Dual role for TWEAK in angiogenic regulation

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Summary

Angiogenic regulators modulate endothelial cell functions, including proliferation, migration, secretion, and adhesion, through their action on endothelial cells or other cell types. TWEAK, a novel member of the tumor necrosis factor family, appears to be a pro-angiogenic agent on the basis of previous studies demonstrating its ability to induce interleukin-8 production by epithelial tumor lines, stimulate proliferation of human vascular cell types and neovascularization in rat corneas. Here, we further characterized the angiogenic potential of TWEAK, revealing a dual role for TWEAK as an angiogenic regulator. We demonstrate that TWEAK is a potent inducer of endothelial cell survival and cooperates with basic fibroblast growth factor to induce the proliferation and migration of human endothelial cells and morphogenesis of capillary lumens. In contrast, TWEAK antagonizes the morphogenic response of endothelial cells to vascular endothelial growth factor (VEGF) without inhibiting VEGF-induced survival or proliferation. Thus, our observations suggest that TWEAK may differentially regulate microvascular growth, remodeling and/or maintenance in vivo, depending upon the angiogenic context.

Key words: TWEAK, Tumor necrosis factor-related, Angiogenesis, Basic fibroblast growth factor, Vascular endothelial growth factor

Introduction

The growth of microvessels is an integral component of tissue remodeling during a variety of normal and pathological events, such as the female reproductive cycle, fetal development, wound healing, inflammation and tumor progression (Folkman, 1995; Han and Liu, 1999). Angiogenesis is a tightly regulated process that involves the coordinated migration, proliferation, differentiation and morphogenetic organization of endothelial cells (EC) into new capillary structures. Further stabilization and maturation of neovessels occurs through the recruitment of adjacent mesenchymal cells to the vessel walls (Bussolino et al., 1997) and the maintenance of developing and quiescent vessels depends on the availability of appropriate survival signals. All of these angiogenic events are orchestrated by a network of extracellular factors, including several classes of cytokines, extracellular matrix and integrins, and by their cognate receptors.

A number of angiogenic regulators belong to the tumor necrosis factor (TNF) family. Ligands of this family are expressed as type II membrane proteins, which may be proteolytically cleaved to produce soluble cytokines (Smith et al., 1994). These ligands trigger biological activities by binding and signaling through their corresponding receptors in the TNF receptor family. The majority of the TNF family members mediate host defense, inflammation and immunological regulation (Locksley et al., 2001). In addition, some of these ligands regulate EC functions. For example, Fas/Fas ligand (FasL) interaction can induce endothelial capillary tube formation in vivo (Biancone et al., 1997), while vascular endothelial growth inhibitor (VEGI) or TL1 inhibits EC survival and proliferation in vitro (Yue et al., 1999; Zhai et al., 1999). TNF-α also modulates EC behavior; however, its effects are complex. TNF-α inhibits EC growth yet induces capillary tube formation in vitro (Frater-Schroder et al., 1987; Yoshida et al., 1997). It also can be antiangiogenic in the context of solid tumors (Ruegg et al., 1998) or angiogenic in corneal settings in vivo (Fajardo et al., 1992; Frater-Schroder et al., 1987; Leibovich et al., 1987; Yoshida et al., 1997).

TWEAK is a novel member of the TNF ligand family (Chicheportiche et al., 1997) that is broadly expressed at the mRNA level in many normal tissues and in nonlymphoid tumor cell lines. TWEAK was named for its TNF-relatedness, proinflammatory properties and weak ability to induce cell death (TNF weak). Although TWEAK protein expression and its physiological role(s) are still undefined, transfection studies suggest that TWEAK can function as a secreted cytokine, like TNF-α and Lymphotoxin α (LTα). The nature of the TWEAK receptor also remains controversial. One report described a biochemical association between TWEAK and death receptor-3 (DR-3) (Marsters et al., 1998), but this interaction has not been confirmed (Kapstein et al., 2000). In addition, TWEAK induces death in cells that lack DR3 mRNA (Schneider et al., 1999), suggesting the existence of an unidentified TWEAK receptor distinct from DR3. Accumulating evidence supports the original characterization of TWEAK as a proinflammatory cytokine. Recently, TWEAK was reported to be expressed by γ-interferon (IFNγ)-stimulated human peripheral blood monocytes and to play a role in monocyte cytotoxicity against tumor cells (Nakayama et al., 2000). TWEAK has also been shown to induce interleukin 8 (IL-8) in some tumor cell lines (Chicheportiche et al., 1997) and IL-6 and IL-8 in human astrocytes in vitro (Saas et al., 2000). However, additional data

have emerged indicating the proangiogenic potential of TWEAK. TWEAK was reported to induce the proliferation of human ECs and smooth muscle cells and neovascularization when implanted in rat corneas (Lynch et al., 1999), suggesting a positive role for TWEAK in vasculature formation in vivo.

In this study we further investigated the angiogenic potential of TWEAK. Our results demonstrate that TWEAK strongly promotes EC survival. Surprisingly, our results also show that TWEAK can be proangiogenic or anti-angiogenic depending upon the context. TWEAK promotes the proliferation, migration and morphogenesis of capillary lumens induced by basic fibroblast growth factor (bFGF). Conversely, TWEAK opposes EC morphogenic responses induced by vascular endothelial growth factor (VEGF). These results indicate that TWEAK may be an important angiogenic regulator that can modulate EC responses to other key angiogenic cytokines. Potential mechanisms underlying the bidirectional effects of TWEAK are discussed.

MATERIALS AND METHODS

Cells and culture conditions

Human umbilical vein endothelial cells (HUVEC) and human dermal microvascular endothelial cells (HDMEC) from Cell System Corporation (CS-C) (Kirkland, WA) or Clonetics (San Diego, CA) were routinely passaged in CS-C Complete medium, which contains 10% FBS and supplier growth supplement. Survival studies were performed in media purchased from CS-C, complete media (with 2% FBS and growth supplements) or incomplete media (0 or 2% FBS without growth supplements). For other experiments, Clonetics media were used: EC Basal Medium (EBM) containing 2% FBS and supplier growth supplements, defined herein as 'complete media' and EBM containing only 2% FBS, defined herein as 'basal media' and EC Basal Medium 2 (EBM-2) as indicated. Bovine brain extract (BBE) (Clonetics) was also used as indicated to supplement the basal media. The MS-1 and M210B4 cell lines were purchased from American Type Culture Collection (Manassas, VA). All other cell types were purchased from Clonetics. Mouse lymphoid cells were obtained 24 to 48 hours after in vivo activation with 200 μg/ml of anti-CD3 mAb (clone 2C11) (Pharmingen, San Diego, CA). Thioglycollate-induced peritoneal macrophages were stimulated in vitro for 48 hours with IFNγ (100 Units/ml), TNFα (10 ng/ml) or lipopolysaccharide (LPS) (1μg/ml).

Reagents and antibodies

Recombinant human bFGF was obtained as a supplier growth supplement (Clonetics), and bFGF and VEGF were purchased from R&D Systems (Minneapolis, MN) or Sigma (St Louis, MO). Annexin V-FITC and propidium iodide (PI) were from Pharmingen. Neutralizing mouse anti-human TNF and mouse anti-human IL-8 mAbs from R&D (Minneapolis, MN) and isotype-matched control Ig MOPC 21 (ICN Biomedicals Inc., Irvine, CA) were used for blocking studies. Biotin-conjugated anti-FLAG was from Eastman Kodak Company (New Haven, CT), and Phycoerythrin-Streptavidin (PE-Streptavidin) was from Southern Biotechnology Associates, Inc. (Birmingham, AL).

TWEAK-specific monoclonal antibodies

BE.B3, AB.G11 and AB.D3 were generated in Armenian hamsters using immunization with soluble human TWEAK protein and standard hybridoma generation procedures. The ability of AB.D3 and AB.G11 to bind to human and murine TWEAK, and the ability of BE.B3 to bind to human TWEAK, was demonstrated in an ELISA assay using recombinant soluble TWEAK proteins immobilized on 96-well microtiter plates. AB.D3 and AB.G11, but not BE.B3, were shown to have blocking activity on the basis of their ability to inhibit soluble FLAG-tagged human TWEAK binding to HT29 cells in a FACS analysis. BE.B3 was biotinylated using ImmunoPure Biotinylation kits following the manufacturer's protocol (Pierce, Rockford, IL). A hamster control Ig (clone Ha4/8-3.1) was obtained from the American Type Culture Collection, and the mAb was purified from culture supernatant using a Protein A Fast Flow column (Pharmacia, Piscataway, NJ).

Recombinant soluble human TWEAK protein

Recombinant soluble human TWEAK (GenBank accession number AF030099), containing amino-acid residues A106-H249, was expressed either with or without an N-terminal FLAG epitope in the yeast *Pichia pastoris* (Carlsbad, CA). The fermentation medium from *Pichia* expressing soluble human TWEAK was concentrated and dia-filtrated in 20 mM Tris-HCl, pH 8.0 and ion-exchanged on a Q Sepharose column prior to loading onto a Zn chelating column. The soluble TWEAK was eluted using an imidazole gradient in 20 mM sodium phosphate, 0.5 M sodium chloride, pH 7.5 prior to final fractionation by size exclusion using a Sephacryl 300 column.

Analysis of apoptosis

1×10^5 HUVEC or HDMEC were seeded in six well plates and incubated overnight in CS-C complete medium. Following a wash in PBS, cells were cultured in CS-C complete media or incomplete media (supplemented with 0% FBS and 0.1% bovine serum albumin (BSA) or 2% FBS) with or without VEGF (10 ng/ml) or TWEAK (200 ng/ml). Where indicated, 2 μg/ml of anti-TWEAK mAb AB.G11 or control Ig was also added. After 48 hours, cells were washed with PBS and detached by incubation with dispase (CS-C) for 15 minutes at 37°C followed by replacement with PBS containing 5mM EDTA and 0.1% BSA for 15 minutes at 37°C. After an additional wash in PBS, cells were stained with FITC-Annexin-V and 5 μg/ml PI according to the supplier. Fluorescence was analyzed using FACStarPLUS (Becton Dickinson, San Jose, CA). Results are reported as the frequency of viable cells (Annexin and PI negative) and apoptotic cells (Annexin positive, PI negative and positive).

Proliferation assay

HUVEC in 96 well microtiter plates at subconfluence (4300 cells per well) were cultured overnight in CS-C medium. The medium was replaced with complete medium or with basal medium with or without BBE, TWEAK (100-200 ng/ml), a 1/500-1/1000 dilution of the bFGF supplement (Clonetics) or 1 ng/ml bFGF (R&D Systems), VEGF (10 ng/ml) or combinations of these factors. Where indicated, 10 μg/ml anti-TWEAK mAbs AB.D3, BE.B3 or hamster control Ig Ha4/8 were also added. Following a three day incubation, proliferation was measured by a 10 hour pulse with [^{3}H]-thymidine.

Endothelial wound repair assay

A standard wound repair assay was employed as previously described (Morales et al., 1995). Briefly, a confluent monolayer of HUVEC was grown in CS-C complete medium in 35×10 mm cell culture dishes with 2 mm grids (Nalge Nunc International, Naperville, IL). The monolayer was wounded by two perpendicular strokes across the diameter of the dish with a 1 mm tip. The consistency of this methodology was shown by directly quantifying the width of the starting wound using a computerized measuring tool; variation was only 7%. Dislodged cells were aspirated, and plates were rinsed with PBS. Cells were cultured for 18 hours in complete media or in basal media as defined above with or without TWEAK (200 ng/ml), bFGF

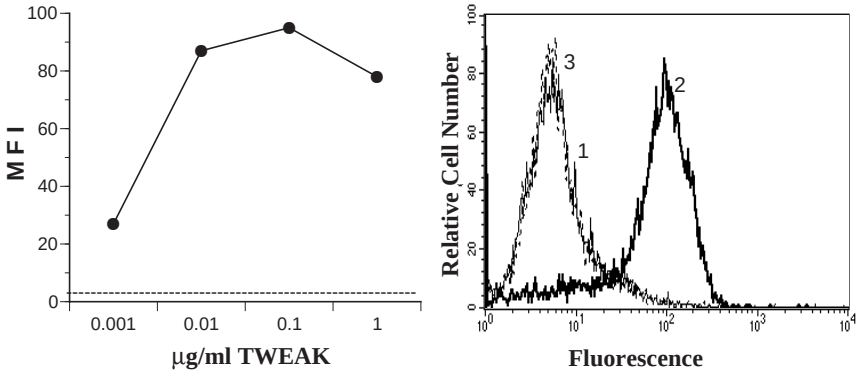

Fig. 1. TWEAK binding to HUVECs is dose-dependent and specific. Mean fluorescence intensity (MFI) versus TWEAK concentration is shown (left), with the dotted line indicating the background MFI with the indirect detection step alone. The traces (right) correspond to (1) background fluorescence with indirect detection step alone, (2) TWEAK binding and (3) inhibition of TWEAK binding by the AB.D3 mAb.

(1/1000 or 1 ng/ml), VEGF (10 ng/ml) or combinations of these before fixing with 1% paraformaldehyde followed by staining with Harris Hematoxylin (Sigma, St Louis, MO). Wound repair was quantified by visually counting the number of grids in which the gap was obscured by migrating cells. This number was divided by the total number of grids that lined the wound, and the results were expressed as mean percentage wound repair ± s.e.m.

Immunofluorescent staining

Cells were analyzed for TWEAK binding by incubation with FLAG-TWEAK, and binding was detected with either biotinylated mouse anti-FLAG mAb or biotinylated BE.B3 and streptavidin-PE. Cold competition was performed with FLAG-TWEAK at 100 ng/ml and increasing concentrations of nontagged TWEAK, and binding was detected with the biotinylated mouse anti-FLAG mAb. Blocking of TWEAK binding by the AB.D3 Ab was performed by preincubation of FLAG-TWEAK with 10 μg/ml of mAb.

Capillary tube formation assay

Capillary tube formation by ECs was analyzed using a fibrin matrix gel assay based on a method that had been previously described (Mach et al., 1999). Briefly, 4 mg/ml plasminogen-free human fibrinogen (Calbiochem, San Diego, CA) was dissolved in serum-free EBM-2 media with heparin and polymixin B both at 1 μg/ml (Sigma) as well as with the supplier supplements except for VEGF and bFGF. The fibrin solution was filter sterilized, and fibrin matrices were prepared by adding thrombin (20-50 milliunits/ml) (Sigma) and distributing 300 μl per well in 24 well plates. HUVEC (4×10^4 cells/cm^2) were seeded onto the gel surfaces and overlayed with EBM-2 media as above, plus 5% FBS in the presence or absence of TWEAK, bFGF, VEGF or combinations of these factors. TWEAK was used at 1 μg/ml or 100 ng/ml, bFGF at 100 ng/ml and VEGF at 50 ng/ml. In some experiments, neutralizing mAbs specific for TNF (1 μg/ml) and IL-8 (10 μg/ml) or isotype control Ig were used. After 48-72 hours of culture, phase-contrast photomicrographs of the gel surface were taken. Gels were examined for the number of capillary sprouts by a blinded investigator. Gels were fixed with 10% ethanol for 10 minutes, transferred from the original wells to new wells, fixed with 4% paraformaldehyde, embedded in paraffin, cross sectioned (5 μm sections) and stained with hematoxylin and eosin.

Results

TWEAK binding is cell-type restricted

In order to determine which primary cells might be targets for TWEAK activity, we surveyed various cell types for their ability to bind to recombinant soluble human TWEAK by immunofluorescent staining. TWEAK did not bind to any leukocytes, neither freshly isolated nor activated under a

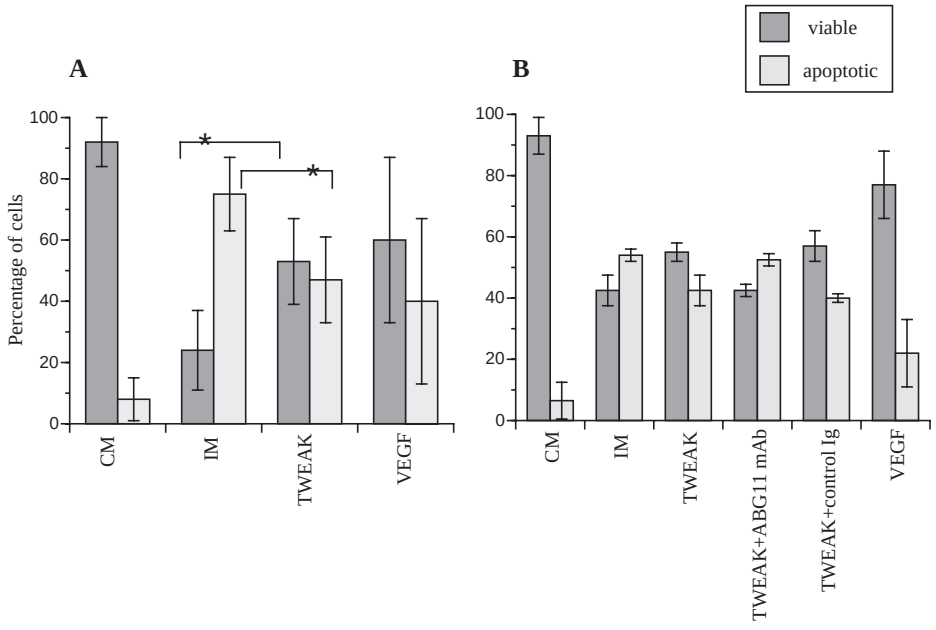

Fig. 2. Effect of TWEAK on survival of (A) HUVEC and (B) HDMEC. ECs were cultured for 48 hours in CS-C complete media (CM) or incomplete media (IM) with or without TWEAK or VEGF. Anti-TWEAK mAb AB.G11 and control Ig were added as specified on the bar graphs. Cells were stained with FITC-Annexin-V and PI, and the percentage of viable and apoptotic cells is indicated. The results with HUVEC and HDMEC are the mean percentages ±s.d. of n=4 and n=2 experiments, respectively. HUVEC cultures with TWEAK versus IM were significantly different with respect to the percentage of viable cells and the percentage of apoptotic cells ($p<0.05$ by Student's t test for each comparison indicated by asterisks).

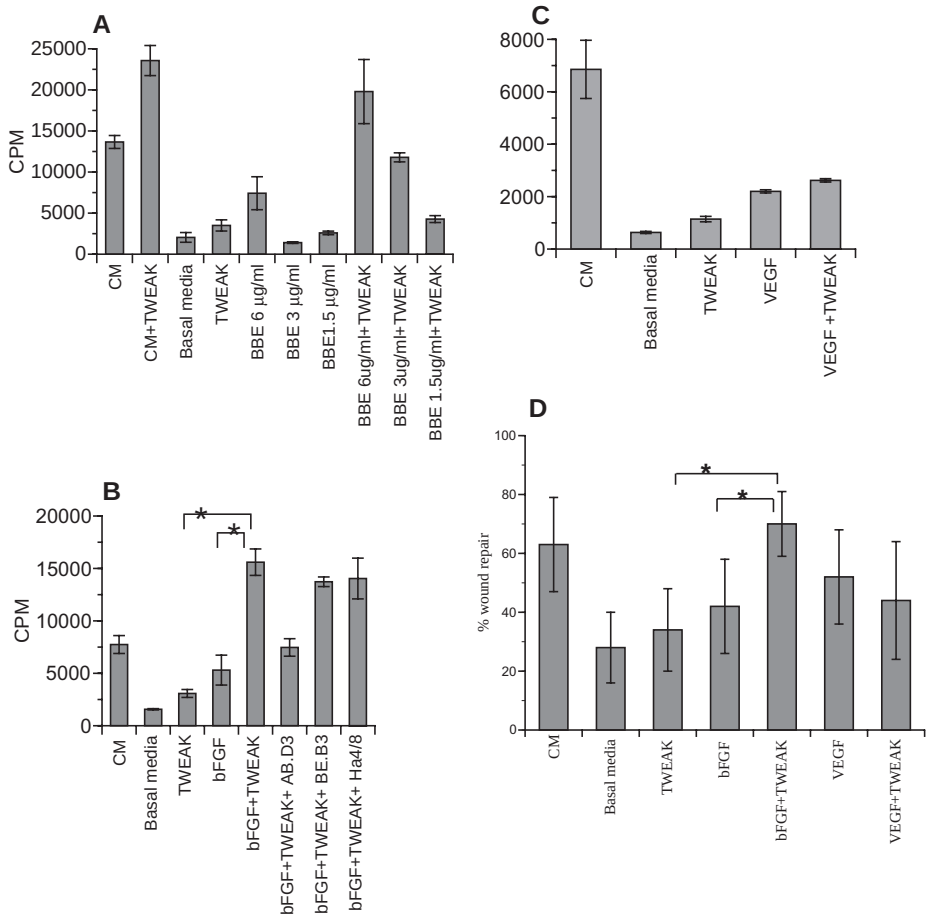

Fig. 3. TWEAK enhances bFGF-dependent proliferation and migration but has no effect on VEGF-dependent proliferation and migration. HUVEC were cultured for three days in complete media (CM) or in basal media with (A) TWEAK, BBE or BBE + TWEAK, and (B) TWEAK, bFGF or combinations of these factors as indicated, and proliferation was measured by [^{3}H]-thymidine incorporation. Data shown are the mean c.p.m. value ±s.d. of triplicate wells. Results in (A) and (B) are representative of two and four independent experiments, respectively. The proliferation in bFGF- + TWEAK-treated cultures was significantly different from that of cultures with bFGF alone or TWEAK alone (indicated by asterisks) or basal media (p values <0.05 by Student's *t* test), and the difference between cultures in basal media with and without TWEAK was not significant. Blocking anti-TWEAK mAb AB.D3, nonblocking anti-TWEAK mAb BE.B3 and a control Ig Ha4/8 were also added where indicated, with results representative of two independent experiments. (C) HUVECs were cultured as described above and TWEAK, VEGF or combinations of these factors were added to basal media as indicated. Results shown are the mean c.p.m. value ±s.d. of triplicate wells and are representative of three independent experiments. The difference between VEGF and VEGF + TWEAK was not significant. (D) Confluent HUVEC monolayers treated with TWEAK, bFGF, VEGF or combinations of these factors were wounded, and repair was measured after 18 hours of culture. Results shown are the average of 6-10 experiments ±s.d. The percentage wound repair was significantly higher (Student's *t* test, p<0.05) with TWEAK + bFGF compared with bFGF or TWEAK alone (indicated by asterisks) or basal media. The percentage wound repair with VEGF was significantly higher than with basal media or TWEAK but not different from that with VEGF + TWEAK.

variety of conditions (Table 1). However, TWEAK bound at relatively high levels to human venous and aortic ECs, aortic smooth muscle cells, embryonic myoblasts and to a lower degree to lung fibroblasts. Similarly, TWEAK bound to murine EC and fibroblast cell lines. As illustrated with HUVECs, FLAG-TWEAK binding was dose-dependent and specific on the basis of blocking with the AB.D3 mAb and by cold competition with an independent TWEAK preparation (Fig. 1 and data not shown). Thus, our results demonstrate that receptors for TWEAK are expressed on a variety of nonhematopoeitic cell types, including ECs.

TWEAK promotion of EC survival

The effect of TWEAK on EC survival was tested by culturing HUVEC in incomplete media in the absence or presence of recombinant soluble TWEAK. Cell viability under these conditions was measured by standard double staining with Annexin-V and PI dye exclusion. As shown in Fig. 2A, cells cultured in CS-C complete media remained viable at 48 hours (92% were viable), whereas in cultures without additional factors the viable cells decreased to 25% and a significant fraction of apoptotic cells appeared (75%). However, in the presence of TWEAK, HUVECs remained largely protected from apoptosis (53% were viable). The difference between cultures treated with TWEAK and without TWEAK was statistically significant, and the degree of EC survival with TWEAK approached that achieved with VEGF. We also found that the frequency of viable cells was somewhat higher in TWEAK + VEGF-treated cultures as compared to TWEAK or VEGF alone (data not shown). The survival of HDMEC in incomplete media also appeared to be enhanced by TWEAK but to a lesser degree than HUVECs, and this activity was specifically inhibited by anti-TWEAK mAb AB.G11 (Fig. 2B). Thus, TWEAK promotes the survival of human EC in vitro.

TWEAK enhances bFGF-dependent EC proliferation and migration

We further examined the effect of TWEAK on proliferation by measuring [^{3}H]-thymidine incorporation in HUVEC cultured in basal media with TWEAK, alone or in combination with key angiogenic factors. We first demonstrated that EC proliferation was enhanced by addition of TWEAK to complete media or

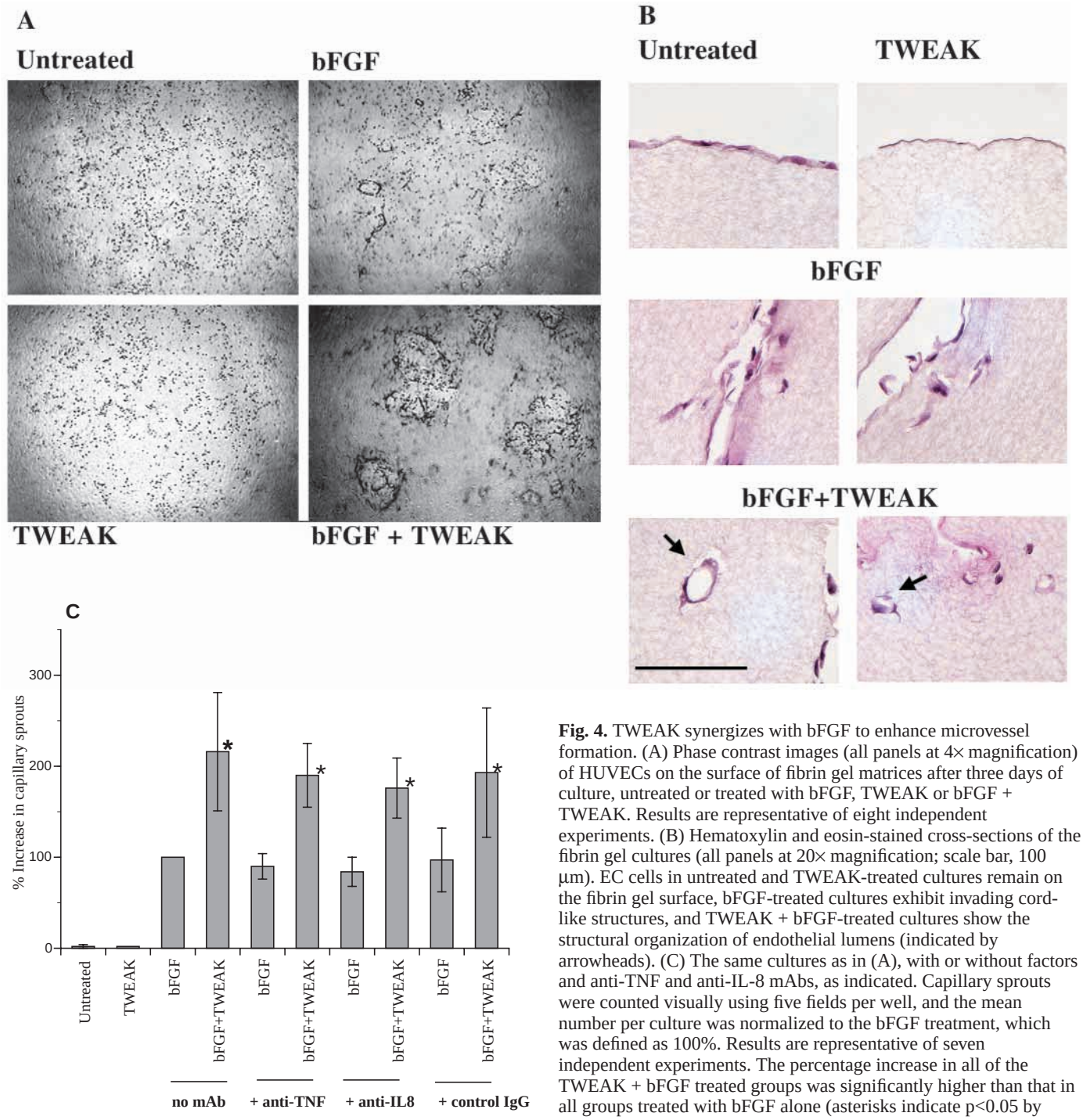

Fig. 4. TWEAK synergizes with bFGF to enhance microvessel formation. (A) Phase contrast images (all panels at 4× magnification) of HUVECs on the surface of fibrin gel matrices after three days of culture, untreated or treated with bFGF, TWEAK or bFGF + TWEAK. Results are representative of eight independent experiments. (B) Hematoxylin and eosin-stained cross-sections of the fibrin gel cultures (all panels at 20× magnification; scale bar, 100 μm). EC cells in untreated and TWEAK-treated cultures remain on the fibrin gel surface, bFGF-treated cultures exhibit invading cord-like structures, and TWEAK + bFGF-treated cultures show the structural organization of endothelial lumens (indicated by arrowheads). (C) The same cultures as in (A), with or without factors and anti-TNF and anti-IL-8 mAbs, as indicated. Capillary sprouts were counted visually using five fields per well, and the mean number per culture was normalized to the bFGF treatment, which was defined as 100%. Results are representative of seven independent experiments. The percentage increase in all of the TWEAK + bFGF treated groups was significantly higher than that in all groups treated with bFGF alone (asterisks indicate $p<0.05$ by Dunnett's method), and there was no significant effect of anti-TNF, anti-IL-8 or control Ig.

basal media supplemented with BBE (Fig. 3A), in agreement with previously reported results (Lynch et al., 1999). However, TWEAK alone did not significantly increase HUVEC proliferation. In order to dissect the basis for the enhanced proliferation by TWEAK in cultures containing growth supplements, we tested the effect of TWEAK in combination bFGF, a known ingredient of BBE. We found that TWEAK + bFGF significantly enhanced EC proliferation compared with bFGF alone (Fig. 3B). The level of [^{3}H]-thymidine incorporation achieved was comparable to or greater than that of ECs cultured in complete media. The synergistic activity of TWEAK with bFGF was completely inhibited by anti-TWEAK mAb AB.D3, indicating that the effect of TWEAK was specific, whereas there was no inhibition by a nonblocking anti-TWEAK mAb BE.B3 or a control Ig. Thus TWEAK enhances bFGF-dependent EC proliferation. By contrast, TWEAK did not affect the proliferative response to VEGF (Fig. 3C).

The ability of TWEAK to affect EC migration also was evaluated in the presence and absence of either bFGF or VEGF. Confluent HUVEC monolayers were wounded and EC migration was monitored within the first 18 hours by determining the degree of wound repair. Addition of TWEAK or bFGF to the basal media induced a low level of wound repair, whereas cultures treated with both TWEAK and bFGF were repaired significantly more than cultures in basal media or with either agent alone (Fig. 3D). There was no significant effect of TWEAK on VEGF-induced wound repair. Thus TWEAK cooperates selectively with bFGF to enhance EC wound repair.

Differential effect of TWEAK on EC morphogenesis induced by bFGF and VEGF

The growth of microvessels involves the coordinated proliferation, migration and morphogenetic organization of ECs into capillary tubes. The effect of TWEAK on morphogenic activity was assessed using EC cultures seeded onto the surface of fibrin gels in the presence or absence of bFGF or VEGF. We found that bFGF, but not TWEAK alone, induced morphological changes in the EC monolayer on the fibrin gel surface as evidenced by phase-contrast microscopy (Fig. 4A). Interestingly, addition of TWEAK to bFGF significantly enhanced these morphogenic changes, inducing about a two-fold increase in the number of capillary sprouts (Fig. 4A,C). Furthermore, histological analysis of cross-sections perpendicular to the matrix surface revealed that bFGF and bFGF + TWEAK, but not TWEAK alone, also promoted EC invasion into the fibrin matrix. However, only TWEAK + bFGF resulted in the formation of lumen-containing structures (Fig. 4B). Specifically, we counted 17 such structures in a total of 30 fields in cross-sections from the TWEAK + bFGF treated cultures versus one lumen-containing structure in those from cultures with bFGF alone. Similar results were obtained with several different EC types, including HUVECs, HDMEC, human pulmonary artery EC and human lung microvascular EC; the effect of TWEAK was specifically blocked by anti-TWEAK mAbs. No stimulation of lumen morphogenesis was observed when TWEAK was substituted for by another TNF family member, CD40L (data not shown). Since TWEAK has been shown to induce the production of IL-8 and TNF in cultured cell lines (Chicheportiche et al., 1997; Schneider et al., 1999), we examined whether the cooperation between TWEAK and bFGF was a secondary effect involving these angiogenic modulators (Koolwijk et al., 1996; Yoshida et al., 1997). As shown in Fig. 4C, the number of capillary sprouts formed by TWEAK + bFGF was not reduced in the presence of neutralizing mAbs for these factors, indicating that neither TNFα or IL-8 are required for the cooperative effect. TNF was produced at <1 ng/ml and IL-8 at 1-2 ng/ml in these cultures, and the ability of the respective mAbs to neutralize these concentrations of TNF and IL-8 independently was confirmed by inhibition of TNF-induced tumor cell killing (data not shown) and neutralization of IL-8 in a cell culture model (Terui et al., 1998).

VEGF also induced EC morphogenesis, as evidenced by the reorganization of the human EC monolayers on the fibrin gel surface. Fig. 5 clearly shows the ECs lining up in an organized fashion in response to VEGF (Fig. 5). However, the appearance

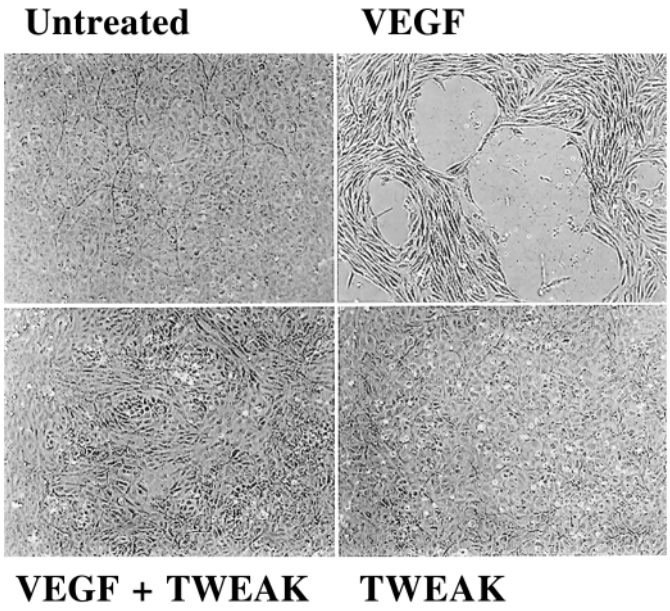

Fig. 5. TWEAK antagonizes the morphogenic response of ECs to VEGF. Phase contrast images (all panels at 10× magnification) of human EC on the surface of fibrin gel matrices after three days of culture, untreated or treated with VEGF, TWEAK or TWEAK + VEGF. Results are representative of 13 independent experiments.

of the structures induced by VEGF was qualitatively different from those induced by bFGF, and VEGF did not induce EC invasion into the fibrin matrix. Interestingly, confluent EC monolayers treated with TWEAK + VEGF did not reorganize and remained as confluent monolayers similar in appearance to untreated cultures and cultures treated with TWEAK alone. Similar results were obtained with HUVEC, human pulmonary artery EC and human lung and dermal microvascular ECs, and the effect of TWEAK was specifically inhibited by anti-TWEAK mAbs (data not shown). Thus TWEAK cooperates with bFGF to induce the formation of capillary-like structures but antagonizes the morphogenic response of HUVECs to VEGF.

Discussion

Previously TWEAK was reported to induce EC proliferation in vitro and corneal neovascularization (Lynch et al., 1999). In this report, we have further characterized the angiogenic potential of TWEAK, revealing for the first time a dual role for TWEAK in angiogenic regulation through its ability to modulate EC responses to key angiogenic cytokines. We demonstrate that TWEAK acts cooperatively with bFGF to promote several aspects of EC behavior that are central to microvascular growth, such as EC proliferation, migration and capillary tube morphogenesis. Conversely, we found that TWEAK antagonizes the morphogenic response of ECs to VEGF but has no measurable effect on EC proliferation in the presence of VEGF. Although TWEAK alone has no significant effect on proliferation, migration or morphogenesis, our studies also reveal that TWEAK can potently induce EC survival. Thus, our studies elucidate three novel findings: TWEAK can promote EC survival, TWEAK can modulate EC responses to

Table 1. TWEAK binding[a] to primary cell types

Human cell types	TWEAK binding
HUVEC	+++
Aortic EC	++
Aortic smooth muscle cells	+++
Lung fibroblasts	+
Embryonic myoblasts	++
Peripheral blood lymphocytes	–
Peripheral blood dendritic cells	–
Murine cell types	
EC cell line (MS-1)	++
Bone marrow fibroblast line (M210B4)	+
Spleen cells with and without anti-CD3 mAb activation	–
Lymph node cells with and without anti-CD3 mAb activation	–
Thymocytes with and without anti-CD3 mAb activation	–
Resident peritoneal macrophages	–
Thioglycollate-induced macrophages (with and without LPS, TNF or IFNγ stimulation)	–

[a]Relative binding compared to HUVECs as measured by mean fluorescence intensity.

other angiogenic cytokines and the action of TWEAK may be pro-angiogenic or anti-angiogenic, depending upon the particular angiogenic context.

Endothelial survival is critical for a sustained angiogenic process, which includes vessel sprouting, maturation and stabilization, and for long-term maintenance of newly formed vessels (Carmeliet, 2000). Factors such as VEGF, bFGF and Angiopoietin-1 have been shown to promote EC survival upon withdrawal of nutrients or serum (Nor et al., 1999). Using similar conditions, we demonstrate that TWEAK also promotes survival of EC. In addition, we found that TWEAK markedly enhances EC proliferation in BBE-supplemented media, in complete agreement with previously reported results (Lynch et al., 1999). In contrast, the effect of TWEAK alone, assessed here for the first time, was not significant. Importantly, using more defined culture conditions our studies reveal that TWEAK enhances EC proliferation induced by bFGF. Thus the proliferative activity of TWEAK under previous conditions presumably reflects its cooperativity with bFGF or other components. It is also possible that cooperativity between TWEAK and another cytokine/growth factor underlies the stimulatory effect of TWEAK observed in the corneal micropocket assay (Lynch et al., 1999). which is characterized by post-surgical inflammation. TWEAK also enhanced bFGF-dependent EC wound repair. This effect is probably due to stimulation of EC migration rather than proliferation, as we found no increase in the number of cells recovered (relative to input) after one day of culture with bFGF alone or bFGF + TWEAK (data not shown). We also found no changes in EC-surface expression of a variety of integrins such as α_v, α_1, α_2, α_5, β_3 and β_1, which have been implicated in angiogenesis (Eliceiri and Cheresh, 1999; Senger et al., 1997), in cultures with the TWEAK/bFGF combination as compared with either agent alone (data not shown). The enhanced organization of ECs into lumen-containing structures within the fibrin gel matrix suggests an additional level of cooperation between TWEAK and bFGF, perhaps through pathways that mediate EC invasion, polarity or the formation of tight intercellular junctions. We propose two possible models to explain the cooperativity between TWEAK and bFGF. bFGF may be required to upregulate the TWEAK receptor, enabling TWEAK to induce a range of angiogenic activities. Alternatively, signaling through the TWEAK receptor may amplify or be amplified by bFGF-induced signals. Interestingly, although the identity of the TWEAK receptor is yet to be reported, our preliminary data indicate that it can activate NFκB and MAP kinase pathways in ECs (data not shown). Further experiments are required to define the mechanism(s) underlying the cooperativity between TWEAK and bFGF, and this will be the subject of future studies.

In contrast with the pro-angiogenic activity of TWEAK in the context of bFGF, we found that TWEAK can inhibit the angiogenic behavior of ECs in other settings. We observed TWEAK inhibition of EC morphogenesis induced by VEGF. We hypothesized that TWEAK might inhibit EC morphogenesis, but not survival or proliferation induced by VEGF, by reducing the expression of VEGF receptors, particularly Flt-1 (VEGFR-1), on the basis of the phenotype of Flt-1-deficient mouse embryos whose ECs fail to organize into tube-like structures (Korpelainen and Alitalo, 1998; Fong et al., 1995). In addition, previous studies have indicated that Flt-1 mediates chemotactic but not proliferative responses, whereas the Flk-1/KDR receptor (also called VEGFR-2) mediates both proliferative and chemotactic signaling (Thomas, 1996). However, we found only a modest 30% reduction in the expression of Flt-1 mRNA and no detectable reduction in mRNA for other angiogenic molecules, including Flt-4, TIE, TIE2, thrombin receptor, CD31 and endoglin, as measured in EC cultures with or without TWEAK, VEGF or VEGF + TWEAK (data not shown). It will be important to examine the effect of TWEAK on Flt-1 protein levels. We also analyzed the cell-surface expression of a variety of integrins (as above) and found them to be similar in cultures treated with TWEAK and VEGF as compared to VEGF alone (data now shown). Further analyses are required to delineate the mechanism of inhibition by TWEAK of VEGF-induced morphogenesis.

The dual role of TWEAK as an angiogenic regulator is reminiscent of the context-dependent role of TNF. Although the role of TWEAK as an angiogenic regulator cannot be deduced fully from in vitro studies, our data, together with the TNF paradigm, support the hypothesis that TWEAK can differentially modulate angiogenesis depending upon the particular angiogenic setting. TWEAK is ubiquitously expressed at the mRNA level. However, we have not detected TWEAK protein in normal tissues, tumor cell lines or HUVECs to date. The reported expression of TWEAK protein by IFNγ-stimulated human monocytes (Mach et al., 1999) supports the possibility that angiogenesis may be modulated by TWEAK produced by infiltrating macrophages in the context of inflammatory responses.

In summary, our studies reveal a dual role for TWEAK in angiogenic regulation. We demonstrate that TWEAK promotes EC survival and regulates EC proliferation, migration, and morphogenesis in vitro through modulation of EC responses to bFGF and VEGF. These data suggest that TWEAK may play a role in the regulation of microvascular growth, remodeling, and/or maintenance in vivo, being pro-angiogenic or anti-angiogenic depending on the angiogenic microenvironment. Agonists or antagonists of the TWEAK pathway may provide useful therapeutic approaches to treatment in settings of ischemic injury, cancer, angioproliferative and inflammatory disorders.

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