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Synergistic Effect of TNF-α in Soluble VCAM-1-Induced Angiogenesis Through α4 Integrins

Shintaro Nakao,* Takashi Kuwano,* Tatsuro Ishibashi,† Michihiko Kuwano,*† and Mayumi Ono2*†

In our present study we focused on soluble VCAM-1 (sVCAM-1)/α4 integrin-induced angiogenesis and found that this type of angiogenesis was mediated through p38 mitogen-activated protein kinase and focal adhesion kinase (FAK). HUVEC expressed both α4 and β1 integrins, and it was reported that expression of α4 integrin and its counterreceptor, sVCAM-1/VCAM-1, was enhanced in response to an inflammatory cytokine, TNF-α. In endothelial cells phosphorylation of p38 and FAK, but not that of extracellular signal-regulated kinase 1/2 was induced by sVCAM-1. Migration of endothelial cells was stimulated in response to sVCAM-1 at similar levels as those induced by vascular endothelial growth factor, and sVCAM-1-induced migration was almost completely blocked by neutralizing Ab against α4 integrin, either by an inhibitor of p38 (SB203580), or by adenovirus containing FAK-related nonkinase. sVCAM-1 also induced the formation of blood vessels in Matrigel plug assay in vivo, and this neovascularization was blocked by SB203580 or neutralizing Ab against α4 integrin. Moreover, we also confirmed that both TNF-α and sVCAM-1 could synergistically induce angiogenesis in the corneas of mice when each factor at used dose could not induce. This angiogenesis by TNF-α and sVCAM-1 was almost completely blocked by coadministration of SB203580 and also by neutralizing Ab against α4 integrin. These results suggest that sVCAM-1/α4 integrin induces angiogenesis through p38 and FAK signaling pathways.

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2 Address correspondence and reprint requests to Dr. Mayumi Ono, Department of Medical Biochemistry, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka, Japan 812-8582. E-mail address: mayumi@biochem1.med.kyushu-u.ac.jp

3 Abbreviations used in this paper: sVCAM-1, soluble VCAM-1; ERK, extracellular signal-related kinase; MAP, mitogen-activated protein; FAK, focal adhesion kinase; VEGF, vascular endothelial growth factor; Adv-FRNK, adenovirus encoding FAK-related nonkinase; moi, multiplicity of infection; Adv-LacZ, adenovirus containing the β-galactosidase gene LacZ.
34. Expression of sVCAM-1 was dramatically enhanced in response to IL-4 or IL-13 by vascular endothelial cells (33). IL-4- or IL-13-elicted angiogenesis could be almost completely blocked by neutralizing Ab against α5 integrin (33), suggesting the involvement of sVCAM-1 in inflammatory angiogenesis. However, it remains unclear which signaling pathway may be involved in the sVCAM-1-induced angiogenesis. In our present study, we considered the mechanism of sVCAM-1-induced angiogenesis through interaction with an α5 integrin, a counterreceptor on the endothelial cells. The possible roles of p38 and FAK activation by sVCAM-1 are discussed.

Materials and Methods

Materials

Recombinant human VEGF, human sVCAM-1, mouse sVCAM-1Fc chimera, and murine TNF-α were purchased from R&D Systems (Minneapolis, MN). SB203580 was purchased from Calbiochem (San Diego, CA). Anti-ERK1/2, phospho-ERK1/2, p38, and phospho-p38 Abs were purchased from Cell Signaling Technology (Beverly, MA). Anti-FAK Abs were purchased from Upstate Biotechnology (Lake Placid, NY). Phospho-FAK Abs at Y397 were purchased from BioSource International (Camarillo, CA). FITC-conjugated anti-human CD31 mAbs, R-PE-conjugated anti-human CD49d (α5 integrin) mAbs, CyChrome-conjugated anti-human CD29 (β3 integrin), R-PE-conjugated anti-human CD61 (β3 integrin) mAbs, and growth factor-reduced Matrigel were purchased from BD Biosciences (San Diego, CA). FITC-conjugated anti-human CD151 (αv integrin) mAbs were purchased from Immunotech (Marseille, France). Rat anti-mouse CD49d (α5 integrin) mAbs and rat IgG2b were purchased from Serotec (Oxford, U.K.).

Cell culture

HUVEC were isolated from individual human umbilical cord veins by collagenase digestion and were routinely cultured on type-I collagen-coated plates in endothelial cell growth medium (Clonetics, San Diego, CA) supplemented with 2% FBS in a humidified incubator under 5% CO2 at 37°C. Tissue samples were obtained under an institutional review board-approved protocol, with subjects providing informed consent.

Flow cytometry

HUVEC were harvested with 0.05% trypsin and washed with PBS. The cells were resuspended in PBS and adjusted to a concentration of 1 × 10^6 cells/ml. Simultaneous staining with various leukocyte surface markers was performed with the following commercial Abs: FITC-conjugated anti-human CD31 mAb, PE-conjugated anti-human CD49d (α5 integrin) mAbs, CyChrome-conjugated anti-human CD29 (β3 integrin), FITC-conjugated anti-human CD51 (αv integrin) mAbs, and R-PE-conjugated anti-human CD61 (β3 integrin) mAbs. These mAbs were added and incubated for 15 min at room temperature. Cells were washed twice with PBS and analyzed in a flow cytometer using FACScs (BD Biosciences). For determination of background fluorescence levels, isotype control Abs were used at the same concentration as the test Abs.

Western blot analysis

HUVEC (passage 3-4) were incubated for 24 h in endothelial cell growth supplement-free medium containing 0.5% serum before addition of VEGF or sVCAM-1. The cells were harvested and were lysed in Triton X-100 buffer (50 μM HEPES, 150 μM NaCl, 1% Triton X-100, and 10% glycerol containing 1 mM PMSE, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM sodium orthovanadate). Cell lysates were collected, electrophoresed by SDS-PAGE on 10% polyacrylamide gel, and were blotted onto nitrocellulose filters. The nitrocellulose filters were developed by chemiluminescence according to the ECL protocol of Amersham Pharmacia Biotech (Piscataway, NJ). For immunoblot analysis with anti-phospho-FAK Abs by HUVEC under suspension culture, the cells were incubated with SB203580 (10 μM) or DMSO for 3 h. Cells were harvested by 0.05% trypsin, washed twice with PBS, and held in suspension for 2 h in M199 with 0.5% serum with or without SB203580 (10 μM), and further incubated with VEGF or sVCAM-1 for 15 min at 37°C.

Cell migration assay

Cell migration activity was measured with a modified Boyden chemotaxis chamber (Kurabo, Tokyo, Japan) (35). Polycarbonate filters with 8-μm pores (Kurabo) in the inner chamber were preincubated for 1 h in a 0.1% solution of collagen type I (Nitta gelatin; Osaka, Japan). Cells (1.5 × 10^5) were trypsinized, suspended with M199 containing 0.5% serum with or without anti-α5 integrin Ab (10 μg/ml) or nonspecific isotype-matched control Ab (10 μg/ml), and placed onto each well in the upper chamber. In the lower chamber, VEGF or sVCAM-1 with or without various concentration of SB203580, or with or anti-α5 integrin Ab (10 μg/ml) or nonspecific isotype-matched control Ab (10 μg/ml), was added. After incubation for 2 h at 37°C in a 5% CO2 incubator, nonmigrated cells on the upper surface of the filter were removed. Filters were mounted onto microscope slides, and stained cells were counted at ×200 magnification in four random fields per well. In each individual experiment, chemotaxis was performed in four separate wells for each concentration of a given test substance under a specified condition. Each n value in the figure legends refers to the number of individual experiments.

An in vivo angiogenesis model using Matrigel

An in vivo angiogenesis model using commercially available basement membrane extracts (Matrigel) was performed as previously described (35, 36). Three hundred microliters of Matrigel supplemented with 100 ng or 500 ng sVCAM-1 with or without SB203580 (10 μM) or anti-α5 integrin Ab (2 μg) or nonspecific isotype-matched control Ab (2 μg) was s.c. inoculated into the back of anesthetized BALB/c mice. Five days after the inoculation, the mice were sacrificed, and the gel was removed. These gels were then fixed in 10% buffered-formalin for at least 24 h, cleared by immersion in Histoclear (National Diagnostics, Atlanta, GA), embedded in paraffin, and sectioned at 5 μm thickness. The sections were then deparaffinized and stained with Masson-Trichrome. The total length of blood vessels was measured for each field using a cosmozone image analyzer (Nikon, Tokyo, Japan).

Corneal micropocket assay in mice

The corneal micropocket assay in mice and quantification of cornea neovascularization was made essentially as previously described (37, 38). In brief, 0.3 μl of Hydron pellets (IPN Sciences, New Brunswick, NJ) containing 100 ng of murine TNF-α, 200 ng or 500 ng of human sVCAM-1, or 100 ng of murine TNF-α and 200 ng of human sVCAM-1 were prepared and implanted in the corneas of male BALB/c mice. SB203580 or anti-α5 integrin Ab (0.5 μg/pellet) or nonspecific isotype-matched control Ab (0.5 μg/pellet) was added directly to cytokine/Hydron solution. After 7 days the animals were sacrificed, and the corneal vessels were photographed. The quantitative analysis of neovascularization in mouse corneas was performed using the software package NIH Image as described previously (37, 38).

Adenoviral constructs

A replication-defective adenosine encoding FAK-related non kinase (AdvFRNK) was kindly provided by Dr. A. M. Samarel (Loyola University Medical Center, Maywood, IL) (39). A replication-defective adenosine containing the β-galactosidase gene LacZ (Adv-LacZ) was used to control for the nonspecific effects of viral infection. Adenoviruses were amplified and purified using human embryo kidney 293 cells. Preliminary experiments determined that a viral concentration of 20 multiplicity of infection (MOI) produced readily detectable proteins within 48 h (Western blot analysis) and also infected >90% of HUVEC (X-gal staining).

Results

Phosphorylation by sVCAM-1 of p38 and FAK, but not ERK1/2

It has been reported that integrin-ligand interactions induced the activation of MAP kinases and FAK (40). We demonstrated the direct effects of sVCAM-1 on the phosphorylation of two MAP kinases (p38 and ERK1/2) and FAK in endothelial cells. p38 was phosphorylated by sVCAM-1, and the maximal phosphorylation of p38 was induced by 100 ng/ml sVCAM-1, comparable to the phosphorylation with 10 ng/ml VEGF (Fig. 1A). In contrast, ERK1/2 was phosphorylated by VEGF, but not by sVCAM-1. We next examined whether FAK was also phosphorylated by sVCAM-1. As shown in Fig. 1B, when endothelial cells were treated with 10 or 100 ng/ml sVCAM-1 for 15 min, FAK was markedly phosphorylated over the unstimulated culture. FAK was also phosphorylated by VEGF in suspended cells.
Relative activity was presented when the migrated cell number (57/H11006 levels (Table I). sVCAM-1 at 10 ng/ml increased cell migration at comparable 1.7-fold over that of the unstimulated control. Both VEGF and sVCAM-1 at 10 ng/ml also enhanced maximum cell migration 1.7- to 1.8-fold increase in endothelial cell migration (Table I). sVCAM-1 at 10 ng/ml, when added for 2 hi nt h elower chamber, induced a tion, the initial step of angiogenesis in endothelial cells. VEGF We next determined whether sVCAM-1 could induce cell migra-
tion is dependent on p38, we examined the effect of a speci fi fi
cation of p38 (30, 31). To determine whether sVCAM-1-induced cell migration is mediated through α4 integrin Ab or p38 inhibitor, SB203580, on cell migration by endothelial cells. SB203580 at 1 μM almost completely inhibited the sVCAM-1-dependent cell migration (Table II). However, SB203580 at 1 and 10 μM inhibited unstimulated cell migration only by 15 and 30%, respectively. SB203580 at up to 100 μM did not inhibit cell proliferation of endothelial cells (data not shown). The sVCAM-1-induced cell migration thus appeared to be depen-
dent on the activation of p38.

α4 integrin is a receptor for sVCAM-1, and we also examined whether sVCAM-1-induced cell migration is mediated through α4 integrin. Administration of an anti-α4 Ab markedly inhibited sVCAM-1-induced cell migration (Table I).

VEGF-induced migration is known to be dependent on activation of p38 (30, 31). To determine whether sVCAM-1-induced cell migration is dependent on p38, we examined the effect of a specific inhibitor of p38, SB203580, on cell migration by endothelial cells. SB203580 at 1 μM inhibited sVCAM-1-dependent cell migration by 70%, and SB203580 at 10 μM almost completely inhibited the sVCAM-1-dependent cell migration (Table II). However, SB203580 at 1 and 10 μM inhibited unstimulated cell migration only by 15 and 30%, respectively. SB203580 at up to 100 μM did not inhibit cell proliferation of endothelial cells (data not shown). The sVCAM-1-induced cell migration thus appeared to be depend-
ent on the activation of p38.

To assess the role of p38 in FAK activation by sVCAM-1, HUVEC were preincubated with SB203580 before sVCAM-1 stimulation. As shown in Fig. 2, neither VEGF nor sVCAM-1-induced phosphorylation of FAK was affected by SB203580 at a concentration of 10 μM. These results indicate that p38 does not affect the FAK signaling transduction pathway induced by sVCAM-1.

Inhibition of sVCAM-1-induced migration by Adv-FRNK

It has been shown that tyrosine phosphorylation of FAK is another key factor in endothelial cell migration (31). We further examined

### Table I. Cell migration by sVCAM-1 in endothelial cells

<table>
<thead>
<tr>
<th>Factors (ng/ml)</th>
<th>Migrated Cell Number</th>
<th>Relative Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>56.8 ± 8.4</td>
<td>100(^a)</td>
</tr>
<tr>
<td>VEGF (10)</td>
<td>98.3 ± 16.9</td>
<td>175(^a)</td>
</tr>
<tr>
<td>sVCAM-1 (0.1)</td>
<td>64.4 ± 4.7</td>
<td>114</td>
</tr>
<tr>
<td>sVCAM-1 (1)</td>
<td>76.8 ± 11.7</td>
<td>136(^a)</td>
</tr>
<tr>
<td>sVCAM-1 (10)</td>
<td>97.3 ± 23.5</td>
<td>172(^a)</td>
</tr>
<tr>
<td>sVCAM-1 (100)</td>
<td>83.5 ± 7.9</td>
<td>148(^a)</td>
</tr>
<tr>
<td>sVCAM-1 (10) + anti-α4 Ab</td>
<td>54.9 ± 14.9</td>
<td>97**</td>
</tr>
<tr>
<td>sVCAM-1 (10) + IgG</td>
<td>101.5 ± 14.9</td>
<td>179</td>
</tr>
</tbody>
</table>

\(^a\) The number of migrated HUVEC was the mean of triplicate dishes (±SD). Relative activity was presented when the migrated cell number (57 ± 8.4) in the absence of factors, was normalized as 100%.

\(^b\) Significant difference (\(\ast, p < 0.05\), **, \(p < 0.01\)) between the presence VEGF or sVCAM-1.

\(^c\) Significant difference (\(\ast, p < 0.05\), **, \(p < 0.01\)) between sVCAM-1 alone and sVCAM-1 with anti-α4 Ab.

### Table II. Inhibition of sVCAM-1-induced cell migration by p38 inhibitor, SB203580

<table>
<thead>
<tr>
<th>sVCAM-1 (ng/ml)</th>
<th>SB203580 (μM)</th>
<th>Migrated Cell Number</th>
<th>Relative Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>55 ± 2.4</td>
<td>100(^a)</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>95 ± 3.2</td>
<td>67.5</td>
</tr>
<tr>
<td>10</td>
<td>0.1</td>
<td>82 ± 7.9</td>
<td>29.7(^a)</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>67 ± 9.2</td>
<td>0(^a)</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>54 ± 8.8</td>
<td>85</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>55 ± 2.4</td>
<td>100(^a)</td>
</tr>
<tr>
<td>0</td>
<td>1</td>
<td>47 ± 13.7</td>
<td>85</td>
</tr>
<tr>
<td>0</td>
<td>10</td>
<td>39 ± 5.1</td>
<td>71</td>
</tr>
</tbody>
</table>

\(^a\) The number of migrated HUVEC was the mean of triplicate dishes (±SD). Soluble VCAM-1-specific migration activity was presented as 100% when the cell number (55 ± 2.4) in the absence of both sVCAM-1 and SB203580 was subtracted from that in the presence of sVCAM-1 alone.

\(^b\) Significant difference (\(\ast, p < 0.01\)) between sVCAM-1 alone and sVCAM-1 with SB203580.

\(^c\) Cell number (55 ± 2.4) in the absence of both sVCAM-1 and SB203580 was presented as 100%.
the involvement of FAK in sVCAM-1-induced cell migration in vitro. As seen in Fig. 3, VEGF at 10 ng/ml as well as sVCAM-1 at 10 or 100 ng/ml induced the phosphorylation of FAK in HUVEC. Moreover, a replication-defective Adv-FRNK was found to almost completely block FAK phosphorylation by HUVEC in response to both VEGF and sVCAM-1. We next examined whether FAK affected the p38 signaling transduction pathway by sVCAM-1. In contrast, Adv-FRNK inhibited sVCAM-1-induced phosphorylation of p38, suggesting that FAK affected the p38 signaling transduction pathway by sVCAM-1.

VEGF or sVCAM-1 at 10 ng/ml stimulated cell migration ~1.8- and 1.7-fold, respectively, and Adv-FRNK almost completely blocked sVCAM-1-induced cell migration (Fig. 4). Adv-FRNK blocked VEGF-induced cell migration to some extent that was ~40% of the initial activity. These results indicate that the activation of FAK is closely associated with sVCAM-1-induced cell migration.

sVCAM-1-induced angiogenesis and inhibition by p38 inhibitor or anti-α4 integrin Ab in Matrigel plug assay

The angiogenic role of sVCAM-1 in vivo was assessed by examining the effect of sVCAM-1 on blood vessel growth in the Matrigel plugs in mice. sVCAM-1 was mixed with Matrigel and injected into mice. After a 5-day incubation, we found that the s.c. tissues attached to the Matrigel/sVCAM-1 mixture as well as the surface of the Matrigel became reddish with fine blood vessels. The Matrigel/sVCAM-1 mixture showed a clear angiogenic response with several vessels, whereas the mice injected with Matrigel alone showed no angiogenic response (Fig. 5). Administration of 500 ng of sVCAM-1 caused a much greater angiogenic response than did administration of 100 ng of sVCAM-1 (data not shown). To examine the effect of p38 inhibitor, SB203580, on sVCAM-1-induced angiogenesis, Matrigel plugs containing 500 ng sVCAM-1 and 10 μM SB203580 were injected into mice. As shown in Fig. 5, coadministration of SB203580 and sVCAM-1

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was found to block sVCAM-1-induced angiogenesis in Matrigel (Fig. 5). The total length of blood vessels was measured for each field, and quantitative analysis with four mice for each assay showed that blood vessel lengths were 100 ± 70 mm (untreated), 730 ± 180 mm (sVCAM-1, 500 ng), and 160 ± 110 mm (sVCAM-1, 500 ng and SB203580, 10 μM), thereby indicating a significant (p < 0.01) decrease by SB203580 in blood vessel length.

We next examined whether sVCAM-1 was able to induce angiogenesis in mouse corneas. We implanted a pellet of Hydron that had been impregnated with sVCAM-1 and/or TNF-α into the corneas of mice. A total of 200 ng of sVCAM-1 alone did not induce angiogenesis, and 500 ng of sVCAM-1 alone induced only a slight degree, if any, angiogenesis in mouse corneas (Fig. 6A). Although TNF-α alone at 100 ng/ml did not induce any significant angiogenic response, coadministration of TNF-α at 100 ng and sVCAM-1 at 200 ng as a pellet did induce a marked increase in neovascularization in mouse corneas. Neovascularization by TNF-α and sVCAM-1 was almost completely blocked by the p38 specific inhibitor, SB203580. TNF-α at 200 ng alone did induce
neovascularization, and this neovascularization was partially inhibited by SB203580 (Fig. 6A). Quantitative analysis demonstrated the synergistic effects of both TNF-α at 100 ng and sVCAM-1 at 200 ng on neovascularization, whereas SB203580 almost completely inhibited the development of neovascularatures by TNF-α and sVCAM-1 (Fig. 6B). TNF-α at 200 ng/ml also induced neovascularization in the mouse cornea, and SB203580 blocked TNF-α-induced neovascularization by ~30%.

We further examined if angiogenesis in mouse corneas by both TNF-α and sVCAM-1 is mediated through αv integrin. Consistent with our angiogenesis assay in Fig. 6, administration of TNF-α at 100 ng and sVCAM-1 at 200 ng induced angiogenesis in mouse cornea (Fig. 7). Either TNF-α alone at 100 ng or sVCAM-1 alone at 200 ng was again found to induce no angiogenic response (data not shown). Administration of anti-αv integrin Ab markedly inhibited the angiogenesis induced by both TNF-α and sVCAM-1. Quantitative analysis also demonstrated that anti-αv integrin Ab almost completely inhibited the development of neovascularization by TNF-α and sVCAM-1.

Up-regulation of αv integrin by TNF-α in vascular endothelial cells

It has been reported that αvβ3 integrin, a receptor for sVCAM-1, is expressed on the surface of endothelial cells (20, 21). By flow cytometric analysis, we confirmed the expression of αvβ3 molecules on the surface of endothelial cells, and the expression of the αvβ3 molecule was affected by TNF-α. Most of the population of the endothelial cells expressed αvβ3 integrin (Fig. 8). Furthermore, αv integrin was expressed in 6.8% of endothelial cells at passage 2 without cytokine stimulation. The population of αv integrin-positive cells increased 3- to 4-fold over that of the unstimulated control by 10 ng/ml TNF-α or 100 ng/ml TNF-α (Fig. 8, c and d) for 24 h.

We next examined whether the expression of β3 integrin in endothelial cells was affected by TNF-α. Almost all of the cell populations (>99%) were positive for β3. TNF-α at 10 or 100 ng/ml did not affect β3-positive cell population (data not shown). Expression of αv integrin rather than β3 integrin was found to be more susceptible to TNF-α in endothelial cells.

Discussion

In this present study, we found that sVCAM-1 enhanced both p38 and FAK phosphorylations in endothelial cells (Fig. 1). Cell migration activity by endothelial cells in vitro and neovascularization in Matrigel in vivo were markedly enhanced in response to sVCAM-1. We also observed marked inhibition of cell migration in response to sVCAM-1 by Adv-FRNA (Fig. 3). Moreover, TNF-α and sVCAM-1 resulted in synergistic development of angiogenesis in mouse cornea. We found that p38 inhibitor blocked these angiogenic processes in vitro as well as in vivo and that sVCAM-1/αv integrin could implement its angiogenic signaling through p38 and/or FAK activation by endothelial cells.

Many laboratories have already demonstrated that integrins are implicated in angiogenesis (41, 42). Friedlander et al. (41) reported that angiogenesis induced by TNF-α or fibroblast growth factor-2 is dependent on αvβ3, and also that angiogenesis induced by VEGF and TGF-α is dependent on αvβ5, suggesting that αv integrin plays a critical role in angiogenesis by potent angiogenic factors. In contrast, sVCAM-1 mainly binds to αv integrins that associate with two β subunits, β1 and β3. We also observed αvβ1 integrin expression in HUVEC, and that αv integrin expression was apparently up-regulated by TNF-α (21) (see also Fig. 8). TNF-α alone at a dose of 200 ng was capable of inducing angiogenesis in the mouse cornea, and this type of TNF-α-induced angiogenesis was partly blocked by SB203580. Although TNF-α-induced angiogenesis is also mediated through up-regulation of various angiogenic factors such as VEGF, IL-8, fibroblast growth factor-2, and metalloproteinases (43, 44, 45), sVCAM-1 is another angiogenic factor induced by TNF-α. We have previously reported that the expression of sVCAM-1 was markedly enhanced in vascular endothelial cells by IL-4 and IL-13 (33, 34). The angiogenesis induced by TNF-α or IL-4/IL-13 might be partly attributable to the sVCAM-1/αv integrin pathway, plausibly under certain inflammatory conditions.

Serum levels of adhesion molecules detectable as soluble forms have been studied (12, 13, 14). In patients with rheumatoid arthritis, circulating concentrations of sVCAM-1 are detected as 827.72 ± 294.33 ng/ml compared with those in normal controls (523.50 ± 156.26 ng/ml) in sera (12). In our present study, we used 0.1–100 ng/ml of sVCAM-1 and confirmed that 10 ng/ml or higher concentrations of sVCAM-1 could induce migration by endothelial cells in vitro. The concentration of sVCAM-1 in sera thus appeared to be enough to induce angiogenic switch by sVCAM-1.

Koch et al. (15) have reported that sVCAM-1 per se induces neovascularization in the rat cornea. Using two in vivo angiogenesis model assays, we examined whether sVCAM-1 was angiogenic in a mouse corneal micropocket assay and in a Matrigel plug assay. sVCAM-1 resulted in the successful induction of angiogenesis in Matrigel. This apparent increase of neovascularization by sVCAM-1 was highly reproducible when three independent assays with Matrigel were performed. In contrast, we did not observe any apparent neovascularization by sVCAM-1 alone in the mouse cornea. Rohan et al. (46) have reported that different strains of inbred mice have an ~10-fold range of response to growth factor-stimulated angiogenesis in the corneal micropocket assay, suggesting

FIGURE 8. Expression of αv integrin in endothelial cells by FACS analysis. Unstimulated HUVEC at passage 2 (c) and cells stimulated with 10 ng/ml (d) or 100 ng/ml (e) TNF-α for 24 h were stained with FITC-conjugated anti-human CD31 mAbs and R-PE-conjugated anti-human CD49d (αv integrin) mAbs. Unstimulated HUVEC were also stained with FITC-conjugated anti-human CD51 (αv integrin) mAbs and CyChrome-conjugated anti-human CD61 (β3 integrin) mAbs (b). For determination of background fluorescence levels, isotype control Abs were used in the same concentrations as those of the test Abs (a).
the presence of genetic factors that control individual angiogenic potential. The difference of angiogenic response to sVCAM-1 in mouse cornea and rat cornea might be caused by such genetic heterogeneity in both strains. Inflammatory cytokines such as TNF-α up-regulate the expression of VCAM-1 and sVCAM-1 (47). We also observed apparent up-regulation of VCAM-1/sVCAM-1 in endothelial cells by TNF-α or IL-1β (S.N. and M.O., unpublished data). Any inflammatory condition that is induced by TNF-α could be necessary for sVCAM-1-induced neovascularization in mouse cornea. TNF-α and sVCAM-1 synergistically induced angiogenesis in mouse corneas when TNF-α or sVCAM-1 alone at the experimental concentrations was unable to induce angiogenesis. Treatment of vascular endothelial cells with TNF-α apparently enhanced expression of α4 integrins, but not that of β1 integrin. Neutralizing Ab against α4 integrins almost completely blocked the angiogenesis induced by both TNF-α and sVCAM-1. The up-regulation of α4 integrins by TNF-α thus appears to play a critical role in the sVCAM-1-induced neovascularization in vivo.

Integrin ligation is known to induce a wide range of intracellular signaling events, including the activation of MAP kinases (48). Members of the MAP kinase family such as ERK1/2, c-Jun N-terminal kinase, and p38 MAP kinase are central elements of post-receptor signal transduction pathways. ERK1/2 activation regulates cell proliferation that is requisite for angiogenesis, and p38 MAP kinase modulates the migration of endothelial cells (49). In our present study, cell migration, but not cell proliferation (S. Nakao and M. Ono, unpublished results), was markedly stimulated by sVCAM-1. Moreover, p38, but not ERK1/2, was markedly phosphorylated when endothelial cells were stimulated with sVCAM-1. A specific inhibitor of p38 almost completely blocked the sVCAM-1-induced migration. These results suggest that p38 MAP kinase is involved in the modulation of migration of endothelial cells in response to sVCAM-1. ERK activation thus appears unlikely to be involved in the sVCAM-1-induced angiogenic switch. Concerning the possible role of p38 kinase in sVCAM-1-induced angiogenesis, we observed the apparent inhibition of a p38 inhibitor, SB203580, in these different angiogenesis model systems. First, SB203580 inhibited the migration of endothelial cells in response to sVCAM-1, as well as in response to VEGF; second, SB203580 almost completely blocked sVCAM-1-induced angiogenesis in the Matrigel plug assay; and third, SB203580 also inhibited neovascularization by sVCAM-1 together with TNF-α in the mouse cornea.

FAK is a nonreceptor protein-tyrosine kinase that is localized to integrin-receptor clustering sites. It has been reported that tyrosine phosphorylation of FAK is important for cell migration (32). FAK was markedly phosphorylated by sVCAM-1 in endothelial cells (Fig. 1B). FAK-related nonkinase overexpression blocked FAK phosphorylation in response to sVCAM-1 as well as in response to VEGF. Cell migration was specifically enhanced in response to sVCAM-1 and VEGF, and both sVCAM-1- and VEGF-induced cell migration was inhibited by Adv-FRNK (Fig. 4). VEGF-induced migration by vascular endothelial cells is known to be mediated through FAK signaling (31). However, Adv-FRNK demonstrated much greater inhibition of sVCAM-1-induced cell migration than did VEGF-induced cell migration. This type of FAK activation appears to be specifically associated with an initial angiogenic switch in cell migration induced by the sVCAM-1/α4 integrin pathway, instead of being specifically associated with the VEGF pathway.

In conclusion, both VEGC-1/sVCAM-1 and α4 integrins are up-regulated by TNF-α in endothelial cells. Endothelial cell migration in vitro, and angiogenesis in the Matrigel plug assay and in the mouse cornea in vivo are all significantly stimulated by sVCAM-1. A specific inhibitor of p38 results in a blockage of cell migration in vitro as well as in vivo by sVCAM-1. FAK is also involved in sVCAM-induced cell migration, because Adv-FRNK almost completely inhibited the cell migration. Taken together, our present study suggests that sVCAM-1-induced angiogenesis is mediated through both p38 and FAK signalings and that this sVCAM-1/α4 integrin pathway might play a role in inflammatory angiogenesis.

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References


