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ORIGINAL ARTICLE

Angiogenesis induced by tumor necrosis factor-α is mediated by α4 integrins

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Tumor necrosis factor-α (TNF-α) and fibroblast growth factor-2 (FGF-2 or bFGF) are potent stimulators of angiogenesis. TNF-α, but not FGF-2, can induce the expression of vascular cell adhesion molecule-1 (VCAM-1) on the surface of endothelial cells. The soluble form of VCAM-1 has recently been demonstrated to function as an angiogenic mediator. Here we demonstrate that monoclonal antibodies directed against VCAM-1 or its α4 integrin counter-receptor inhibited TNF-α-induced endothelial cell migration *in vitro*. Angiogenesis induced *in vivo* in rat corneas by TNF-α was inhibited by a neutralizing antibody directed against the rat α4 integrin subunit. A peptide antagonist of the α4 integrins blocked TNF-α-induced endothelial cell migration *in vitro* and angiogenesis in rat corneas *in vivo*. No inhibition by the antibodies or peptide antagonist was observed either *in vitro* or *in vivo* when FGF-2 was used as the stimulus. The peptide antagonist did not inhibit TNF-α binding to its receptor nor did it block the function of αvβ3, an integrin previously implicated in TNF-α and FGF-2 mediated angiogenesis. These results demonstrate that angiogenic processes induced by TNF-α are mediated in part by α4 integrins possibly by a mechanism involving the induction of soluble VCAM-1.

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Introduction

Angiogenesis is the growth of new blood vessels from preexisting vessels. This process is essential for proper development and wound healing, yet is also characteristic of certain pathological conditions including arthritis, psoriasis, retinopathy and tumor growth.[1] TNF-α is one of the primary angiogenic mediators derived from activated macrophages and as such is thought to play a key role in chronic inflammatory diseases such as rheumatoid arthritis.[2]

TNF-α was first identified as the substance mediating necrosis of certain tumors that were treated with endotoxin.[3] Although TNF-α inhibits endothelial cell growth *in vitro*,[4,5] it has been shown to induce endothelial cell migration *in vitro*[6] and cause angiogenesis *in vivo* when implanted into corneas or choriallantoic membranes.[5,6] It has been proposed that the mechanism of TNF-α mediated angiogenesis may be indirect and may depend on the synthesis of secondary mediators including prostaglandins, platelet activating factor, the Eck receptor ligand B61, and more recently interlukin-8, vascular endothelial growth factor (VEGF) and FGF-2.[7–10] Friedlander *et al.*[11] demonstrated that angiogenesis induced by TNF-α or FGF-2 is dependent on the integrin αvβ3 whereas that mediated by two other angiogenic mediators, VEGF and transforming growth factor-α, is αvβ5 dependent.

TNF-α can induce the expression of cell adhesion molecules, including VCAM-1 and

E-selectin, on the surface of endothelial cells.[12,13] Soluble forms of VCAM-1 (sVCAM-1) and E-selectin have recently been demonstrated to function as angiogenic mediators.[14] The α4 integrins serve as counter-receptors for VCAM-1.[15,16] Expressed abundantly on the surface of leukocytes, the α4 integrin subunit has been detected at lower levels on the surface of endothelial cells as well.[14,17] In fact, neutralizing antibodies directed against the α4 integrin receptors inhibit the migration of endothelial cells toward sVCAM-1.[14]

We have used endothelial cell migration assays and an *in vivo* model of angiogenesis to demonstrate that TNF-α-induced angiogenesis is dependent, in part, on functions mediated by α4 integrins. These data implicate sVCAM-1 as a potential secondary mediator in angiogenic processes induced by TNF-α.

Materials and methods

Materials. Recombinant human TNF-α and recombinant human FGF-2 were purchased from R&D Systems, Inc. (Minneapolis, MN) and Upstate Biotechnology (Lake Placid, NY), respectively. Anti-human VCAM-1 was from Genzyme (Cambridge, MA) and anti-rat α4 (TA-2) was from Seikagaku America, Inc. (Rockville, MD). Anti-human α4 (L25) and anti-human β1 (mAb13) were from Becton Dickinson (San Jose, CA). Anti-human β1 (33B6) and anti-Leu 13 (35F9) were generated and maintained in the McIntyre laboratory. Anti-CD3 mAb OKT3 was obtained from the American Type Culture Collection (Rockville, MD). Anti-human αvβ3 (23C6) was from Pharmingen (San Diego, CA). Anti-human αvβ5 (P1F6) ascites and the cyclic RGD peptide (GPenGRGDSPCA) were from Life Technologies (Grand Island, NY). FITC-conjugated goat anti-mouse IgG was obtained from Organon Teknika Corp. (West Chester, PA). Human plasma vitronectin was purchased from Sigma Chemical Co. (St. Louis, MO) and BSA fraction V was from Calbiochem (LaJolla, CA). The α4 integrin antagonist peptide, TBC772 (CWLDVC cyclic disulfide), and the scrambled control peptide, TBC1194 (CDLVWC, cyclic disulfide), have been previously described.[18]

Cells. Human umbilical vein endothelial cells (HUVEC) were purchased from Cascade Biologics, Inc. (Portland, OR) and maintained on gelatin coated plates in medium 199 supplemented with 15% FBS and 50 μg/ml endothelial mitogen (Biomedical Technologies Inc., Stoughton, MA) at 37°C in 5% CO_2. HL-60 cells (human promyelocytic leukemia) were obtained from the American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640 supplemented with 10% FBS at 37°C in 5% CO_2.

Animals. Male Sprague-Dawley rats (300 g) were purchased from Harlan (Indianapolis, IN). Housing and anesthesia concurred with the guidelines established by the University of Texas Health Science Center Institutional Animal Welfare Committee, in accordance with the Public Health Service Guide for the Care and Use of Laboratory Animals, United States Department of Agriculture regulations, and the American Veterinary Medicine Association's Panel on Euthanasia guidelines. Rats were housed individually, fed standard rat chow (Standard Laboratory Rodent Diet No. 5001; Ralston Purina, Richmond, IN) and water *ad libitum*, and were on a 12 hour light/dark cycle.

Migration assays. Assays were carried out in a 48-well chamber (NeuroProbe, Cabin John, MD) using collagen coated polycarbonate filters following procedures similar to those previously described.[19] Briefly, polycarbonate filters (8 μm pore size; Poretics Corp., Livermore, CA) were treated with 0.5 M acetic acid overnight, rinsed with distilled water, and incubated for 48 h at room temperature with 10 μg/ml collagen (Vitrogen 100; Cetrix Laboratories; Palo Alto, CA). Filters were air dried prior to placing into the chamber. Subconfluent HUVEC (passage 1–4) were harvested with 0.05% trypsin/EDTA, washed with M199 containing 10% fetal calf serum and resuspended in M199 containing 0.1% FCS at 5×10^5 cells/ml. TNF-α at 50 ng/ml or FGF-2 at 10 ng/ml in M199/0.1% FCS was placed in the bottom wells of the chamber and 150 μl of HUVEC (75,000 cells) were placed in the upper wells. Antibodies (10 μg/ml) or peptides (10 μM) were added to both the top and bottom chambers.

The chamber was incubated for 4 h at 37°C in a 5% CO_2 incubator. Solution in the top wells was aspirated and nonmigrated cells were removed from the filter with a wet cotton swab. Migrated cells were fixed in 100% ethanol for 30 min at room temperature and stained with a modified Wright-Giemsa stain (Diff-Quik, Baxter Diagnostics, Chicago, IL). Migrated cells were counted at 400 × magnification in four random fields per well. Each treatment group was performed in triplicate wells. Statistical analysis of the data was performed using a paired Student's t-test with $p < 0.05$ considered statistically significant.

Flow Cytometry. HUVEC were harvested as described above and resuspended in RPMI 1640 supplemented with 10% FCS (3×10^6 cells/ml). mAbs (0.1 μg/ml) were added and incubated for 30 min at room temperature. Cells were washed twice with PBS containing 1% BSA and incubated with goat anti-mouse FITC for 30 min at room temperature. Cells were washed with PBS and analyzed in an EPICS Profile flow cytometer (Coulter Corp., Hialeah, FL). At least 10,000 events were analyzed for every test.

Quantification of soluble VCAM-1. Confluent monolayers of HUVEC in 35 mm plates were treated with 50 ng/ml TNF-α or 10 ng/ml FGF-2 in M199/0.1% FCS for 4 h at 37°C. Media was collected, centrifuged for 5 min at 1000 × *g*, and concentrated using Centricon-3 filters (Amicon, Beverly, MA). Samples were assayed for the presence of human soluble VCAM-1 using an immunoassay kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Corneal angiogenesis assays. Assays were performed similarly to those previously described.[6] Pellets were made by mixing equal volumes of TNF-α or FGF-2 (300 ng in PBS containing 0.1% BSA) and a 12% solution of Hydron (Interferon Sciences, New Brunswick, NJ) in ethanol. Antibodies (1.75 μg/pellet) or peptides (100 ng/pellet) were added directly to the cytokine/hydron solution. Five μl were placed on the ends of 2 mm diameter teflon rods and allowed to dry for 30 min in a cell culture hood. Pellets were implanted in the corneas of 300–400 g Sprague-Dawley rats approximately 1 mm from the limbus. After 7 days, rats were euthanized with CO_2, the vasculature perfused with buffer (PBS containing 10 U/ml heparin) followed by colloidal carbon, and eyes excised and fixed in 10% formalin.

TNF-α receptor assay. Anti-Human TNF Receptor (p55) IgG (Austral Biologics, San Ramon, CA) was coated onto Immulon-4 plates at 1 μg/well in PBS overnight at 4°C. Wells were washed three times with PBS containing 0.1% Tween-20 followed by three washes with PBS. Wells were blocked with 1% BSA in PBS for 1 h at 22°C and then washed as described above. Recombinant human TNF-α receptor extracellular domain (Austral Biologics) was coated onto the wells at 10 ng/well in PBS overnight at 4°C. Wells were washed, blocked and rewashed as described above. ^{125}I-TNF-α (Biomedical Technologies Inc., Stoughton, MA) in PBS containing 0.1% BSA and 25 mM HEPES was added to the wells at a concentration of 300 pg (2×10^4 cpm)/well. In certain wells, unlabeled TNF-α (300 ng/well, PeproTech Inc., Rocky Hill, NJ) or TBC772 (1 mM) were also added. Plates were incubated for 90 min at 22°C and washed three times with PBS. Wells were separated, placed in vials containing scintillation fluid and counted using a Beckman LS6000TA scintillation counter.

αvβ3 assay. Human plasma vitronectin was coated onto 96-well Probind plates at 5 μg/ml in TBS (50 mM Tris, pH 7.5, 150 mM NaCl) for 16 h at 4°C. Control wells were coated with the same concentration of BSA in TBS. Plates were washed three times with TBS, blocked with TBS containing 2% BSA at room temperature for 4 h, then washed three more times with TBS prior to assay. HUVEC grown to confluency were harvested as described above and resuspended in M199 containing 0.1% BSA. Cells were fluorescently labeled with calcein AM (Molecular Probes, Eugene, OR) and resuspended at 4×10^6 cells/ml in M199, 0.1% BSA, 1 mM $MnCl_2$. Calcein-AM is a non-fluorescent substrate taken up and processed by viable cells to yield a fluorescent, membrane impermeable product. Labeled HUVEC were added to the wells (2×10^5 cells/well)

and incubated at 37°C for 30 min. Following three washes with TBS containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mM $MnCl_2$, adherent cells were lysed with 50 µl of 1% Nonidet P-40 in 50 mM Tris, pH 7.5 and 5 mM EDTA. The number of cells bound was quantitated using a Millipore Cytofluor 2350 fluorometer (Millipore Corp., Bedford, MA). Antibodies (10 µg/ml) or ascites (1:100 dilution) were preincubated with labeled HUVEC for 20 min at 37°C prior to addition of cells to the wells. Peptide inhibitors at the indicated concentrations were mixed with HUVEC immediately prior to addition of cells to the wells.

E-selectin assay. Assays were performed as previously described.[20] Briefly, 10 µl of fluorescently labeled HL-60 cells (10^7 cells/ml) were added to wells of a 96 well plate that had been previously blocked with 3% BSA. This was followed by the addition of 10 µl of magnetic beads (4×10^6 beads/ml) coated with an E-selectin IgG fusion protein. After a 10 min incubation at room temperature, the beads were placed on a magnetic seperator and unbound cells were removed with two washes of the wells with PBS. Bound cells were lysed with a 1% NP-40 solution in PBS and quantitated using a Millipore Cytofluor 2350 fluorimeter. The TBC772 peptide was added to the cells immediately prior to the addition of beads. Binding of HL-60 cells to E-selectin beads previously has been shown to be blocked by sLe^x tetrasaccharide and anti-sLe^x antibodies.[20]

Results

Inhibition of α4 integrins or VCAM-1 inhibits TNF-α– but not FGF-2-induced endothelial cell migration. HUVEC migration across collagen coated polycarbonate membranes was measured in response to the cytokines TNF-α or FGF-2. The optimal doses for HUVEC migration were determined to be 50 ng/ml and 10 ng/ml for TNF-α and FGF-2, respectively (data not shown). Stimulation with cytokines resulted in an ~1.5–2.0 fold increase in cell migration as compared to unstimulated controls (Figures 1A–C). For ease of comparison, the y-axis of each graph in Figures 1A–C spans an equal-fold range of cell counts.

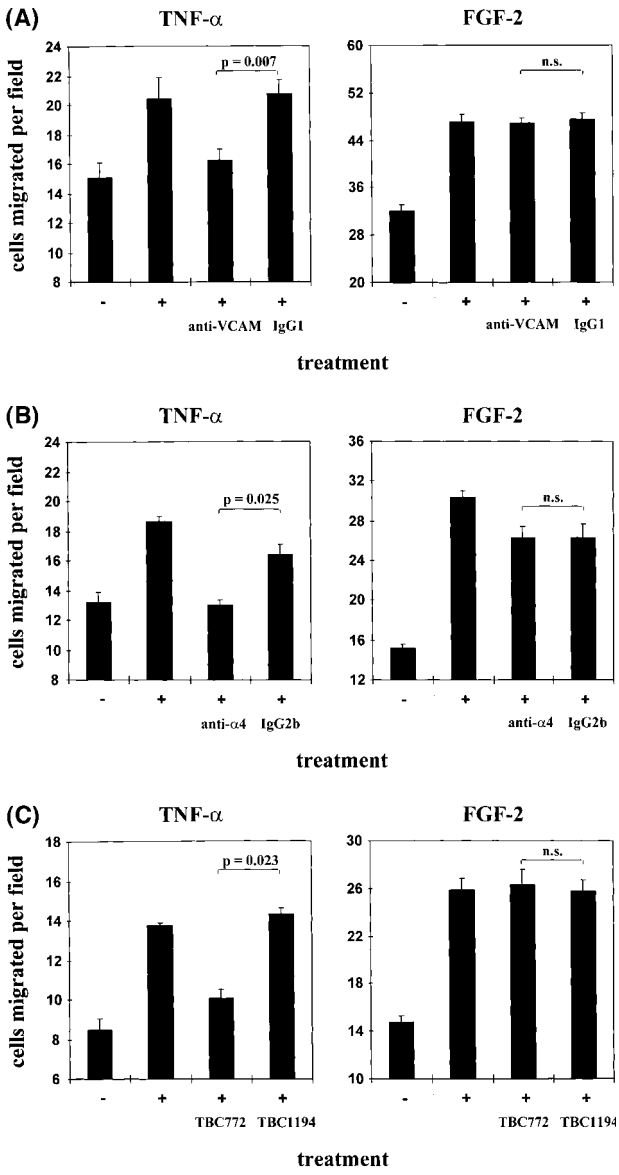

Figure 1. *TNF-α- but not FGF-2-induced HUVEC migration is inhibited by anti-VCAM-1, anti-α4 or a peptide antagonist of α4 integrins.* HUVEC migration was measured in a 48-well chamber using collagen-coated polycarbonate filters in the absence (–) and presence (+) of TNF-α or FGF-2. Cytokine-induced migration was determined in the presence of: (A) anti-VCAM-1 or IgG1 isotype control antibodies, (B) anti-α4 or IgG2b isotype control antibodies, (C) an α4 integrin peptide antagonist TBC772 or a scrambled peptide control TBC1194. Migrated cells were counted in four random fields per well and each treatment group was performed in triplicate wells. Data are presented as the average number of cells counted per field ± S.E.M. In each case, the y-axis represents a two-fold range of cell counts. Statistical analysis of the data was performed using a paired student's t-test with $p < 0.05$ considered statistically significant (n.s. = not significant).

TNF-α-induced migration was inhibited greater than 75% in the presence of an anti-VCAM-1 antibody ($p = 0.007$ relative to an isotype control) (Figure 1A). Similarly, HUVEC migration in response to TNF-α was completely inhibited in the presence of an anti-α4 antibody ($p = 0.025$ relative to an isotype control) (Figure 1B). Significant inhibition of migration also was demonstrated with a cyclic peptide antagonist of α4 integrins, TBC772,[18] included in the assay at a concentration of 10 μM ($p = 0.023$ relative to a scrambled peptide control TBC1194) (Figure 1C). In contrast, no significant inhibition of FGF-2-induced HUVEC migration was seen relative to controls in the presence of anti-VCAM-1, anti-α4 or TBC772 (Figures 1A–C).

HUVEC treated with TNF-α generate sVCAM-1. The above results suggested that HUVEC migration induced by TNF-α may be mediated by the interaction between VCAM-1 and its α4 integrin counter-receptor. TNF-α induces VCAM-1 expression on the surface of endothelial cells[12] whereas FGF-2 does not.[21] Since a soluble form of VCAM-1 previously has been demonstrated to induce endothelial cell migration,[14] levels of sVCAM-1 in culture media from cells treated with TNF-α were determined. Confluent monolayers of HUVEC in 35 mm plates were treated with cytokines under the same conditions used in the migration assays (4 h at 37°C with 50 ng/ml TNF-α or 10 ng/ml FGF-2). Culture media were collected and assayed for the presence of sVCAM-1 by ELISA. No sVCAM-1 was detected in media derived from untreated cells or cells treated with FGF-2. sVCAM-1 was detected in media from TNF-α treated cells at a concentration of 1.76 ± 0.06 ng/ml or ~20 pM.

Although α4 integrin has previously been reported to be expressed on the surface of HUVEC,[14,17] the presence of α4 on the HUVEC used for the experiments described herein was verified by flow cytometry. Figure 2 shows the relative fluorescence intensity profiles obtained with HUVEC in the presence of various antibodies. The profiles of HUVEC with mAbs directed against the leukocyte antigens CD3 (OKT3) (2.8% positive cells) or Leu 13 (35F9) (3.1% positive) served as negative controls and were essentially indistinguishable from that of FITC alone (2.2% positive). In contrast, there was a significant shift in the profile with a mAb against the α4 integrin subunit (L25) (28.1% positive). The anti-β1 mAb, 33B6, was used as a positive control (84.2% positive). These results demonstrate that α4 integrin receptors are indeed expressed on the surface of the HUVEC that were used for the experiments detailed above.

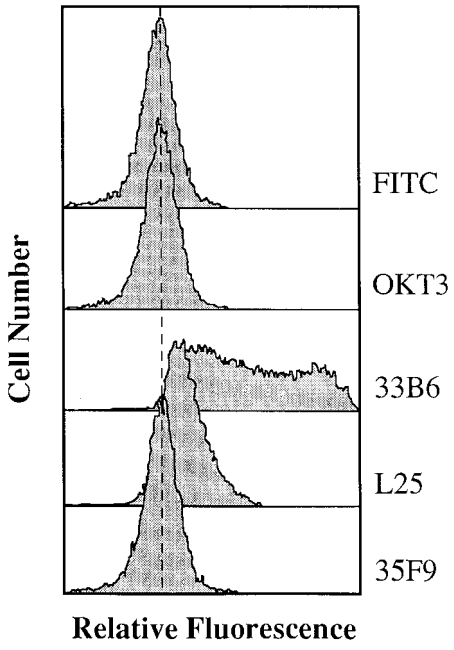

Figure 2. *HUVEC express α4 integrin.* HUVEC were incubated with anti-CD3 mAb (OKT3), anti-β1 mAb (33B6), anti-α4 mAb (L25), anti-Leu 13 mAb (35F9), or no primary mAb (FITC). Binding of mAbs to HUVEC was determined by FACS analysis.

Inhibition of α4 integrins inhibits TNF-α– but not FGF-2-induced angiogenesis in the rat cornea. The rat corneal model was used to determine if α4 integrins were involved in TNF-α-induced angiogenesis *in vivo*. Hydron pellets containing cytokines and either an anti-rat α4 integrin monoclonal antibody or an IgG1 control were implanted into rat corneas and analyzed 7 days later following perfusion of the blood vessels with India ink. The anti-α4 antibody blocked the angiogenic response induced by TNF-α in all six corneas tested (0 of 6 corneas were positive) whereas the control IgG1 had a negligible effect on the angiogenic response (7 of 9 corneas posi-

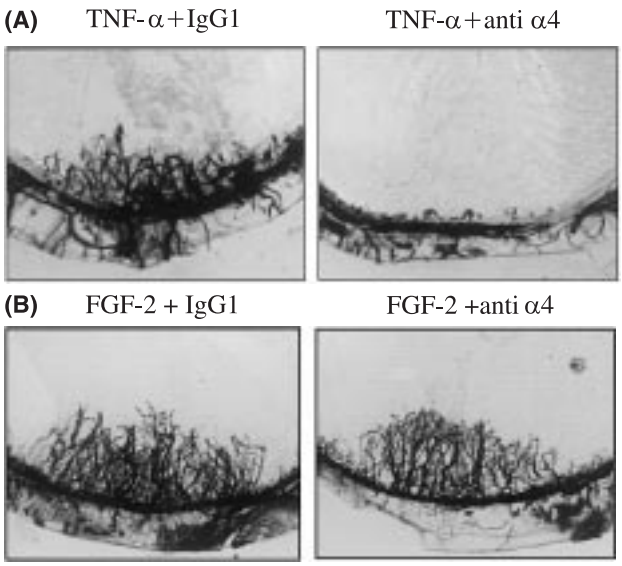

Figure 3. *Angiogenesis induced in rat corneas by TNF-α is inhibited by an anti-rat α4 antibody.* Hydron pellets containing 300 ng of TNF-α (A) or FGF-2 (B) were implanted into the corneas of Sprague-Dawley rats. Pellets also contained anti-rat α4 antibody or an IgG1 isotype control antibody (1.75 μg/pellet) as indicated. Seven days later, rats were perfused with colloidal carbon and the eyes excised and fixed.

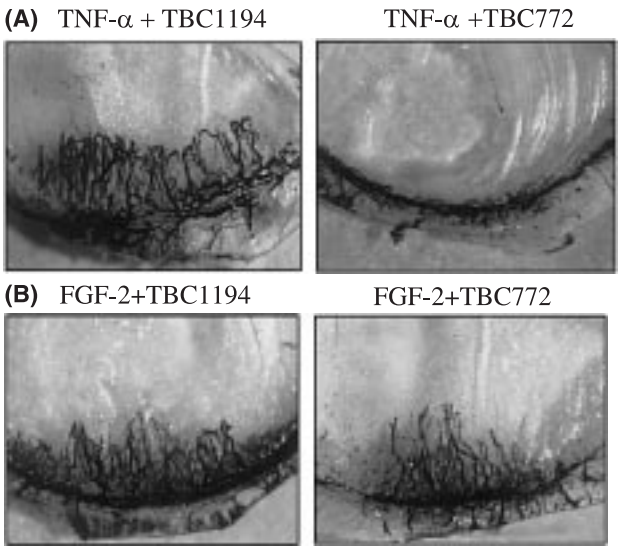

Figure 4. *Angiogenesis induced in rat corneas by TNF-α is inhibited by a peptide antagonist of α4 integrins.* Hydron pellets containing 300 ng of TNF-α4 (A) or FGF-2 (B) were implanted into the corneas of Sprague-Dawley rats. Pellets also contained a peptide antagonist of α4 integrins, TBC772, or a scrambled peptide control, TBC1194 (100 ng/pellet) as indicated. Seven days later, rats were perfused with colloidal carbon and the eyes excised and fixed.

Table 1. Results of rat cornea angiogenesis model using inhibitors of α4 integrins

Treatment	Cytokine (# of positive corneas/ total # of corneas tested)	
	TNF-α	FGF-2
Anti-α4 mAb	0/6	6/6
IgG1 control	7/9	5/5
TBC772	0/8	7/8
TBC1194	7/8	7/8

tive) (Figure 3A, Table 1). Similar results were obtained with the α4 peptide antagonist and control; angiogenesis induced by TNF-α was effectively inhibited with TBC772 (0 of 8 corneas positive) but not with the control peptide TBC1194 (7 of 8 corneas positive) (Figure 4A, Table 1). Conversely, neither the anti-rat α4 antibody nor TBC772 were effective inhibitors of angiogenesis induced by FGF-2 (Figures 3B, 4B and Table 1). The antagonist peptide did not prevent angiogenesis by non-specifically inhibiting TNF-α binding to its receptor; since no inhibition was detected in a TNF-α receptor binding assay at concentrations as high as 1 mM TBC772 (Figure 5A).

TNF-α-induced angiogenesis has been shown to be dependent on the integrin $\alpha v\beta 3$.[11] Although the peptide TBC772 is selective for the $\alpha 4$ integrins over $\alpha 5\beta 1$,[18] an assay was developed to determine if the peptide demonstrated any activity towards $\alpha v\beta 3$. The binding of HUVEC to human plasma vitronectin was determined to be inhibited by an anti-$\alpha v\beta 3$ antibody ($p = 0.0008$) and completely eliminated by a cyclic RGD peptide specific for the vitronectin receptor at a concentration of 10 μM ($p = 0.0001$) (Figure 5B). No inhibition of $\alpha v\beta 3$-dependent binding of HUVEC to vitronectin was detected in the presence of 1 mM TBC772 (Figure 5B).

Finally, TNF-α induces the expression of E-selectin on the surface of endothelial cells.[13] A soluble form of E-selectin has also been demonstrated to function as an angiogenic mediator with sLe^x being the likely counter-receptor.[14] As an additional analysis of specificity, TBC772 at 1 mM had no effect on the binding of sLe^x-expressing HL-60 cells to purified E-selectin (Fig-

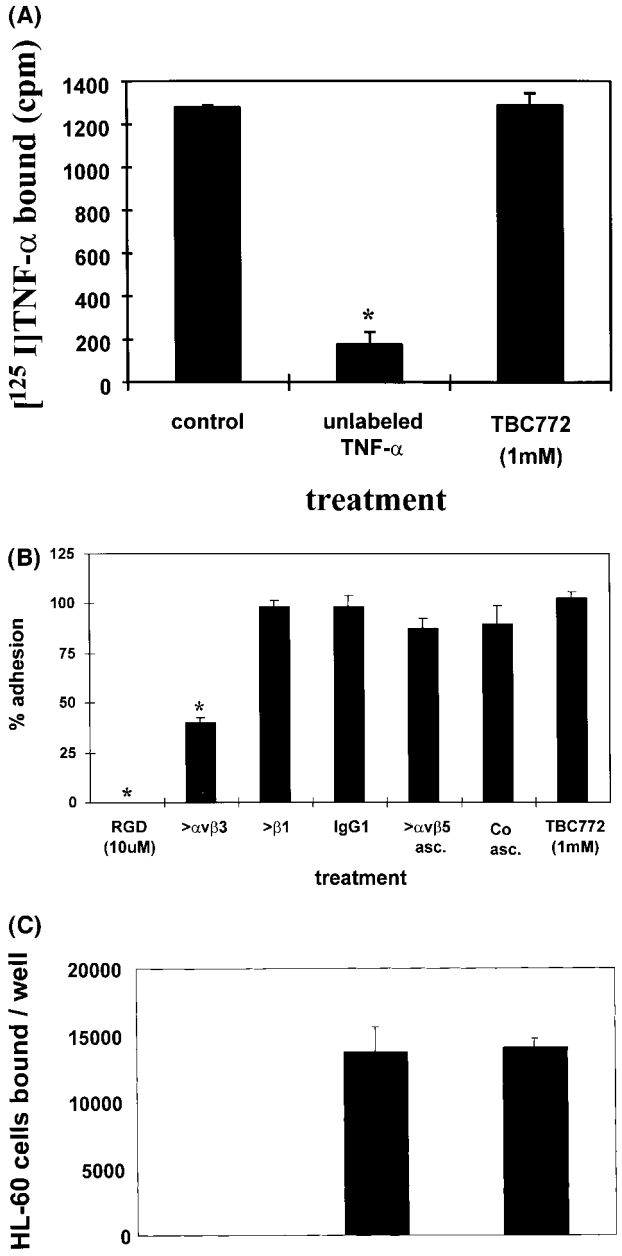

Figure 5. *TBC772 does not inhibit ligand binding to the TNF receptor, αvβ3, or E-selectin.* All assays were performed as described in 'Materials and methods'. Statistical analysis of the data in Figures 5A and 5B was performed using a paired Student's t-test. Treatment groups with p values less than 0.05 when compared to untreated controls were considered statistically significant and denoted with an asterisk (*). (A) [^{125}I] TNF-α binding to recombinant human TNF-α receptor extracellular domain was measured in the absence or presence of unlabeled TNF-α or 1 mM TBC772. TNF-α binding is expressed as the average cpm ± SD of duplicate wells. (B) Binding of fluorescently labeled HUVEC to human plasma vitronectin immobilized on a 96 well plate was measured in the absence or presence of 10 μM RGD peptide (GpenGRGDSPCA), 1 mM TBC772, anti-αvβ3 (10 μg/ml), anti-β1 (10 μg/ml), anti-αvβ5 ascites (1:100 dilution) or the proper isotype controls. The data are expressed as % adhesion relative to untreated HUVEC bound to vitronectin (typically 1600 cells bound/mm^2) versus bound to BSA (typically <10 cells bound/mm^2). The data represent the average % adhesion ± SD of triplicate wells. (C) Binding of fluorescently labeled HL-60 cells to an E-selectin-IgG fusion protein bound to magnetic beads was measured in the absence or presence of 1 mM TBC772. Background fluorescence of control mock beads (~10% of E-selectin beads) was subtracted from all values. The binding of the HL-60 cells to the E-selectin in this assay format previously has been shown to be blocked by sLe^x tetrasaccharide and anti-sLe^x antibodies.[20] The data represent the average number of cells bound per well ± SD of duplicate wells.

Table 2. Activity of TBC772 in cell adhesion assays[a]

Receptor/Counter-receptor	IC_{50} (μM)	Reference
α4β1/VCAM-1	0.4	18
α4β1/CS1	0.05	18
α4β7/MAdCAM-1	10	18
α5β1/Fibronectin	>1000	18
αvβ3/Vitronectin	>1000	This paper
sLe^x/E-selectin	>1000	This paper

[a] The cell adhesion assays measure the binding of integrin- or sLe^x -expressing cell lines to purified forms of the counter-receptors. All cell lines and counter-receptors are of human origin except the α4β7-expressing cell line and the MAdCAM-1 which are both of murine origin. IC_{50} values represent the concentration of compound required for inhibition of maximal cell binding by 50%.

ure 5C). The binding of the HL-60 cells to the E-selectin in this assay format has previously been shown to be blocked by sLe^x tetrasaccharide and anti-sLe^x antibodies.[20]

A summary of the activity of TBC772 in various adhesion assays is given in Table 2. Although the peptide antagonist is quite selective for the α4 class of integrins, the possibility remains that it may be active in the migration and corneal assays by interacting with other endothelial cell adhesion molecules or receptors yet to be analyzed.

Discussion

TNF-α is a pluripotent mediator effecting several cellular processes including adhesion, migration, angiogenesis and apoptosis.[6,12,22] The angiogenic potential of TNF-α may be due to the generation

of secondary mediators, including prostaglandins,[7] platelet activating factor,[8] the ligand for the Eck receptor tyrosine kinase, B61[9] as well as interlukin-8, VEGF and FGF-2.[10] The data presented here suggest that sVCAM-1 may also play a key role in TNF-α-induced angiogenesis.

Using endothelial cell migration assays as well as a rat corneal angiogenesis assay, we have demonstrated a role for the VCAM-1/α4 integrin pathway in TNF-α-mediated angiogenesis. HUVEC treated with TNF-α generated concentrations of sVCAM-1 (~20 pM) previously shown to induce significant levels of endothelial cell migration.[14] Antibodies directed against VCAM-1 and the α4 integrin subunit significantly inhibited the migration of HUVEC across collagen coated filters in response to TNF-α. *In vivo*, antibodies directed against rat α4 integrin effectively blocked TNF-α-induced angiogenesis in rat corneas. A peptide antagonist selective for the α4 integrins was also found to inhibit both HUVEC migration *in vitro* and corneal angiogenesis *in vivo* mediated by TNF-α. These data support the hypothesis that TNF-α can mediate angiogenic processes by inducing the production of sVCAM-1 from endothelial cells which can then function as an angiogenic stimulus via interaction with an α4 integrin counter-receptor on the endothelium.

Integrins, including αvβ3, have been clearly linked to TNF-α-induced angiogenesis.[11] How might the α4 integrins also be involved in regulating this process? sVCAM-1 binding to α4 integrin receptors on endothelial cells may lead to integrin-dependent signaling pathways regulating chemokinetic or chemotactic mechanisms. Here we show that inhibitors of the α4 integrins can inhibit the migration of HUVEC across collagen coated membranes. This class of integrins do not bind collagen suggesting an indirect role for the α4 integrins in this process. Recent reports have demonstrated that the engagement of one integrin can regulate the function of another in several diverse settings.[23–27] Antibodies to αvβ3 have been shown to block α5β1-dependent phagocytosis.[23] T cell coactivation induced by either β2 or β7 integrins could be inhibited by an anti-β1 antibody.[24] Engagement of αIIbβ3 inhibited the adhesive functions of the integrins α2β1 or α5β1 in transfected chinese hamster ovary cells.[25] Finally, ligation of LFA-1 on T-cells was found to regulate α4β1 and α5β1-mediated adhesion.[26] Migration of endothelial cells, a critical component of angiogenesis, is a complex process involving a series of integrin-mediated adhesion and de-adhesion events. Engagement of α4 integrins by sVCAM-1 may lead to cross-talk with other key integrins and play a role in coordinating these events. In fact, cross-talk between αvβ3 and α4β1 has recently been demonstrated in the regulation of lymphocyte migration on VCAM-1.[27]

Angiogenesis is also characterized by the production and activation of proteinases. Huhtala *et al.*[28] have demonstrated in rabbit synovial fibroblasts that the ligation of α4β1 can regulate the expression of matrix metalloproteinases induced by the interaction of the integrin α5β1 with fibronectin. Additionally, TNF-α induces the expression of urokinase plasminogen activator by endothelial cells which may promote angiogenesis.[29] The urokinase receptor has been demonstrated to associate with and regulate the function of certain integrins.[30] Reciprocally, integrins associated with the urokinase receptor, a glycosyl-phosphatidylinositol-linked protein, may serve as a means for urokinase receptor signal transduction. In this regard, it is important to note that it has yet to be determined if α4 integrins are associated with the urokinase receptor on endothelial cells. At the level of migration and proteolysis, the α4 integrins may be involved at several key steps in regulating angiogenesis induced by TNF-α. Further research is required to dissect the molecular details of such a mechanism.

The α4 integrins and/or their ligands have been implicated in several inflammatory disease states.[31] Monoclonal antibodies to the α4 subunit have proven to be effective treatments in animal models of asthma,[32] multiple sclerosis,[33] diabetes,[34,35] inflammatory bowel disease,[36] coronary allograft rejection[37] and rheumatoid arthritis.[38,39] Presumably these treatments have been successful by either preventing the adhesion and subsequent migration of inflammatory cells or by inhibiting activation of cell types critical for the progression of the inflammatory cascade. The data reported here suggest an additional mechanism by which an α4 integrin antagonist may be functioning in those inflammatory diseases that contain an an-

giogenic component. For example, rheumatoid arthritis is characterized not only by infiltration of inflammatory cells into the synovial tissue but also by proliferation and neovascularization of cells lining the synovium.[40] TNF-α has been reported to be one of the primary angiogenic mediators derived from monocytes/macrophages obtained from rheumatoid synovial tissue[2] and treatment of patients with recombinant soluble TNF-α receptor has been associated with improvement in the symptoms of rheumatoid arthritis.[41] The expression levels of VCAM-1 have been demonstrated to be upregulated in rheumatoid synovium.[42,43] Elevated levels of sVCAM-1 have been detected in the plasma and synovial fluid from arthritic patients.[44,45] Interestingly, the angiogenic potential of human rheumatoid synovial fluid as measured in the rat corneal model could be attenuated with antibodies directed against VCAM-1.[14] These results suggest that the production of sVCAM-1 by TNF-α may play a prominent role in the progression of angiogenic, inflammatory diseases.

Conclusion

TNF-α-induced endothelial cell migration *in vitro* and angiogenesis in rat corneas *in vivo* were inhibited by antibodies or by a small peptide antagonist directed against the VCAM-1/α4 integrin adhesion pathway. These data support a mechanism by which TNF-α induces angiogenesis via the generation of sVCAM-1 which then acts through an α4 integrin receptor expressed on the endothelial cell surface. Our results extend the findings of Koch *et al.*[14] implicating sVCAM-1 as an angiogenic mediator and strengthen the link between inflammatory cytokines, cellular adhesion, and angiogenesis.

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