Angiogenesis mediated by soluble forms of E-selectin and vascular cell adhesion molecule-1

Alisa E. Koch*†, Margaret M. Halloran*, Catherine J. Haskell*, Manisha R. Shah* & Peter J. Polverini‡

* Department of Medicine, Section of Arthritis and Connective Tissue Diseases, Northwestern University Medical School, 303 E. Chicago Avenue, Chicago, Illinois 60611, USA
† The Veteran's Administration Lakeside Medical Center, 333 E. Huron Street, Chicago, Illinois 60611, USA
‡ The Laboratory of Molecular Pathogenesis of the University of Michigan Dental School, Ann Arbor, Michigan 48109, USA

ENDOTHELIAL adhesion molecules facilitate the entry of leukocytes into inflamed tissues. This in turn promotes neovascularization, a process central to the progression of rheumatoid arthritis, tumour growth and wound repair[1]. Here we test the hypothesis that soluble endothelial adhesion molecules promote angiogenesis[2–4]. Human recombinant soluble E-selectin and soluble vascular cell adhesion molecule-1 induced chemotaxis of human endothelial cells *in vitro* and were angiogenic in rat cornea. Soluble E-selectin acted on endothelial cells in part through a sialyl Lewis-X-dependent mechanism, while soluble vascular cell adhesion molecule-1 acted on endothelial cells in part through a very late antigen (VLA)-4 dependent mechanism. The chemotactic activity of rheumatoid synovial fluid for endothelial cells, and also its angiogenic activity, were blocked by antibodies to either soluble E-selectin or soluble vascular cell adhesion molecule-1. These results suggest a novel function for soluble endothelial adhesion molecules as mediators of angiogenesis.

First, we showed that recombinant human soluble E-selectin stimulated chemotaxis of human umbilical vein endothelial cells (HUVECs) (Fig. 1*a*) and human dermal microvascular endothelial cells (HMVECs) (Fig. 1*b*). Soluble E-selectin was potently chemotactic for HUVECs in the picomolar range, with 0.01 pM soluble E-selectin inducing chemotaxis equivalent to 60 nM control angiogenic cytokine basic fibroblast growth factor (bFGF). Recombinant human soluble vascular-cell-adhesion molecule-1 (soluble VCAM-1) was also chemotactic, with 1 nM soluble VCAM-1 inducing HUVEC chemotaxis equivalent to 60 nM bFGF (Fig. 1*c*). Similar results were obtained with HMVECs (Fig. 1*d*), indicating that these effects were not restricted to HUVECs. Checkerboard analysis, incorporating varying concentrations of chemoattractant in the upper and lower chemotaxis chambers, indicated that the effects of soluble

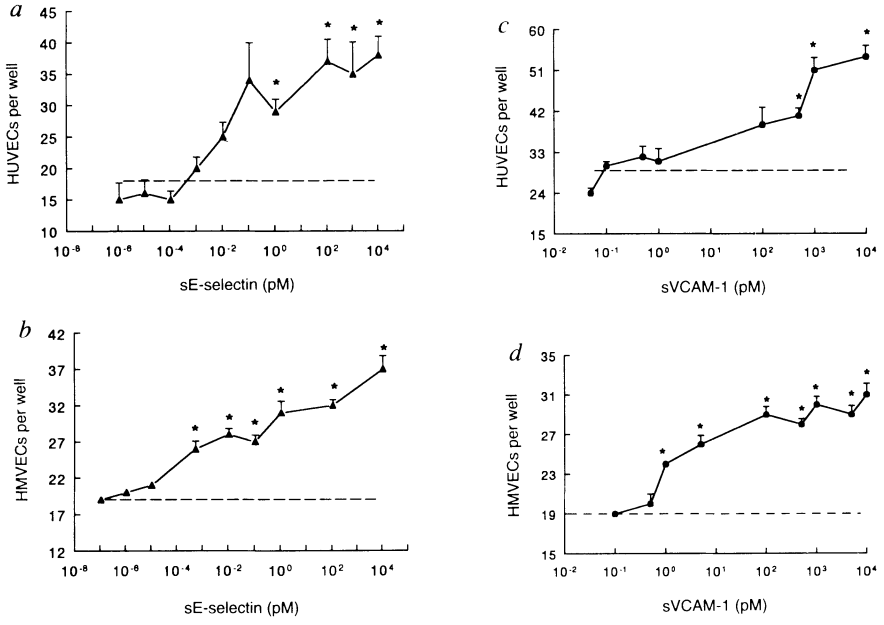

FIG. 1 Chemotaxis of endothelial cells. Chemotaxis of HUVECs or HMVECs (Clonetics, San Diego) was performed in 48-well blind-well chemotaxis chambers with polycarbonate membranes of 8 μm pore size (Neuroprobe, Cabinjohn, MD)[5,17]. HUVECs (2.5×10^4 cells per well) or HMVECs (3.75×10^4 cells per well) in 25 μl of Roswell Park Memorial Institute (RPMI) media containing +0.1% fetal calf serum were added to the bottom wells of the chambers. Inverted chambers were incubated at 37 °C for 2 h, allowing endothelial cell attachment. The following were added to the top half of reinverted chambers: PBS with or without recombinant human soluble E-selectin (sE-selectin) (*a*, *b*), or soluble VCAM-1 (s-VCAM-1) (*c*, *d*) (Biogen, Cambridge, MA), or bFGF (60 nM) (R&D Systems, Minneapolis). The sE-selectin was biologically active, functioning as an adhesion molecule[2]. This binding was blocked by EDTA (R. Lobb, personal communication). After incubation for 2 h, the membranes were removed, fixed in methanol and stained with Diff-Quik (Baxter Diagnostics, Chicago). Each test group was assayed in quadruplicate. Three high-power microscope fields were counted in each replicate well and results were expressed as cells per well. Statistical analysis was done with an unpaired Student's *t*-test without correcting for multiple comparisons. Values significantly different ($P<0.05$) from the PBS control (horizontal broken line) are indicated by stars. Chemotaxis in response to bFGF was: *a*, 25±2 cells per well (±s.e.m.); *b*, 37±2; *c*, 48±1; *d*, 31±1. Endotoxin concentrations in the soluble adhesion preparations did not exceed 0.04 EU mg^{-1} in the limulus amebocyte lysate assay (Associates of Cape Cod, Woods Hole, MA). PBS controls containing up to 0.4 EU mg^{-1} endotoxin did not affect the assay. Results represent 1 of at least 3 experiments. *e*, *f*, The results of representative checkerboard analyses using HUVECs and varying concentrations of sVCAM-1 or sE-selectin in the upper or lower compartment of the chemotaxis chamber[20]. Both sE-selectin (*e*) and sVCAM-1 (*f*) induced chemotactic rather than chemokinetic responses. Results are expressed as the number of cells ±s.e.m. per replicate well.

e

	Upper			
Lower	0	0.5 pM	110 pM	1.1 nM
0	19.3 ± 0.2	23.0 ± 1.2	28.5 ± 1.1	27.3 ± 2.1
0.5 pM	19.3 ± 0.7	20.8 ± 1.2	22.5 ± 1.1	27.3 ± 1.4
110 pM	17.3 ± 0.2	22.0 ± 1.7	26.3 ± 0.8	25.0 ± 1.6
1.1 nM	16.5 ± 1.2	22.3 ± 1.5	24.3 ± 1.0	22.8 ± 0.4

f

	Upper			
Lower	0	100 pM	1 nM	5 nM
0	13.3 ± 0.7	16.0 ± 1.1	25.8 ± 1.6	29.0 ± 2.8
100 pM	13.0 ± 0.7	11.8 ± 0.5	21.0 ± 1.1	28.5 ± 1.7
1 nM	12.8 ± 1.2	14.0 ± 0.9	13.3 ± 1.1	27.5 ± 2.2
5 nM	11.7 ± 0.5	14.3 ± 0.7	12.5 ± 0.4	12.3 ± 0.2

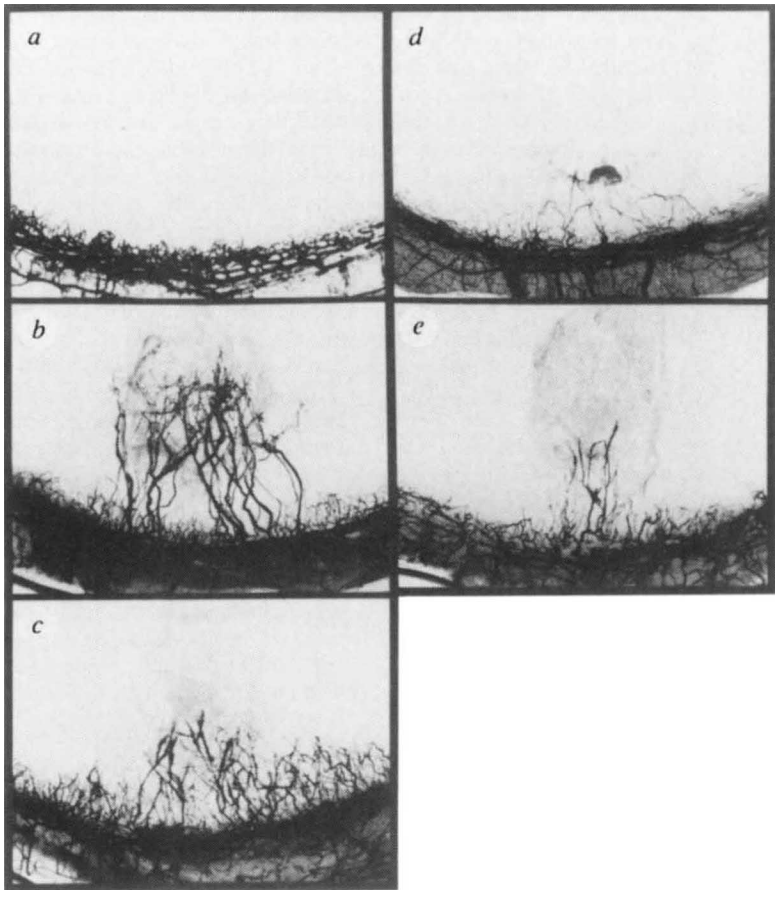

FIG. 2 *a*, Negative angiogenic response induced control PBS; *b*, positive angiogenic response induced by soluble E-selectin (10 nM); *c*, positive angiogenic response induced by soluble VCAM-1 (10 nM); *d*, minimal angiogenic response induced by rheumatoid arthritis (RA) synovial fluids (SFs) incubated with anti-E-selectin; *e*, markedly suppressed angiogenic response induced by RA SFs incubated with anti-VCAM-1.
METHODS. Test substances were combined 1:1 with Hydron (Interferon Sciences, New Brunswick, NJ) and implanted into the normally avascular corneal stroma of the rat several mm from the limbus[5,21]. Corneas were perfused with colloidal carbon after 7 days to provide a permanent record of the angiogenic response (33×). No cornea exhibited histological evidence of nonspecific inflammation. All human samples were obtained with Institutional Review Board approval. SFs were obtained from arthrocentesis of patients with RA. SFs were diluted 1:50 with PBS and incubated with either anti-E-selectin, anti-VCAM-1, or isotype-matched control monoclonal antibody (mAb) for 1 h at 37 °C. The mAbs used were: mAb BB11, which recognizes E-selectin; mAb 4B9, which recognizes VCAM-1 domain 1; and mAb GH12, which recognizes VCAM-1 domain 4 (refs 22, 23). All test mAbs were used at 10 μm ml^{-1} and obtained from Biogen (Cambridge, MA). Control mAbs (10 μl ml^{-1}) were obtained from Coulter Diagnostics (Hialeah, FL); mAbs 4B9 and GH12 were used simultaneously.

E-selectin or soluble VCAM-1 were chemotactic, not chemokinetic, for HUVECs or HMVECs (Fig. 1*e*, *f*). Neither soluble adhesion molecule (SAM) induced mitogenesis of HUVECs or HMVECs, suggesting that they mediated angiogenesis independently of stimulating endothelial cell proliferation (results not shown).

We next determined whether soluble E-selectin and soluble VCAM-1 were angiogenic *in vivo*. SAMs were incorporated into Hydron pellets and implated into rat corneas. Soluble E-selectin (10 nM) or soluble VCAM-1 (10 nM) induced an angiogenic response in 3 out of 4 and 4 out of 4 corneas, respectively (Fig. 2*a*–*c*).

To determine whether the SAMs were acting *in vivo* by inducing the release of angiogenic cytokines by HUVECs[5], these cells were incubated with either soluble E-selectin (up to 1.1 nM) or soluble VCAM-1 (up to 50 nM), at concentrations that met or exceeded those necessary to induce HUVEC chemotaxis *in vitro*. Treatment of HUVECs with these SAMs did not increase their production of interleukin-8 (IL-8), tumour necrosis factor-α (TNF-α), or bFGF, as measured by enzyme-linked immunosorbent assay (ELISA), indicating that they did not act as indirect angiogenic mediators (results not shown).

To investigate the mechanism of action of soluble E-selectin on endothelial cells, HUVECs were incubated with a monoclonal antibody to the E-selectin ligand, sialyl Lewis-X, which is expressed on a variety of cells, including endothelial cells[6,8] (Fig. 3). We detected the sialyl Lewis-X antigen on HUVECs by immunoperoxidase histochemistry (results not shown). In the presence of anti-sialyl Lewis-X, chemotaxis of HUVECs in response to soluble E-selectin was significantly reduced ($P<0.05$), indicating that soluble E-selectin induces HUVEC chemotaxis in part through binding endothelial sialyl Lewis-X. Similar experiments involved incubating HUVECs with monoclonal antibodies to the soluble VCAM-1 ligand VLA-4, which

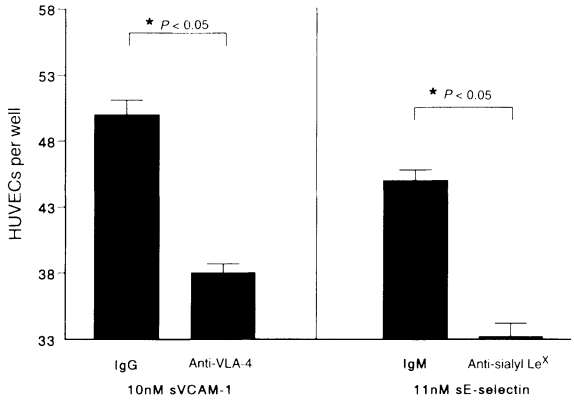

FIG. 3 Soluble E-selectin and soluble VCAM-1 mediate HUVEC chemotaxis by sialyl Lewis-X and VLA-4-dependent mechanisms, respectively.
METHODS. We determined whether HUVECs migrated in response to sE-selectin or sVCAM-1 when the ligands for these molecules on target HUVECs were blocked. RPMI (0.5 ml) containing 10% fetal calf serum and 10 μg ml^{-1} mAb or isotype-matched control mAb were placed in replicate wells of confluent HUVEC-containing 24-well plates. The mAbs used were: mAb CSLEX1, which recognizes sialyl Lewis-X (Le^X) (Becton Dickinson, San Jose, CA); and mAb HP 1/2, which recognizes the α chain of VLA-4 (Biogen)[24]. Isotype-matched control mAbs were obtained from Coulter Diagnostics. After incubation for 1 h at 37 °C in a humidified incubator gassed with 5% CO_2, HUVECs were collected and resuspended in RPMI containing 0.1% fetal calf serum and 10 μg ml^{-1} mAb, and chemotaxis was performed as described in Fig. 1. Results represent 1 of 3 experiments, each performed in quadruplicate. Additionally, in 3 experiments, concentrations of sE-selectin down to 1.1 nM or sVCAM-1 down to 5 pM showed similar results. Statistical analysis was done with an analysis of variance[25].

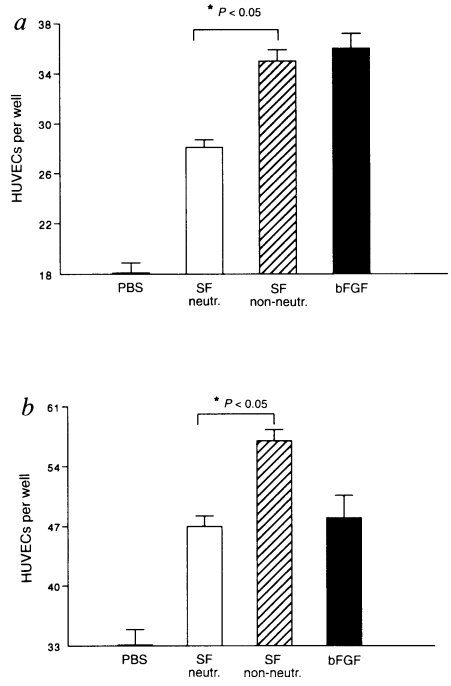

FIG. 4 Soluble E-selectin and soluble VCAM-1 account for a large portion of the RA SF chemotactic activity for HUVECs.
METHODS. RA SFs were obtained from 9 patients, diluted 1:50 with PBS and depleted of sE-selectin or sVCAM-1 as described in Fig. 2. Chemotaxis with SFs was done as described in Fig. 1. SF neutr., neutralized RA SFs; SF non-neutr., SFs incubated with isotype-matched control mAbs. Results represent the means±s.e.m. for 9 patients. Depleted SFs contained no detectable antigenic sE-selectin or sVCAM-1 as measured by ELISA (R&D Systems, Minneapolis) (results not shown). Anti-E-selectin (*a*) or anti-VCAM-1 (*b*) completely eliminated the chemotactic activity for HUVECs induced by 11 nM sE-selectin or 10 nM sVCAM-1, respectively. None of the mAbs had any effect on bFGF-induced HUVEC chemotaxis.

is also expressed on endothelial cells and was detected on HUVECs by immunoperoxidase histochemistry (results not shown)[9]. Incubation of HUVECs with anti-VLA-4 significantly diminished ($P<0.05$) the chemotactic activity for HUVECs in response to soluble VCAM-1, indicating that soluble VCAM-1 was acting on HUVECs to induce their chemotaxis in part by a VLA-4-dependent mechanism (Fig. 3).

We then determined whether SAMs contributed to the angiogenic activity of rheumatoid synovial fluid. Incubation with anti-E-selectin or anti-VCAM-1 significantly attenuated the rheumatoid synovial fluid chemotactic activity for HUVECs (Fig. 4*a*, *b*). Rheumatoid synovial fluid (5 μg protein) was potently angiogenic in all 3 corneas examined. Neutralization of soluble E-selectin or soluble VCAM-1 diminished or completely abolished the angiogenic response (1 out of 11 positive corneas (Fig. 2*d*), and 2 out of 14 positive corneas and 3 out of 14 more or less weakly positive corneas (Fig. 2*e*), respectively). Incubation with control isotype-matched monoclonal antibody had no effect on the angiogenic activity present in the synovial fluid (6 out of 6 positive corneas). These results indicate that soluble E-selectin and soluble VCAM-1 account for a large portion of the angiogenic activity found in rheumatoid synovial fluids.

Cellular adhesion and angiogenesis, often regarded as separate processes, may be linked. We have shown that the β3 integrin subunit is expressed in rheumatoid arthritis angiogenesis-rich synovial tissue, but virtually absent in normal angiogenesis-deficient synovial tissue[10]. Interestingly, the adhesion molecule αvβ3 integrin has recently been identified as a marker of angiogenic vascular tissue in wound granulation tissue[11]. Paradoxically, addition of anti-αvβ3 promoted tube formation in fibrin gels and inhibited endothelial cell proliferation on a fibrinogen matrix[12]. Anti-α2β1 integrin enhanced the number of capillary tubes formed by HUVECs *in vitro*[12]. Similarly, sialyl Lewis-X/A has been implicated in bovine capillary morphogenesis *in vitro*[13].

It has been shown that E-selectin and VCAM-1 are upregulated both in rheumatoid synovial fluid *in situ* and in soluble form in synovial fluid from rheumatoid arthritis compared with osteoarthritis[4,14–16]. Despite the increased quantities of SAMs in angiogenic disease states, their main known functions have included mediating cellular adhesion when immobilized to plastic, and, for soluble E-selectin, recruiting neutrophils *in vitro*[2,3]. We have shown that soluble E-selectin and soluble VCAM-1 are angiogenic in the 10 nM range in the cornea. This compares with amounts reported for the induction of angiogenesis by IL-8, TNF-α, aFGF and bFGF, angiotropin, angiogenin and vascular endothelial growth factor[5,17–19]. The studies described here suggest a proangiogenic role for soluble E-selectin and soluble VCAM-1. It is possible that when leukocytes bind to endothelial cells with concomitant release of cytokines, these molecules expressed on endothelial cells are then shed. The shed molecules in turn bind adjacent endothelial cells via their respective ligands, sialyl Lewis-X and VLA-4, exert a direct angiogenic effect on these endothelial cells and mediate inflammation. Our results therefore demonstrate a link between cellular adhesion and angiogenesis, and suggest a novel function for soluble E-selectin and soluble VCAM-1 in angiogenesis. □

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