Angiogenic Activity of Human Soluble Intercellular Adhesion Molecule-1

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Abstract

Serum levels of soluble intercellular adhesion molecule-1 (sICAM-1) are elevated in a number of pathological conditions associated with angiogenesis, including tumor growth. Because the increased levels of sICAM-1 suggested that it may be angiogenic, we tested the ability of sICAM-1 to promote angiogenesis. Human recombinant sICAM-1 stimulates chemokinetic endothelial cell migration, endothelial cell tube formation on Matrigel, and sprouting of aortic rings. sICAM-1 also mediates angiogenesis in the chik chorioallantoic membrane assay. Additionally, we found a Mr 49,000 molecule that binds to sICAM-1 that may be the surface ligand on endothelial cells. The evidence that sICAM-1 has angiogenic activity suggests a possible role linking inflammation and neovascularization. Furthermore, sICAM-1 may enhance tumor growth by promoting angiogenesis and escape from immunosurveillance.

Introduction

Although well-regulated angiogenesis is critical in a number of physiological processes, abnormally regulated angiogenesis plays a prominent role in the development and progression of a variety of pathological processes including tumor growth, rheumatoid arthritis, and diabetic retinopathy (1, 2). In many of these and other angiogenesis-associated diseases, increased levels of cytokines, inflammatory cells, and angiogenic factors are present. In turn, these factors increase the expression of endothelial CAMs. CAMs mediate cellular interactions with other cells as well as with extracellular matrix proteins. They play an important role in regulating both the migration of WBCs into inflamed tissues and interactions between T cells and antigen-presenting cells. ICAM-1, a member of the immunoglobulin superfamily, is present on resting endothelial cells. However, its expression on activated endothelial cells and on various other cell types is markedly induced during inflammation. ICAM-1 mediates binding to the integrin lymphocyte function-associated antigen present on leukocytes and is particularly important for the attachment and subsequent transendothelial migration of leukocytes to sites of inflammation. The migration and activation of leukocytes and other inflammatory cells can consequently initiate angiogenesis (3).

In addition to the membrane forms, CAMs can be shed from the cell surface and circulate in the blood. Serum levels of many CAMs, including sICAM-1, are elevated in inflammation, infection, and cancers, although their pathological significance has yet to be determined. sICAM-1 levels are elevated 2-fold in diabetes, 3-fold in septic shock, and 3–5-fold in metastatic cancer (4, 5). Although the physiological role of soluble CAMs is incompletely understood, previous reports have hypothesized that they promote angiogenesis (6). Recently, it was demonstrated that soluble forms of vascular cell adhesion molecule-1 and E-selectin mediated angiogenesis (7), and P-selectin was reported to induce migration of HUVECs (8).

Present theories indicate that in pathological states, the elevated levels of soluble CAMs may reach adhesion-blocking concentrations (9). Soluble CAMs can act as either agonists or antagonists and compete with membrane-bound forms in binding to their respective counter receptor. Elevated levels of sICAM-1 have been reported in several human malignancies (4, 5) and can be detected in the cultured medium of human prostatic cancer and melanoma lines in vitro (10, 11). Until now, investigators have proposed that sICAM-1 could either reflect an immune response to chronic inflammation and tissue destruction or may act as an active immunomodulator. Thus, the soluble CAMs may be used by certain tumors, for example, to bind to circulating cytotoxic lymphocytes, thereby helping tumors to escape immune recognition (4). We proposed that in addition to these functions, sICAM-1 may itself promote angiogenesis. Because angiogenesis involves cell migration, proliferation, and tube formation, we assayed for these activities to define a mechanism of action. Our findings demonstrate that sICAM-1 has angiogenic activities both in vitro and in vivo in a variety of assays.

Materials and Methods

Cell Culture. HUVECs were isolated from freshly delivered umbilical cords and grown to passages four to six (12). Human fibrosarcoma (HT1080) and immortalized HMEC (13) cells were grown in RPMI 1640 containing 10% fetal bovine serum.

Preparation of Immuno-depleted and Heat-inactivated sICAM-1. The purity of recombinant human sICAM-1 (R&D Systems, Minneapolis, MN) was checked by SDS-PAGE with GelCode Blue Staining (Pierce, Rockford, IL). There were only two protein bands with similar molecular weight when up to 10 μg of sICAM-1 were visualized. These two bands were also detected by Western blotting (data not shown). We used three different lots of sICAM-1 in all of the experiments.

A mixture of sICAM-1 (5 μg) and 20 μg of anti-ICAM-1 monoclonal antibody or control isotype-matched mouse IgG1 (R&D Systems) in 0.25 ml of RPMI 1640 containing 0.1% BSA was incubated for 2 h and then incubated with 1:1 slurry of protein G-agarose beads (0.2 ml) for 4 h. Then, the supernatants were reincubated with 20 μg of anti-ICAM-1 antibody or control IgG1 for 2 h, and 0.2 ml of protein G-agarose beads was added to the mixture. After overnight incubation, the supernatant was aliquoted and stored at −80°C until use. All experiments were carried out at 4°C. For heat inactivation, 10 μg of sICAM-1 (1 mg/ml in PBS) were boiled for 10 min, placed on ice, and then stored at −80°C until use.

Boyden Chamber Assay. Migration and checkerboard assays were performed as described previously (7) in a 48-well microchemotaxis chamber (Neuro Probe, Inc., Cabin John, MD). Polyester membranes with 10 μm pores (Neuro Probe, Inc.) were coated with 0.1 mg/ml of collagen IV (Trevigen, Gaithersburg, MD) in double-distilled water and then dried for 1 h. HUVECs and HT1080 cells were harvested using Versene (Life Technologies, Inc., Gaithersburg, MD) and resuspended in RPMI 1640 containing 0.1% BSA. The bottom chamber was loaded with 30,000 cells, and the filter was laid over the cells. The microchamber was then inverted and incubated at 37°C for 2 h. After reinserting the chamber to its upright position, the upper wells were then loaded with RPMI 1640 containing 0.1% BSA and sICAM-1. bFGF was added at a concentration of 5 ng/ml as a positive control. The chamber was then
reincubated at 37°C for 2 h, and the filters were fixed and stained using Diff-Quick (Baxter Healthcare Corp., McLean, IL). The cells that migrated through the filter were quantitated by counting the center of each well in 36-box grid at ×20 using an Olympic CK2 microscope. Each condition was studied in triplicate wells, and each experiment was performed three times.

Checkerboard assays were carried out as described above, except various amounts of sICAM-1 were placed in the top and/or bottom wells (see Table 1).

**Tube Assay.** The tube formation assay was performed as described previously (14). Plates (24-well) were coated with 320 μl of 1:1 mixture of RPMI 1640 and Matrigel (12 ng/ml; Collaborative Biomedical Products, Bedford, MA) and incubated at 37°C for 1 h to promote gelling. HUVECs (40,000) in 0.5 ml of serum-free RPMI 1640 medium with varying concentrations of sICAM-1 were added to each well. For a positive control, bFGF was added at a concentration of 5 ng/ml. All test samples were performed in triplicates. After a 6-h incubation, the plates were fixed with Diff-Quick, and images were transferred from the Hamamatsu Newvision camera to a computer via Universal Software Metamorph programming (Universal Imaging Corp., West Chester, PA) for NIH image analysis. Total pixels of three randomly chosen fields (×10) from each sample were analyzed by the NIH Image program. This assay was performed twice.

**Aortic Ring Assay.** As described previously (15), aortas were harvested from Sprague Dawley rats 6 weeks of age. Plates (48-well) were coated with 110 μl of Matrigel; after gelling, the rings were placed in the wells and sealed in place with an overlay of 40 μl of Matrigel. Various amounts of sICAM-1 were added to the wells in a final volume of 200 μl of human endothelial serum-free media (Life Technologies). As controls, medium alone and medium containing 200 μg/ml of ECGS (Collaborative Research) were assayed. Additional sICAM-1 and ECGS were added on day 3, and the assay was fixed and stained with Diff-Quick on days 5–7. Each data point was assayed in sextuplets, and each experiment was repeated three times. A blinded observer scored outgrowth by comparing responses with media alone (background levels) to that observed with the sICAM-1 levels and with ECGS (positive control). Results were scored from 0 (least positive) to 5 (most positive).

**CAM Assay.** The CAM assay was carried out to determine the in vivo angiogenic activity of sICAM-1 (16). Briefly, 5 μl of salt-free aqueous solution containing varying amounts of sICAM-1 or bFGF (10 μg/ml) were loaded onto a one-fourth piece of a 15-mm Thermonox disc (Nunc, Naperville, IL), and the sample was dried under sterile air. The disc was then applied to the CAM of an embryo 10 days of age. After 70 ± 2 h incubation, the negative or positive response was assessed under a microscope. Assays for each test sample were carried out twice, and each experiment contained 10–15 eggs/data point.

**Ligand Overlay and Affinity Chromatography.** Cell surface molecules of HUVECs and HT1080 cells (4 × 150-mm dishes) were biotinylated using sulfo-NHS-biotin (Pierce) according to the manufacturer’s instructions. A crude cell membrane fraction was prepared by hypotonic lysis in 20 mM Tris/HCl (pH 7.4), containing 10 mM KCl, 1 mM EDTA, and a protease inhibitor mixture (Boehringer Mannheim, Indianapolis, IN). After Dounce homogenization, the nuclei were removed by centrifugation (1500 × g for 5 min at 4°C). The NaCl concentration of the supernatant was increased to 150 mM, and the cell membranes were pelleted at 50,000 × g for 30 min at 4°C. The cell membrane pellet was resuspended in 2 ml of PBS containing 2% Triton X-100 (pH 7.4). After centrifugation at 14,000 × g for 20 min at 4°C, the supernatant was aliquoted and stored at −80°C until use.

Crude membrane extract (100 μg) was separated by SDS-PAGE (4–20% gels, nonreducing) and transferred to nitrocellulose filter (Novex, San Diego, CA). The filters were blocked in TBST (TBS [Tris-buffered saline] + 0.05% Tween 20) containing 3% BSA and incubated with biotinylated sICAM-1 (1 μg/ml) in TBS containing 1% BSA for 2 h at room temperature. After washing three times with TBS (5 min each), the filter was incubated with streptavidin-
We next investigated whether sICAM-1 has a mitogenic effect on endothelial cells using the 5-bromo-2′-deoxy-uridine and 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-based cell proliferation kit I (Boehringer Mannheim). The presence of sICAM-1 at concentrations up to 4 μg/ml did not show any effect on DNA synthesis and proliferation of HUVECs and immortalized HMECs (data not shown). These results indicate that sICAM-1 may not act as a mitogen on endothelial cells.

**sICAM-1 Stimulates Chemokinetic Migration of HUVECs.** We next determined whether sICAM-1 was stimulating migration by chemotaxis (directional migration) or chemokinesis (random motility). Checkerboard assays were performed with various concentrations of sICAM-1 in the top chamber, in the bottom chamber, or in both chambers. sICAM-1 stimulates the random movement of HUVECs (Table 1). These results indicate that sICAM-1 induces the migration of HUVECs in a chemokinetic, not a chemotactic, manner.

**sICAM-1 Stimulates Endothelial Cell Differentiation.** We next examined the ability of sICAM-1 to promote the formation of a capillary-like structure of HUVECs on basement membrane Matrigel. This assay measures some of the steps in angiogenesis including migration and differentiation (14). sICAM-1 stimulated tube formation of HUVECs in a dose-dependent manner (Fig. 2). The presence of sICAM-1 at 100 ng/ml and 1000 ng/ml showed a significant 1.5- and 1.8-fold increase in tube area ($P = 0.047$ and $P = 0.034$, respectively) over control containing medium alone. bFGF (5 ng/ml) stimulated only a 1.1-fold increase in tube area ($P = 0.013$) compared with that of baseline ($P = 0.047$ and $P = 0.034$).

**Results**

**sICAM-1 Stimulates HUVEC but not HT1080 Migration.** The migratory effect of sICAM-1 on HUVECs and HT1080 cells was tested using Boyden chambers. sICAM-1 significantly induced migration of HUVEC in a dose-dependent manner over migration in the presence of medium alone, whereas HT1080 migration did not exceed that of baseline (Fig. 1A). HUVEC migration stimulated by 10 ng/ml of sICAM-1 was similar to levels observed for the bFGF (5 ng/ml) positive control. sICAM-1 also induced the migration of immortalized HMECs with a response comparable with that observed with HUVECs (data not shown). As shown in Fig. 1B, immunodepleted sample did not induce the migration of endothelial cells. However, isotype-matched mouse IgG1-treated sICAM-1 still induced the migration. Heat treatment inactivated the ability of sICAM-1 to stimulate migration (Fig. 1C).

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angiogenesis from aortic rings was used to determine whether sICAM-1 stimulated angiogenic. sICAM-1 can promote tube formation, suggesting that it may also stimulate tube formation (2.2-fold increase; Columns, means of the percentage of eggs that showed positive response in two assays; 1,000 ng/egg).

Discs and surrounding CAMs were photographed. A, water; B, bFGF (50 ng/egg); C, sICAM-1 (100 ng/egg); D, sICAM-1 (1,000 ng/egg). Bottom, quantification of angiogenesis induced by bFGF or sICAM-1. Columns, means of the percentage of eggs that showed positive response in two assays; bars, SE. Each assay used 10–15 eggs. A positive response is indicated by a typical spokewheel pattern of new blood vessels around the loaded samples. Ps versus water control were as follows: bFGF (50 ng/egg), 0.034; sICAM-1 (100 ng/egg), 0.013; sICAM-1 (1000 ng/egg), 0.011.

sICAM-1 Promotes Vessel Sprouting. The sprouting of vessels from aortic rings was used to determine whether sICAM-1 stimulated angiogenesis *ex vivo*. sICAM-1 stimulated vessel sprouting above background levels at all doses but was significant at 750 ng/ml (2–3-fold increase; *P* = 0.015; Fig. 3). The observation that the most significant sprouting occurred at 750 ng/ml may correlate with serum levels of sICAM-1 in a number of pathological conditions (4, 5).

sICAM-1 Induces Angiogenesis *in Vivo*. Because sICAM-1 stimulated migration and differentiation of HUVECs and sprouting from explanted aorta rings *in vitro*, the ability of sICAM-1 to induce neovascularization was investigated in the chicken CAM assay. As shown in Fig. 4, 100 and 1000 ng of sICAM-1 per egg induced neovascularization in 48.4% (*P* = 0.013) and 55.8% (*P* = 0.011) of the eggs, respectively. These values are significantly higher than the value obtained with water alone (28.4% positive response). bFGF (50 ng/egg) also induced angiogenesis in 50.6% of tested eggs (*P* = 0.034). These results indicate that sICAM-1 is a potent angiogenic factor *in vivo*, and angiogenic activity observed with sICAM-1 is comparable with that of the potent angiogenic molecule bFGF.

Identification of a Putative sICAM-1 Binding Protein from the Endothelial Cell Surface. Our data demonstrate that sICAM-1 induces the migration of endothelial cells but not of HT1080 human fibrosarcoma cells. Because HUVECs do not express β2 integrin, which binds to ICAM-1 (17), other cell surface molecules may be involved in the induction of endothelial cell migration by sICAM-1. To identify these molecules, we carried out ligand overlay and affinity chromatography using surface-biotinylated membrane fractions. By ligand overlay, several bands were observed from membrane fractions of HUVECs (Fig. 5A, Lane 2). Four different molecules are the putative sICAM-1 binding protein, because the others were also identified by incubation in the absence of biotinylated sICAM-1 (Fig. 5A, Lane 3). Among them, only the M, 49,000 molecule was identified by sICAM-1 affinity chromatography (Fig. 5B, Lane 2). The M, 49,000 was also present in the eluate from sICAM-1 affinity beads and migrated similarly under nonreducing and reducing condition (data not shown). This molecule was not detected when excess sICAM-1 was added to the binding solution (Fig. 5B, Lane 3). In addition, the M, 49,000 band was not identified in the HT1080 cell surface molecules in both experiments (Fig. 5A, Lane 2, and Fig. 5B, Lane 5) as expected because sICAM-1 has no effect on the migration of these cells. These results indicate that the M, 49,000 protein is the
putative sICAM-1 binding protein, which is present on the endothelial cell surface.

Discussion

Because the circulating levels of CAMs are increased during the progression of many angiogenesis-dependent diseases, such as rheumatoid arthritis, diabetic retinopathy, and cancer (4, 5), our work focused on determining the angiogenic activity of sICAM-1. In light of recent reports that other soluble forms of CAMs mediate angiogenesis including vascular cell adhesion molecule-1 and E-selectin (7, 8), our results in this report further support the angiogenic activity of the cell adhesion molecules and indicate clearly that sICAM-1 mediates angiogenic activity in vitro and in vivo. Here, we demonstrate that sICAM-1 stimulates: (a) chemokinetic endothelial cell migration; (b) endothelial cell differentiation; (c) vessel sprouting from explanted aortic rings; and (d) neovascularization in the chick CAM assay. However, sICAM-1 does not act as an endothelial cell mitogen. Also, sICAM-1 does not support the adhesion of endothelial cells (data not shown). Thus, the chemokinetic, and not the mitogenic or adhesive, effect of sICAM-1 may be responsible for its angiogenic activity. Furthermore, we identified a putative sICAM-1 binding protein (Mr, 49,000) from the HUVEC endothelial cell surface. Interestingly, this molecule is not present on the surface of HT1080 cells in which sICAM-1 does not induce migration.

ICAM-1 has been identified on the surface of endothelial, epithelial, fibroblast-like, and many other tumor cells (4, 5). sICAM-1, shed from the cell membrane, is present in normal human plasma, and the mean level ranges from 10^2 to 450 ng/ml (4). sICAM-1 is produced by cultured endothelial, epithelial, and ICAM-1-positive tumor cells, including melanoma and prostatic carcinoma (4, 5, 10, 11). Many studies have reported that there is a close relationship between the elevated circulating sICAM-1 levels and the progression of malignancy, especially in metastatic cancers where sICAM-1 levels are elevated 3–5-fold (4, 5). The serum levels of human sICAM-1 from nude mice bearing human melanoma tumors show a positive correlation with tumor weight (18). On the other hand, ICAM-1-negative human tumor cells can up-regulate significantly the release of sICAM-1 by endothelial cells in culture via IL-1α (19). Therefore, our finding that sICAM-1 exerts angiogenic activity suggests that it may play an important role in tumor-induced angiogenesis, regardless of whether the tumor is ICAM-1 positive or negative.

For tumor growth and metastasis, escape from immune surveillance and angiogenesis are necessary (20). Many studies have reported that sICAM-1 plays an important role in tumors escaping from immune response (21–23). sICAM-1 binds to lymphocyte function-associated antigen and inhibits lymphocyte attachment to endothelial cells (21). Circulating sICAM-1 from human melanoma cells can block natural killer cell-mediated cytotoxicity (22) and MHC-restricted specific T cell-tumor interaction (23). Therefore, sICAM-1 may be one of the mechanisms by which tumor cells escape from immune surveillance. Our results show an additional activity of angiogenesis. Thus, sICAM-1 may perform dual functions that are essential for tumor growth and metastasis: escape from immunosurveillance and angiogenesis.

References

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