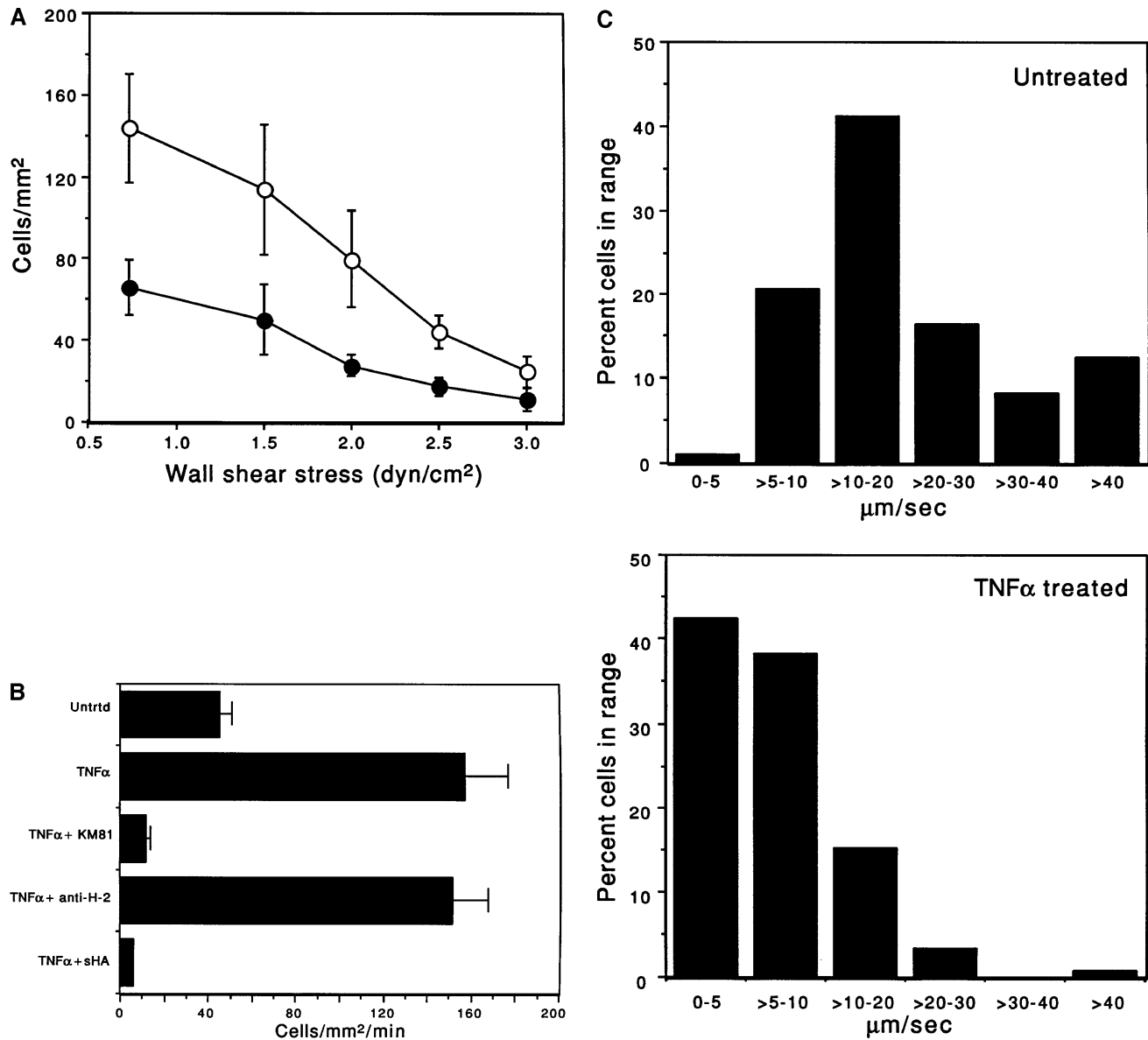


Figure 6. Characteristics of the rolling interaction of BW5147 on TNF α treated and untreated SVEC4-10 monolayers. (A) Initial attachment (tethering) of BW5147 cells at varying wall shear stress (WSS). BW5147 cells were applied to feed solution already equilibrated under flow at each WSS, which was incrementally adjusted by decreasing the outlet pump speed. The number of cells attaching to the SVEC4-10 monolayer during the first 30 s for each shear force was scored and are reported as the number of tethering cells per mm². Closed circles, untreated SVEC4-10; open circles, TNF α treated SVEC4-10. $P < .005$ at all WSS. (B) BW5147 cells were applied to feed solution already equilibrated under flow at a WSS of 2.0 dyn/cm² and perfused over EC monolayers in the parallel plate flow assay. Rolling was analyzed as described and the number of cells/mm² per minute rolling across each monolayer was determined. For blocking, KM81 anti-CD44, anti-H-2, or soluble HA (sHA) was added to the BW5147 cell suspension before their introduction in the flow system. (C) Velocity distribution of rolling BW5147 cells at a wall shear stress of 2.0 dyn/cm². The frequency of cells within each velocity range was calculated as a percent of the total number of cells evaluated (100 from two separate experiments for each experimental condition).

rolling velocities were markedly decreased after treatment, no significant conversion of this primary adhesion interaction to firm adhesion was noted with this cell line. The increased tethering rates, decreased average velocity, and downward shift in velocity distribution suggest that changes in levels of HA on EC do in fact influence the efficiency of CD44 mediated attachment and primary lymphocyte adhesion, and suggests that elevated HA expression in inflamed vascular beds potentially

could enhance and prolong leukocyte interactions with the vessel wall at such sites.

Changes in the expression of HA by EC do not appear regulated by HA synthase mRNA levels or by mRNA levels of described HA degradative enzymes. The major pathway for HA synthesis uses the enzyme hyaluronate synthase (HAS) (41). To determine whether changes in cell surface HA is dependent on simple modulation of HA synthesis in EC via message

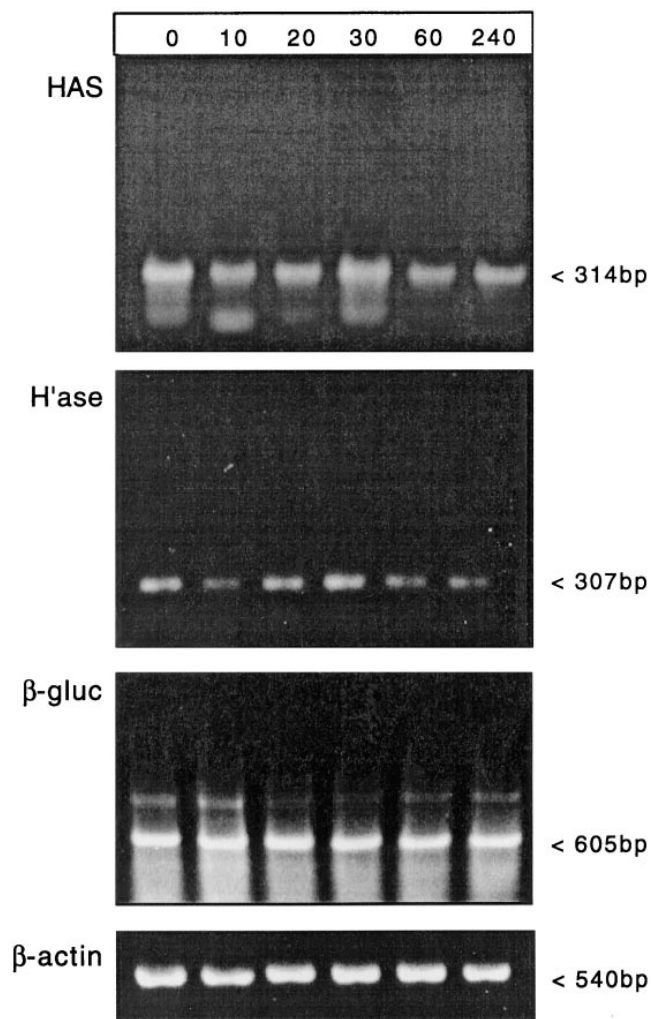


Figure 7. RT-PCR amplification of RNA from SVEC4-10 cells treated with TNF α for 0, 10, 20, 30, 60, or 240 min. Amplification was done with primers specific for murine hyaluronate synthase (*HAS*), hyaluronidase (*H'ase*), and β -D-glucuronidase (*β -gluc*), as shown. No differences in *HAS*, hyaluronidase, or β -D-glucuronidase PCR products are seen following treatment with TNF α . Cycling conditions were 95°C/60 s, 47°C/90 s, 72°C/120 s for *HAS* and 95°C/60 s, 55°C/90 s, 72°C/120 s for hyaluronidase and β -glucuronidase. Amplification of β -actin RNA under the same conditions from the same samples was carried out for each reaction set. For comparison, results from amplification reactions with β -actin primers run in parallel with β -D-glucuronidase are shown in the lower panel.

levels of this enzyme, we stimulated SVEC4-10 cells with TNF α and analyzed for changes in RNA levels using RT-PCR. Fig. 7 shows the results of SVEC4-10 stimulation with TNF α at various time increments, from 10 min to 4 h. No significant changes in the level of *HAS* product are detected at any time point. Additionally, no changes were seen at any time point when cells were treated with IL1 β or LPS (data not shown). Thus, changes in the level of message for the central enzyme in the HA biosynthetic pathway do not appear responsible for increased HA expression on EC.

Since the increase in detectable HA could also be due to a decrease in degradation, rather than an increase in de novo

synthesis, treated cells were also evaluated for changes in mRNA levels for a number of HA degradative enzymes. The enzymatic degradation of hyaluronan in mammalian tissue results primarily from the combined action of three enzymes: hyaluronidase, β -D-glucuronidase, and β -N-acetyl-D-hexosaminidase, although the relative contribution by each is not well characterized (42). Of the three, hyaluronidase has the greatest affinity for intact hyaluronan and most likely initiates degradation of HA. While RNA for hyaluronidase and β -D-glucuronidase could be detected within SVEC4-10, no changes were detected in RNA for either enzyme over the 4-h time course of treatment with TNF α (Fig. 7), IL1 β , or LPS (data not shown). In these same preparations, β -N-acetyl-D-hexosaminidase α and β chains were not detectable in SVEC4-10 by PCR either before or after treatment with TNF α , IL1 β , or LPS, although amplification was robust in control liver mRNA (data not shown). Thus, changes in the level of mRNA for HA degradation enzymes also do not appear responsible for increased HA expression. Together, the data suggest that if the mechanism of HA regulation in EC is due to biosynthesis or degradation of HA, it does not appear to be closely related to the levels of mRNA of the major known synthetic and degradative enzymes. Thus, regulation could be more complex than changes in HA biosynthesis or degradation.

Discussion

The regulated expression of cell adhesion molecules on endothelial cells in response to microenvironmental changes is pivotal to the recruitment of leukocytes, and hence, the evolution of inflammatory responses. A variety of adhesion receptors belonging either to the immunoglobulin gene superfamily or selectin family have been described to be regulated on EC by cytokines or other inflammatory stimuli (43, 44). For example, E-selectin expression is limited to endothelium stimulated with such inflammatory agents as TNF α , IL-1, or bacterial LPS (45). VCAM-1 can be induced by IL-1, TNF α , and IL-4 (46, 47), and levels of ICAM-1 have also been shown to be influenced by cytokines on a variety of EC (48–51). Thus, proinflammatory stimuli exert some of their effects by regulating the adhesion and thus recruitment of leukocytes to inflamed sites, and this provides a commonly exploited theme for the induction of endothelial adhesion molecule expression.

While investigations thus far have centered on the inducibility of endothelial cell surface glycoproteins, our demonstration that activated CD44 can mediate primary adhesion on its carbohydrate ligand HA in analogous fashion to selectins adds the glycosaminoglycan hyaluronan as a potential new target for regulation. Our proposal that the CD44/HA interaction participates in lymphocyte localization at inflamed sites has certain implied characteristics: (a) T cell sensitization to antigen results in the induction of the activated HA-binding form of CD44; (b) activation is followed by mobilization of the stimulated cells bearing activated CD44 into the peripheral circulation; (c) activated CD44 on these cells subsequently facilitates entry into inflammatory sites; and (d) HA expression levels are regulated on the endothelium of appropriate vascular beds to permit such entry. We have recently demonstrated that the activated form of CD44 on T lymphocytes is an early activation marker induced directly by T cell receptor triggering (4, 7). Additionally, in an in vivo model of activated T cell extravasation, entry into an inflamed site is both CD44 and HA

dependent (11). Particularly considering the very wide distribution of both CD44 and HA throughout the organism, the need for stringent regulation of CD44/HA interactions might well be anticipated. The studies presented here establish that HA surface levels can also be modulated by proinflammatory stimuli *in vitro*, supporting the notion that such regulation may have direct relevance *in vivo* as well.

The proinflammatory cytokines TNF α and IL-1 β , and the bacterial cell wall component LPS, generally associated with increasing endothelial adhesion receptor expression, all induced elevated HA cell surface expression. These observations are similar to findings of Carley et al. (52), who demonstrated increased HA secretion by human synovial endothelial cells in response to IL-1 α and acidic fibroblast growth factor, but not TNF α or IFN γ . The pace of the upregulation of HA is of further interest, as the time required for induction and the duration of expression of adhesion receptors bears a relationship to the contributions made to the acute and chronic phases of responses, and to the composition of the infiltrate recruited to the site (48, 53). With all stimuli tested, appreciable HA expression was not induced until about 4 h of stimulation, and the duration of this expression appears quite long, extending at least 72 h in the continual presence of these stimuli. This time course is comparable to the induced expression of VCAM-1, which is frequently associated with chronic inflammation, and has been shown to be induced for 72 h, and in human dermal microvascular endothelium perhaps longer (48, 51). Enhanced ICAM-1 expression has also been shown to persist for 72 h (49, 51, 54). Thus, the duration of elevated HA expression is prolonged in a manner similar to other adhesion receptors associated with chronic inflammatory states.

To maintain homeostasis, it would be expected that return of a vascular bed to a noninflamed state would result in resetting of HA expression levels back to baseline. Thus, it is of further note that elevated HA expression is dependent on the continual presence of the inflammatory stimulus (Fig. 2 D). The relatively rapid return to baseline is further consistent with the known rather surprisingly high turnover rate of HA normally (55, 56). Indeed, the need for dynamic modulation at endothelial surfaces may help to explain the requirements for such rapid turnover. In addition, stimuli such as thrombin and histamine which induce rapid mobilization of P-selectin had no effect on the expression of endothelial HA (data not shown). The temporal features of this regulation therefore seem quite consistent with a role for the CD44/HA interaction in chronic inflammatory states where regulation is modified by local microenvironmental conditions.

By all of the criteria examined, increased HA expression resulted in enhanced interactions with lymphoid cells (Fig. 6), suggesting that initial contact, and therefore recruitment at the endothelial–blood interface, as well as subsequent rolling interactions, permitting more sustained sampling of the microenvironment, would potentially result from such increased expression. It should be noted that although rolling interactions were markedly enhanced by increased HA expression, these interactions were not converted to firm adhesion as a result. These observations are consistent with the CD44/HA interaction serving primarily as a mediator of tethering and rolling, as do selectins. It is clear that secondary adhesion must follow for extravasation to occur, and the molecule(s) acting in conjunction with CD44 to effect this remain to be defined. However, our recent demonstration that T cell extravasation into an in-

flamed site *in vivo* is in fact dependent on CD44 interactions with HA implies such mechanisms do exist (11).

It has been clear that large vessel endothelium, particularly HUVEC, frequently used as well-characterized general model systems for investigating endothelial cell biology (43, 57), may differ markedly phenotypically and functionally from small vessel endothelium. Moreover, it is small vessel endothelium, primarily postcapillary venules, which is the principal location at which recruitment of circulating leukocytes occurs during inflammation. We have shown in these studies that SV40 transformed microvascular endothelial lines from lymph node, and primary cultures from murine lymph nodes and of human dermal microvascular endothelium respond to proinflammatory stimuli with increased HA expression. However, none of several endothelia derived from large vessels, nor two lung capillary endothelial cell lines, were induced to elevate surface HA expression. Therefore the pattern suggests that the ability to regulate HA levels may be confined primarily to small venular types of endothelium. In addition, this evidence is consistent with a previous report demonstrating inducibility of HA secretion by another microvascular endothelial cell type, human synovial endothelium (52). These observations are particularly striking given the ample evidence demonstrating modulation of glycoprotein adhesion receptors using large vessel sources. Thus, regulation preferentially at sites of small venular endothelium would be further consistent with a role in leukocyte recruitment to inflammatory sites.

Some endothelial adhesion molecules, for example, ICAM-1 and VCAM-1, are constitutively expressed at low levels and dramatically enhanced on activation. The situation is less clear for HA. Endothelial cells clearly can produce cell surface HA in culture, and CD44/HA interactions have previously been shown to mediate static binding of lymphocytes to cultured endothelial cell lines in both mouse and human (25, 58–60). However, the expression of HA on normal endothelium *in vivo* has been less extensively examined, although it has been described in chronic psoriatic lesions in the human (26). Consistent with this, we have observed in some murine models that only under conditions of chronic inflammation do we see significant endothelial HA expression (unpublished observations). Further investigations will be required to determine whether *in vivo* HA is regulated in an on/off fashion as are other adhesion receptors such as the endothelial selectins, or whether constitutive basal levels are normally expressed.

Alterations in the level of expression of HA either within tissues or in body fluids has been frequently associated with inflammatory and degenerative arthropathies and other chronic inflammatory conditions in humans (61–66) and in animal models (67, 68). The relationship of the association of elevated circulating HA levels in autoimmune states to the findings presented here bears further investigation, but it is attractive to speculate that this may in part derive from elevated HA expression and production on endothelial surfaces at chronically inflamed sites, and could potentially be involved in the regulation of adhesion interactions *in vivo*.

Questions remain as to the biochemical basis for changes in HA expression by cytokine-stimulated EC. While the relative contributions of each enzyme to HA catabolism has not been clarified, the high K_m of hyaluronidase for hyaluronan makes it the best candidate for initial attack. In our studies, by RT-PCR analysis, we find no detectable changes in message levels of the synthetic or degradative enzymes to explain surface HA ex-

pression. Of course, activity of these enzymes could be regulated other than transcriptionally, e.g., translational events such as enzyme modification by phosphorylation or glycosylation could regulate enzyme activity, or the enzymes could be particularly stable proteins or otherwise stored in inactive form. It is also possible that other uncharacterized synthetic or degradative enzymes are involved. Hence, the regulation of HA may lie at the level of rates of transcription or in more subtle mechanisms relating to enzyme stability and activation, chain elongation and retention, or changes in the presenting core polypeptide.

In summary, we have begun to systematically address the endothelial side of a model of CD44-mediated lymphocyte extravasation, and have shown that HA expression on endothelial cells can be upregulated by proinflammatory stimuli, that this occurs over an appropriate interval and can be sustained for a long enough period to enable the recruitment of cells to a chronically inflamed site, and that such changes result in enhanced adhesion interactions. These observations further support a role for these interactions in chronic inflammatory and autoimmune conditions and provide new impetus for the understanding of the mechanism of HA regulation and the role of the CD44/HA interaction in chronic inflammation and autoimmunity.

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