

TWEAK Is an Endothelial Cell Growth and Chemotactic Factor That Also Potentiates FGF-2 and VEGF-A Mitogenic Activity

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Objective—TWEAK, a member of the tumor necrosis factor superfamily, binds to the Fn14 receptor and stimulates angiogenesis in vivo. In this study, we investigated Fn14 gene expression in human endothelial cells (ECs) and examined the effect of TWEAK, added either alone or in combination with fibroblast growth factor-2 (FGF-2) or vascular endothelial growth factor-A (VEGF-A), on EC proliferation, migration, and survival in vitro. We also determined whether a soluble Fn14-Fc fusion protein could inhibit TWEAK biologic activity on ECs and investigated TWEAK signal transduction in ECs.

Methods and Results—We found that both FGF-2 and VEGF-A could induce Fn14 mRNA expression in ECs. TWEAK was a mitogen for ECs, and this proliferative activity could be inhibited by an Fn14-Fc decoy receptor. Furthermore, TWEAK treatment activated several intracellular signaling pathways in ECs and potentiated FGF-2- and VEGF-A-stimulated EC proliferation. TWEAK also had EC chemotactic activity, but it did not promote EC survival.

Conclusions—These results indicate that TWEAK is an EC growth and migration factor but not a survival factor. TWEAK can also enhance both FGF-2 and VEGF-A mitogenic activity on ECs. Thus, TWEAK may act alone as well as in combination with FGF-2 or VEGF-A to regulate pathological angiogenesis. (*Arterioscler Thromb Vasc Biol.* 2003;23:594-600.)

Key Words: TWEAK ■ Fn14 ■ fibroblast growth factor ■ vascular endothelial growth factor ■ endothelial cells

TWEAK was first described by Chicheportiche et al¹ in 1997 as a new member of the tumor necrosis factor (TNF) superfamily of structurally related cytokines.^{2,3} The human TWEAK gene is expressed in many different cell types and encodes an ≈30-kDa type II transmembrane protein that can be cleaved to generate an ≈18-kDa soluble factor with biologic activity.^{1,4} TWEAK has been shown to promote various cellular responses in vitro, including cell proliferation,⁵⁻⁷ migration,⁶ survival,⁸ apoptosis,^{1,4,9-13} and necrosis.¹⁰ TWEAK was recently identified as a ligand for Fn14,¹⁴ a growth factor-inducible type I transmembrane protein initially discovered by our group several years ago.^{15,16} Fn14 is the smallest reported member of the TNF receptor (TNFR) superfamily, with a single cysteine-rich domain in its 53-amino acid extracellular region and a short 28-amino acid cytoplasmic tail.¹⁴⁻¹⁶

Several previous studies have indicated that TWEAK and its receptor Fn14 may play a role in vascular endothelial cell (EC) biology. Lynch et al⁵ reported in 1999 that TWEAK was a mitogenic factor for human ECs and could stimulate angiogenesis in the rat cornea. In 2001, Wiley et al¹⁴ reported that TWEAK-Fn14 interactions were important for phorbol myristate acetate- and epidermal growth factor-stimulated EC migra-

tion in vitro as well as fibroblast growth factor-2 (FGF-2)-mediated angiogenesis in the mouse cornea. Jakubowski et al⁸ reported in 2002 that TWEAK was a survival factor but neither a mitogen nor a chemotactic factor for ECs. They also reported that TWEAK cotreatment potentiated FGF-2 activity but inhibited vascular endothelial growth factor-A (VEGF-A) activity in an in vitro angiogenesis assay. Finally, Harada et al⁶ recently reported that TWEAK could stimulate EC proliferation and migration and that these effects could be inhibited by an anti-Fn14 monoclonal antibody.

In this article, we report our findings regarding Fn14 gene regulation, TWEAK biologic activity, and TWEAK signal transduction in human ECs cultured in vitro. Our results indicate that TWEAK is an EC growth and migration factor that can also potentiate FGF-2 and VEGF-A mitogenic activity.

Methods

Cell Culture, RNA Isolation, and Northern Blot Hybridization Analysis

Please see the expanded Methods section (available online at <http://www.ahajournals.org>).

Received January 22, 2003; revision accepted February 5, 2003.

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Arterioscler Thromb Vasc Biol. is available at <http://www.atvbaha.org>

DOI: 10.1161/01.ATV.0000062883.93715.37

Proliferation Assays

Cells were seeded at a density of either 5.3×10^2 cells/cm² (human umbilical vein ECs [HUVECs]) or 15.9×10^2 cells/cm² (human microvascular ECs [HMECs]) in EC growth medium and allowed to attach overnight. The next day, the medium was aspirated and replaced with endothelial proliferation medium (EPM) composed of endothelial cell basal medium (EBM)-2, 5% FBS, and $1 \times$ ascorbic acid (BioWhittaker) for 24 hours. On days 0 and 2, the cells were either left untreated in EPM or treated with EPM containing 1 or more of the following: 10, 50, 100, or 150 ng/mL TWEAK; 30 ng/mL VEGF-A; 10 ng/mL FGF-2; 2.5 μ g/mL mouse Fn14-Fc soluble receptor (see below); or 2.5 μ g/mL mouse IgG1 (BD PharMingen). TWEAK was used at 50 ng/mL in both the growth factor and the Fn14-Fc coaddition experiments. On day 3, the medium was aspirated, and the cells were rinsed briefly with PBS containing 1 mmol/L CaCl₂ and 1 mmol/L MgCl₂, blotted dry, and then stored at -80°C until cell number was determined with a commercially available kit (CyQUANT cell proliferation assay kit; Molecular Probes).

Construction of the Fn14-Fc Expression Plasmid, Isolation of Stably Transfected 293T Cell Lines, and Purification and Characterization of the Fn14-Fc Soluble Receptor

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Western Blot Analysis

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Migration Assays

HUVEC migration assays were performed with the use of modified Boyden chambers as described previously.¹⁷ In brief, the cells were cultured in serum-free M199 medium (CellGro) supplemented with 0.5% BSA (Roche) for 3 hours, harvested, and then plated on polycarbonate filters precoated with 25 μ g/mL fibronectin (Invitrogen). The upper compartments contained serum-free medium while the lower compartments contained serum-free medium alone or serum-free medium supplemented with 10 ng/mL FGF-2, 30 ng/mL VEGF-A, or 50 ng/mL TWEAK, added either alone or in combination with one another. The cells were allowed to migrate for 3 hours at 37°C , and then the stationary cells on the upper side of each filter were removed with a cotton swab. Cells that had migrated to the lower side of each filter were stained with 0.1% crystal violet, eluted with 10% acetic acid into 96-well plates, and quantified by measuring light absorbance at 575 nm with a 96-well plate reader.

Apoptosis Assays

HUVECs were seeded at a density of 2.7 to 5.4×10^3 cells/cm² in EC growth medium and allowed to attach for at least 12 hours. The medium was aspirated, and then the cells were briefly rinsed with EBM-2 medium containing 0.1% BSA. Cells were then incubated in EBM-2/BSA alone or EBM-2/BSA supplemented with 1 or more of the following: 100 ng/mL TWEAK, 30 ng/mL VEGF-A, or 10 ng/mL FGF-2. After 24 hours of incubation, the detached, floating cells were collected and combined with the adherent cells recovered by trypsin treatment. The cells were pelleted, briefly washed with PBS, pelleted again, and then resuspended in PBS containing 25 μ g/mL propidium iodide (Sigma), 0.3% saponin (Sigma), 5 mmol/L EDTA, and 50 μ g/mL DNase-free RNase (Sigma). Apoptosis was quantified by calculating the percentage of hypodiploid cells as measured by flow cytometry analysis.

Statistical Analysis

Please see the expanded Methods section (available online at <http://www.ahajournals.org>).

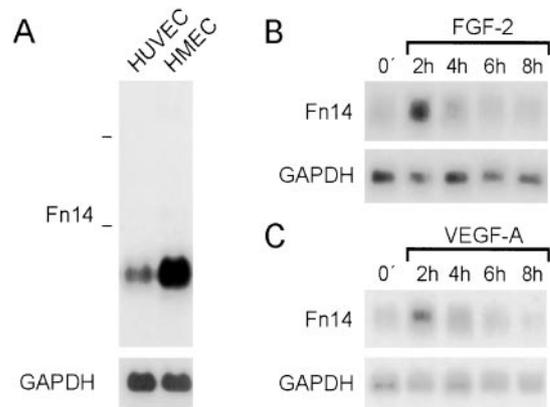


Figure 1. Regulation of Fn14 mRNA expression in human ECs. A, RNA was isolated from HUVEC or HMEC cultures and subjected to Northern blot analysis with the cDNA probes indicated on the left side of each panel. The tick marks on the left side of the upper panel indicate the relative positions of 28S and 18S rRNA. B, HUVECs were serum starved for 2 days and stimulated with 10 ng/mL FGF-2 for the times indicated, and Northern blot analysis was performed. C, HUVECs were serum starved for 2 days and stimulated with 30 ng/mL VEGF-A for the times indicated, and Northern blot analysis was performed.

Results

FGF-2 and VEGF-A Induce Fn14 mRNA Expression in HUVECs

We performed Northern blot hybridization analysis and found that subconfluent proliferating HUVECs and HMECs expressed Fn14 mRNA (Figure 1A). Furthermore, FGF-2 or VEGF-A stimulation of serum/growth factor–starved HUVECs transiently increased Fn14 mRNA levels, with maximal expression (4.2- and 4.4-fold induction, respectively) detected after 2 hours of growth factor treatment (Figure 1B, 1C). FGF-2 or VEGF-A treatment also increased Fn14 mRNA expression in HMECs (data not shown).

TWEAK Stimulates EC Proliferation and Potentiates FGF-2 and VEGF-A Mitogenic Activity

We found that TWEAK increased HUVEC proliferation in a dose-dependent manner: at the highest TWEAK concentration used, 150 ng/mL, there was a 3.2-fold increase in cell number compared with the no-addition control (Figure 2A). Because FGF-2 or VEGF-A treatment of HUVECs can upregulate Fn14 gene expression, we also investigated whether TWEAK cotreatment could potentiate FGF-2- or VEGF-A–stimulated EC proliferation. At the concentrations used, TWEAK, FGF-2, or VEGF-A added individually stimulated HUVEC growth by 1.9-, 4.1-, and 1.4-fold, respectively (Figure 2B). The combination of TWEAK and FGF-2 had an additive effect, increasing HUVEC growth by 5.9-fold. The combination of TWEAK and VEGF-A increased HUVEC growth 2.6-fold, $\approx 20\%$ less than that predicted for an additive proliferative effect. We also treated HUVECs with both FGF-2 and VEGF-A; in this case, HUVEC growth increased 4.6-fold, but this proliferative response was not significantly different from the response noted with FGF-2 addition alone ($P=0.5$).

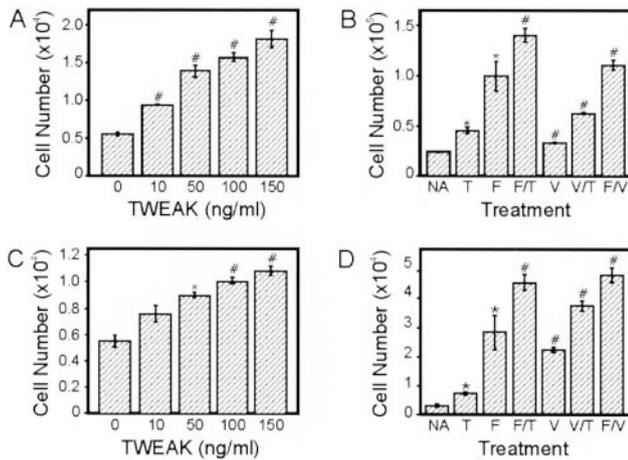


Figure 2. Effect of TWEAK, FGF-2, and VEGF-A on human EC proliferation. A, HUVECs were seeded at low density, placed into growth factor-reduced medium for 1 day, and then either left unstimulated or stimulated with the indicated concentrations of TWEAK for 3 days. B, HUVECs were treated as above and then either left unstimulated (NA; no addition) or stimulated with 50 ng/mL TWEAK (T), 10 ng/mL FGF-2 (F), or 30 ng/mL VEGF-A (V) alone or in combinations for 3 days. C, HMECs were treated as above and then either left unstimulated or stimulated with the indicated concentrations of TWEAK for 3 days. D, HMECs were treated as above and then either left unstimulated (NA; no addition) or stimulated with 50 ng/mL TWEAK (T), 10 ng/mL FGF-2 (F), or 30 ng/mL VEGF-A (V) alone or in combinations for 3 days. In all panels, cell numbers are expressed as mean \pm SD of triplicate wells from 1 representative experiment of 2 independent experiments. * P <0.05 and # P <0.001 compared with untreated control.

TWEAK also had a modest but nevertheless dose-dependent and reproducible stimulatory effect on HMEC growth: at 150 ng/mL, TWEAK increased HMEC number by 2.0-fold compared with the no-addition control (Figure 2C). The ability of TWEAK to enhance FGF-2- or VEGF-A-stimulated HMEC growth was also investigated. TWEAK, FGF-2, or VEGF-A added individually stimulated HMEC growth by 2.2-, 8.7-, and 6.9-fold, respectively (Figure 2D). The combination of TWEAK and FGF-2 increased HMEC growth by 14.0-fold, whereas the combination of TWEAK and VEGF-A increased growth by 11.6-fold. These 2 growth factor combinations stimulated a greater response than the sum of the effects observed with each growth factor added alone; thus, TWEAK can act in concert with either FGF-2 or VEGF-A to stimulate a synergistic growth response in microvascular ECs. FGF-2 and VEGF-A cotreatment increased HMEC growth to the greatest extent, 14.8-fold, a stronger response than that observed when each growth factor was added alone but less than that predicted for an additive or synergistic proliferative effect.

An Fn14-Fc Soluble Receptor Can Inhibit TWEAK-Stimulated HUVEC Proliferation

Human ECs express the Fn14 gene (shown above), and Fn14 is capable of initiating a proliferative signal in these cells.^{6,14} These findings indicate that TWEAK-stimulated EC proliferation is likely mediated through binding to Fn14 cell-surface receptors. Therefore, we generated an Fn14-Fc fusion protein, in which the extracellular, ligand-binding domain of

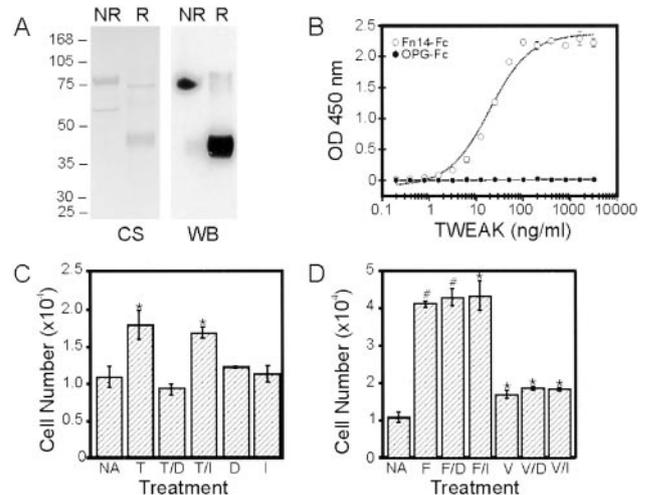


Figure 3. Effect of an Fn14-Fc fusion protein on TWEAK-, FGF-2-, or VEGF-A-stimulated HUVEC proliferation. A, Fn14-Fc protein purified from the conditioned medium of stably transfected human 293T cells was incubated with either nonreducing (NR) or reducing (R) gel loading buffer and then subjected to SDS-PAGE. The samples were run in duplicate. One portion of the gel was stained with Coomassie Blue (CS), and the other portion was transferred to nitrocellulose for subsequent Western blot analysis with anti-Fn14 antiserum (Western blot [WB]). B, TWEAK binding to immobilized Fn14-Fc protein was quantified with an ELISA. OPG-Fc was used as a control for nonspecific binding. Each data point represents the mean \pm SD of triplicate wells from 1 representative experiment of 4 independent experiments. The solid and broken curves represent the best fit determined by nonlinear regression analysis. C, HUVECs were seeded at low density, placed into growth factor-reduced medium for 1 day, and then either left unstimulated (no addition [NA]) or stimulated with 50 ng/mL TWEAK (T), 2.5 μ g/mL Fn14-Fc decoy receptor (decoy [D]), or 2.5 μ g/mL mouse IgG isotype control (IgG [I]) alone or in combinations for 3 days. D, HUVECs were treated as above and then either left unstimulated (NA) or stimulated with 10 ng/mL FGF-2 (F), 30 ng/mL VEGF-A (V), 2.5 μ g/mL Fn14-Fc decoy receptor (D), or 2.5 μ g/mL mouse IgG control (I) alone or in combinations for 3 days. In C and D, cell numbers are expressed as mean \pm SD of triplicate wells from 1 representative experiment of 2 independent experiments. * P <0.05 and # P <0.001 compared with untreated control.

Fn14 was fused to the Fc portion and hinge region of the IgG1 heavy chain, and tested whether it could neutralize TWEAK mitogenic activity on HUVECs. First, Fn14-Fc protein was purified from the conditioned medium of stably transfected human 293T cells and then analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) under reducing and nonreducing conditions; as expected, Fn14-Fc was present in the conditioned medium as an \approx 80-kDa disulfide-linked dimer that could be converted to monomeric form by using a reducing agent (Figure 3A). Second, we determined whether TWEAK could bind to the Fn14-Fc fusion protein by using an ELISA. In brief, an ELISA plate was coated with either purified Fn14-Fc protein or, as a control for nonspecific binding, purified osteoprotegerin (OPG)-Fc protein (OPG is a natural decoy receptor for the TNF superfamily members RANKL and TRAIL¹⁸), and then serial dilutions of FLAG epitope-tagged TWEAK were applied to the wells. This assay demonstrated that TWEAK could specifically bind to the Fn14-Fc protein with an affinity constant (K_d) of \approx 1.1

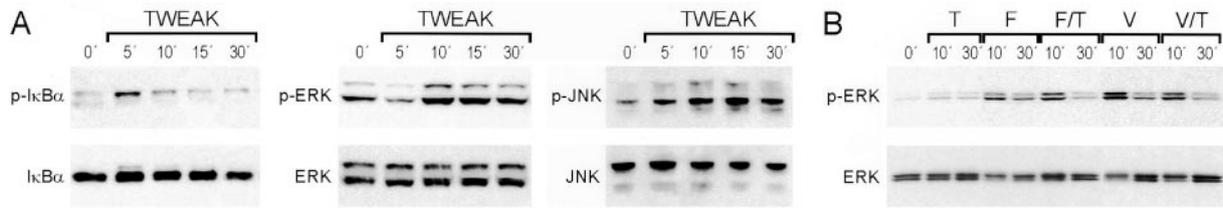


Figure 4. Effect of TWEAK addition on I κ B α , ERK, and JNK phosphorylation. A, Serum-starved HUVECs were either left untreated (0') or treated with 100 ng/mL TWEAK for the indicated periods of time. The cells were harvested, and equivalent amounts of protein were subjected to SDS-PAGE and Western blot analysis with antibodies that detect phospho-I κ B α , total I κ B α , phospho-ERK1/2, total ERK1/2, phospho-JNK1/2, or total JNK1/2. B, Serum-starved HUVECs were either left untreated (0') or treated with 100 ng/mL TWEAK (T), 10 ng/mL FGF-2 (F), or 30 ng/mL VEGF-A (V) alone or in combination for the indicated periods of time. Western blot analysis was conducted with antibodies that detect phospho-ERK1/2 or total ERK1/2.

nmol/L (Figure 3B). Third, we tested whether the Fn14-Fc protein could inhibit TWEAK activity in an EC proliferation assay. HUVECs were incubated in basal medium containing 5% serum alone or 5% serum supplemented with either TWEAK, TWEAK plus a 25-fold molar excess of Fn14-Fc, TWEAK plus control IgG, Fn14-Fc alone, or control IgG alone. After 3 days of incubation, the cells were collected and cell number was determined. TWEAK increased HUVEC growth by 1.6-fold under these experimental conditions, and this effect was significantly inhibited when the cytokine was first preincubated with the Fn14-Fc soluble receptor before its addition to the culture medium ($P=0.015$; TWEAK vs TWEAK/Fn14-Fc; Figure 3C). This same concentration of Fn14-Fc protein had no significant effect on FGF-2- or VEGF-A-stimulated HUVEC proliferation ($P=0.50$, FGF-2 vs FGF-2/Fn14-Fc; $P=0.26$, VEGF-A vs VEGF-A/Fn14-Fc; Figure 3D).

TWEAK Treatment of HUVECs Stimulates I κ B α , ERK, and JNK Phosphorylation

The Fn14 cytoplasmic tail can bind 4 members of the TNFR-associated factor (TRAF) family of adaptor proteins.^{14,19} TNFR superfamily members that associate with TRAF proteins generally function through activation of the nuclear factor (NF)- κ B, extracellular signal-regulated kinase (ERK), c-Jun NH₂-terminal kinase (JNK), and/or the p38 mitogen-activated protein kinase (MAPK) signal transduction pathways.²⁰ We examined TWEAK signaling in HUVECs by performing Western blot analyses with lysates from TWEAK-treated cells and antibodies that specifically recognize phosphorylated forms of various signaling proteins. We found that TWEAK treatment of serum-starved HUVECs stimulated a rapid and transient increase in the level of phosphorylated I κ B α (an indicator of NF- κ B pathway activation²¹), phosphorylated ERK1/2 (p44/p42), and phosphorylated JNK1/2 (p54/p46; Figure 4A). TWEAK-stimulated phosphorylation of p38 MAPK was not detected under these experimental conditions (data not shown).

FGF-2 and VEGF-A each bind to specific receptor tyrosine kinases and activate several intracellular signaling pathways,^{22,23} but ERK function appears to be particularly important for their mitogenic activity on HUVECs.^{24–26} Because TWEAK can potentiate FGF-2- and VEGF-A-stimulated HUVEC proliferation (shown above), we investigated whether it had an effect on the pattern of FGF-2- or

VEGF-A-mediated ERK phosphorylation in these cells. TWEAK, FGF-2, or VEGF-A treatment increased ERK1/2 phosphorylation to differing degrees, with TWEAK showing the weakest stimulatory activity (Figure 4B). TWEAK/FGF-2 or TWEAK/VEGF-A cotreatment also increased ERK1/2 phosphorylation, but the phosphorylation level and the kinetics of phosphorylation were not significantly different from those observed when either FGF-2 or VEGF-A was added alone to these cells.

TWEAK Is A HUVEC Chemotactic Factor but Not a Survival Factor

We next determined whether TWEAK could promote HUVEC migration by using modified Boyden chambers. TWEAK had a dose-dependent stimulatory effect on migration; at the highest dose tested, 150 ng/mL, there was a 2.4-fold increase in HUVEC migration (Figure 5A). Migration assays were also performed with FGF-2 and VEGF-A, added either alone or in combination with TWEAK or one

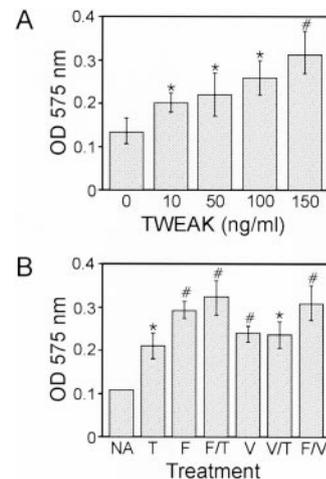


Figure 5. Effect of TWEAK, FGF-2, and VEGF-A on HUVEC migration. A, HUVECs were either left untreated or treated with the indicated concentrations of TWEAK for 3 hours. B, HUVECs were allowed to migrate in the absence (no addition [NA]) or presence of 50 ng/mL TWEAK (T), 10 ng/mL FGF-2 (F), or 30 ng/mL VEGF-A (V) added alone or in combination. In both A and B, data are expressed as an optical density reading at 575 nm, which corresponds to the number of cells migrated. Results represent the mean \pm SD of quadruplicate chambers from 1 representative experiment of 2 independent experiments. * $P<0.05$ and # $P<0.001$ compared with untreated control.

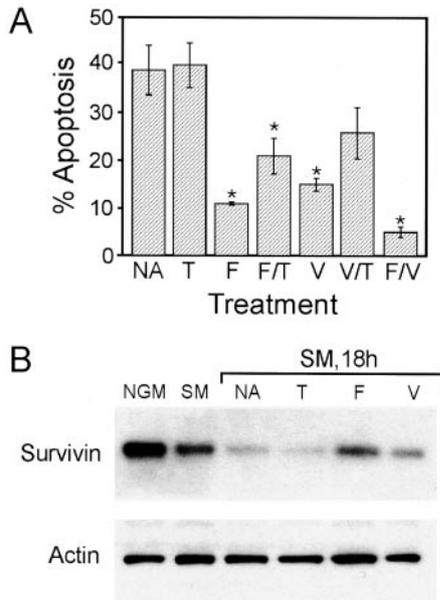


Figure 6. Effect of TWEAK, FGF-2, and VEGF-A on HUVEC survival and survivin expression. **A**, HUVECs were seeded and allowed to attach for 24 hours. Cells were then rinsed briefly and incubated in growth factor/serum-free medium in the absence (no addition [NA]) or presence of 100 ng/mL TWEAK (T), 10 ng/mL FGF-2 (F), or 30 ng/mL VEGF-A (V) added alone or in combination for 24 hours. The proportion of apoptotic cells was determined by fluorescence-activated cell sorting analysis and is expressed as the mean \pm SEM of 3 independent experiments. * $P < 0.05$ and # $P < 0.001$ compared with untreated control. **B**, HUVECs were first cultured in normal growth medium (NGM) and then incubated in starvation medium (SM) for 24 hours. They were then either left untreated (NA) or treated with 100 ng/mL TWEAK (T), 10 ng/mL FGF-2 (F), or 30 ng/mL VEGF-A (V) for 18 hours. The cells were harvested, and equivalent amounts of protein were subjected to SDS-PAGE and Western blot analysis with anti-survivin or anti-actin antibodies.

another. At the concentrations used, TWEAK, FGF-2, or VEGF-A added individually stimulated HUVEC migration by 1.9-, 2.5-, and 2.2-fold, respectively (Figure 5B). FGF-2/TWEAK or VEGF-A/TWEAK cotreatment did not have either an additive or a synergistic effect on HUVEC migration ($P = 0.27$ and 0.79 , respectively), nor did the FGF-2/VEGF-A combination have a significantly greater effect than FGF-2 treatment alone ($P = 0.60$).

Human ECs cultured in medium devoid of serum and growth factors will rapidly undergo apoptosis, and this process can be inhibited by adding EC survival factors, such as FGF-2 or VEGF-A, to the starvation medium.²⁷ To determine whether TWEAK had antiapoptotic activity, we incubated HUVECs in serum/growth factor-free medium in the absence or presence of TWEAK for 24 hours. HUVECs were also treated with FGF-2 or VEGF-A alone as well as with each of these factors in combination with TWEAK or one another. At the end of the incubation period, both the detached and adherent cells were harvested, permeabilized, and stained with propidium iodide. The percentage of cells in the sub-G1 population was calculated by flow cytometry. We found that FGF-2 or VEGF-A addition, but not TWEAK addition, could significantly increase HUVEC survival under these experimental conditions (Figure 6A). Dose-response

experiments with a range of TWEAK concentrations (10 to 200 ng/mL) confirmed that this cytokine had no significant antiapoptotic activity in this assay (data not shown). When TWEAK was added in combination with either FGF-2 or VEGF-A, it doubled the percentage of apoptotic cells, but this effect was not statistically significant ($P = 0.068$, FGF-2 vs FGF-2/TWEAK; $P = 0.150$, VEGF-A vs VEGF-A/TWEAK). FGF-2 and VEGF-A cotreatment inhibited HUVEC apoptosis to the greatest extent (87% inhibition), and this effect was significantly greater than that observed when either factor was added alone ($P = 0.0038$, FGF-2 vs FGF-2/VEGF-A; $P = 0.0030$, VEGF-A vs FGF-2/VEGF-A).

Previous studies have demonstrated that FGF-2 and VEGF-A promote EC survival, at least in part, by activation of the phosphatidylinositol 3-kinase/Akt signaling pathway.^{27,28} Several candidate molecules that may act as downstream effectors of this pathway have been identified, including the antiapoptotic protein survivin.^{27,29,30} FGF-2 and VEGF-A have been shown to induce survivin expression when added to ECs cultured in serum/growth factor-deficient medium.^{30–32} We predicted that because TWEAK was not an antiapoptotic factor, it would not upregulate survivin levels. To test this, HUVECs were incubated in basal medium containing 0.1% FBS and then either left untreated or treated for 18 hours with TWEAK, FGF-2, or VEGF-A. Cell lysates were prepared, and Western blot analysis was performed to assay survivin expression levels. We found that FGF-2 or VEGF-A treatment, but not TWEAK treatment, induced survivin expression in HUVECs (Figure 6B).

Discussion

TWEAK is a TNF superfamily member reported to stimulate neovascularization in the rat cornea angiogenesis assay.⁵ This biologic effect is likely mediated through binding to Fn14, although the existence of additional TWEAK receptors has not been ruled out. The Fn14 gene was initially described as an FGF-1- and FGF-2-inducible gene in murine NIH 3T3 fibroblasts.¹⁵ Subsequent studies revealed that FGF-1 could also induce Fn14 gene expression in human fibroblasts¹⁶ and that FGF-2 had the same effect when added to rat aortic smooth muscle cells.¹⁴ The effect of VEGF-A on Fn14 gene expression has not been previously described. We now report that both FGF-2 and VEGF-A can increase Fn14 mRNA expression in HUVECs and HMECs. These data provide 1 possible mechanistic explanation for TWEAK potentiation of FGF-2 and VEGF-A mitogenic activity on ECs (see below).

TWEAK stimulated HUVEC and HMEC proliferation in a dose-dependent manner. In these experiments, the cell culture medium contained 5% FBS, and cell proliferation was monitored by a sensitive assay for determining actual cell numbers. The ability of TWEAK to promote human EC proliferation has been examined previously by 3 groups that used different cell culture conditions and experimental assays than those used in our study. Both Lynch et al⁵ and Harada et al⁶ reported that TWEAK was a mitogenic factor for human ECs; however, the cell culture medium used by the first group contained bovine brain extract, a rich source of FGF-1 and FGF-2, and the medium used by the second group contained low concentrations of several mitogenic polypeptides, includ-

ing FGF-2 and VEGF-A. Jakubowski et al⁸ reported that TWEAK was not an EC mitogen when the cells were cultured in medium containing 2% FBS. Our results, in combination with these earlier findings, indicate that TWEAK can function as an EC mitogen in the absence of other exogenously added growth factors when an adequate concentration of FBS is present in the basal cell culture medium. Indeed, we have shown that FBS can induce Fn14 gene expression,^{14–16} and this may provide 1 explanation for the serum requirement.

We compared the relative mitogenic potencies of TWEAK, FGF-2, and VEGF-A and also determined whether TWEAK could potentiate FGF-2- or VEGF-A-stimulated EC proliferation. FGF-2 was the most potent HUVEC and HMEC mitogen, followed by either TWEAK (in HUVECs) or VEGF-A (in HMECs). This result is consistent with earlier studies demonstrating that FGF-2 is more effective than VEGF-A in stimulating HUVEC^{33–35} and HMEC³⁵ proliferation. We also found that TWEAK could potentiate both FGF-2 and VEGF-A mitogenic activity on HUVECs and HMECs, but to differing degrees. Indeed, TWEAK/FGF-2 cotreatment had an additive growth effect on HUVECs but a synergistic growth effect on HMECs. TWEAK also enhanced VEGF-A mitogenic activity on HUVECs, but the response was not additive or synergistic; in contrast, TWEAK/VEGF-A cotreatment had a synergistic effect on HMEC proliferation. Our results demonstrating TWEAK potentiation of FGF-2-stimulated HUVEC proliferation are consistent with the findings of Jakubowski et al;⁸ however, those investigators did not detect TWEAK potentiation of VEGF-A-stimulated HUVEC proliferation under their experimental conditions.

The molecular basis for TWEAK potentiation of FGF-2- or VEGF-A-stimulated EC proliferation is not yet known, but it does not appear to be due to TWEAK enhancement of FGF-2- or VEGF-A-mediated ERK1/2 activation. There are at least 2 other possible mechanisms for the observed potentiation. First, FGF-2 or VEGF-A treatment of ECs can upregulate Fn14 gene expression; therefore, if TWEAK mitogenic efficacy varies as a function of Fn14 levels, then one would predict an increased TWEAK response in the presence of FGF-2 or VEGF-A. Second, potentiation may occur because the TWEAK mitogenic signaling pathway is not completely overlapping with the FGF-2 or VEGF-A signaling pathways. For example, although TWEAK promotes NF- κ B activation when added to HUVEC cultures (discussed below), it has been reported that FGF-2 treatment of these cells does not activate this signaling pathway.³⁶

We produced a soluble Fn14-Fc fusion protein and found that it could act as a decoy receptor and inhibit TWEAK mitogenic activity on HUVECs. Wiley et al¹⁴ reported that an Fn14-Fc soluble receptor could inhibit (1) phorbol myristate acetate- or epidermal growth factor-stimulated HMEC migration in an in vitro monolayer wound closure assay and (2) FGF-2-stimulated angiogenesis in the mouse cornea. Those investigators proposed that Fn14-Fc was interfering with a TWEAK-Fn14 autocrine loop that was required for maximal biologic activity of these other EC stimuli. We found that Fn14-Fc cotreatment did not inhibit FGF-2- or VEGF-A-stimulated HUVEC proliferation; therefore, it appears that a

TWEAK autocrine effect is not required for FGF-2 or VEGF-A activity in this assay.

TWEAK treatment of HUVECs stimulated I κ B α , ERK1/2, and JNK1/2 phosphorylation; thus, TWEAK activity on this cell type is likely mediated by the NF- κ B, ERK, and JNK intracellular signaling pathways. TWEAK activation of the ERK or JNK pathways has not been previously reported; however, TWEAK has been shown to induce NF- κ B DNA-binding activity in human embryonic kidney 293 cells,⁴ HUVECs,⁶ and NIH 3T3 cells.¹⁹ The ability of TWEAK to promote NF- κ B activation in HUVECs is consistent with previous studies indicating that TWEAK-treated HUVECs express elevated levels of the NF- κ B-regulated interleukin-8, monocyte chemotactic protein-1, intercellular adhesion molecule-1, and E-selectin genes.^{5,6}

TWEAK was able to stimulate HUVEC migration when tested with the modified Boyden chamber assay. FGF-2 and VEGF-A also stimulated migration, consistent with previous reports,^{33,34} but FGF-2/TWEAK or VEGF-A/TWEAK cotreatment did not promote an additive or a synergistic migratory response. The effect of TWEAK on HUVEC migration was examined previously by 2 groups using a monolayer wound repair assay. Jakubowski et al⁸ found that TWEAK did not significantly increase EC migration; in contrast, Harada et al⁶ reported that TWEAK could stimulate EC migration in this assay.

It has been shown previously that ECs incubated in serum/growth factor-deficient cell culture medium undergo apoptosis, and this response can be inhibited by adding purified FGF-2 or VEGF-A to the medium.^{37–39} TWEAK did not inhibit HUVEC apoptosis under our experimental conditions; in contrast, FGF-2 and VEGF-A were effective EC survival factors, as expected from previous results. Also, TWEAK treatment of HUVECs did not promote expression of survivin, an FGF-2- and VEGF-A-inducible antiapoptotic protein implicated in growth factor-mediated EC survival.^{27,29–32} Our results differ from those of Jakubowski et al,⁸ who reported that TWEAK was a HUVEC survival factor. However, the experimental protocol used by those investigators was quite different from ours; for example, they incubated the ECs in serum/growth factor-deficient medium for a longer period of time, and the proportion of apoptotic cells was calculated by using the adherent cell population only.

In summary, we have demonstrated that TWEAK can stimulate EC proliferation and migration in vitro, 2 essential steps in the angiogenic process. TWEAK bioactivity on ECs is likely mediated through Fn14 binding and activation of the NF- κ B, ERK, and JNK signaling pathways. In addition, TWEAK can potentiate FGF-2 and VEGF-A activity in EC proliferation assays; therefore, this cytokine may act alone as well as in concert with FGF-2 and/or VEGF-A to regulate physiological and pathological angiogenesis.

Acknowledgments

This research was supported in part by National Institutes of Health grants HL-39727 (to J.A.W.) and HL-67330 (to T.H.), an Arthritis Foundation Investigator Award (to P.J.D.), an American Heart Association Established Investigator Grant (to T.H.), and an American Heart Association Scientist Development Grant (to M.S.W.). We thank Mehrdad Tondravi for providing the OPG-Fc expression

plasmid and purified OPG-Fc decoy receptor, Bob Hawley for the 293T cell line, and Bonnie Van Veldhuizen for excellent secretarial assistance.

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Arteriosclerosis, Thrombosis, and Vascular Biology



JOURNAL OF THE AMERICAN HEART ASSOCIATION

TWEAK Is an Endothelial Cell Growth and Chemotactic Factor That Also Potentiates FGF-2 and VEGF-A Mitogenic Activity

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Arterioscler Thromb Vasc Biol. 2003;23:594-600; originally published online February 20, 2003;

doi: 10.1161/01.ATV.0000062883.93715.37

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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I. METHODS

Cell Culture

Human umbilical vein endothelial cells (HUVEC) and human microvascular endothelial cells (HMEC) isolated from adult dermis were obtained from Biowhittaker and cultured in the EC growth medium EGM-2-MV (Biowhittaker). Human embryonic kidney 293T cells (kind gift of Dr. R. Hawley, Holland Laboratory) were grown in Eagle's minimum essential medium (Biowhittaker), 1 mM sodium pyruvate (Biowhittaker), 1X non-essential amino acids (Mediatech), and 10% fetal bovine serum (FBS; Hyclone Laboratories).

RNA Isolation and Northern Blot Hybridization Analysis

Sub-confluent HUVEC and HMEC were harvested, RNA was isolated, and Northern blot analysis performed as previously described.¹ For the growth factor time course experiments, HUVEC were incubated in endothelial basal medium-2 (EBM-2; Biowhittaker), 0.5% FBS for 24 hours and then EBM-2, 0.25% FBS for an additional 24 hours to induce cellular quiescence. The cells were then either left untreated or treated for various time periods with either 10 ng/ml recombinant human FGF-2 (157-amino acid form; R&D Systems) or 30 ng/ml recombinant human VEGF-A (165-amino acid form; R&D Systems). Cells were harvested and Northern blot analysis was performed as above. The cDNA hybridization probes were human Fn14, ~1.0-kb EcoR1/Xho1 fragment of pBluescript/hFn14² and human glyceraldehyde 3-phosphate dehydrogenase (GAPDH), ~0.8-kb Pst1/Xba1 fragment of pHcGAP (American Type Culture Collection). Hybridization signals were quantitated by densitometry using a Bio-Rad

GS-800 Densitometer, and the Fn14 mRNA signals were normalized to the GAPDH mRNA signals to correct for slight differences in the amount of RNA per gel lane.

Western Blot Analysis

For the TWEAK time-course experiments, HUVEC were grown to confluence and then incubated in EBM-2, 0.1% BSA for 18 hours. The cells were then either left untreated or treated with 100 ng/ml TWEAK, 10 ng/ml FGF-2, or 30 ng/ml VEGF-A alone or in combination for various time periods. Cells were washed with cold phosphate-buffered saline (PBS), harvested by scraping, and then incubated for 15 minutes at 0°C in 50 mM Tris/HCl pH 7.4, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 1X protease inhibitor cocktail set #1 (Calbiochem), and 1X phosphatase inhibitor cocktail set #2 (Calbiochem). Lysates were clarified by centrifugation and protein concentrations were determined using the BCA Protein Assay Kit (Pierce). Equivalent amounts of protein were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred to Protran nitrocellulose membranes (Schleicher and Schuell) by electroblotting. Membranes were stained with Ponceau S (Sigma) to verify that equivalent amounts of protein were present in each gel lane. The membranes were then blocked for 2 hours at room temperature in TBST buffer (50 mM Tris/HCl pH 7.7, 150 mM NaCl, 0.1% Tween-20) containing 5% BSA and then incubated overnight at 4°C in TBST/5% BSA containing a 1:1000 dilution of rabbit phospho-I κ B α (Ser32), total I κ B α , phospho-p44/p42 ERK (Thr202/Tyr204), total p44/p42 ERK, phospho-p54/p46 JNK (Thr183/Tyr185), or total p54/p46 JNK antiserum (all antibodies obtained from Cell Signaling Technology). The membranes were then washed three times in TBST/5% BSA

for 5 minutes each and then incubated in TBST/5% BSA containing a 1:5000 dilution of goat anti-rabbit Ig-horseradish peroxidase (HRP) (Santa Cruz) for 30 minutes at room temperature. The membranes were then washed once with TBST/5% BSA for 5 minutes and three times in TBST for 5 minutes each. Bound secondary antibodies were detected using the SuperSignal chemiluminescence reagent (Pierce).

For the survivin expression experiments, HUVEC were grown to 50% confluence and then incubated in EBM-2, 0.1% FBS for 24 hours. The cells were then either left untreated or treated with 100 ng/ml TWEAK, 10 ng/ml FGF-2, or 30 ng/ml VEGF-A for 18 hours. Cells were washed with cold PBS, harvested by scraping, and lysed in PBS containing 4% SDS and 1X protease inhibitor cocktail set #1 (Calbiochem). Nuclear DNA was sheared by passage through a 25 gauge needle. Equivalent amounts of protein were subjected to SDS-PAGE and transferred to nitrocellulose membranes as described above. The membranes were first blocked for 2 hours at room temperature in TBST buffer containing 5% nonfat dry milk (NFDM) and then incubated for either 2 hours at room temperature in TBST/5% NFDM containing a 1:500 dilution of a mouse anti-actin monoclonal antibody (Sigma) or overnight at 4°C in TBST/5% NFDM containing a 1:1000 dilution of a rabbit anti-survivin polyclonal antibody (Novus Biologicals). The membranes were then washed two times in TBST/5% NFDM for 5 minutes each and then incubated in TBST/5% NFDM containing a 1:5000 dilution of either goat anti-mouse or anti-rabbit Ig-HRP (Santa Cruz) for 30 minutes at room temperature. The membranes were then washed once with TBST/5% NFDM for 5 minutes and three times in TBST for 5 minutes each. Bound secondary antibodies were detected as described above.

Construction and Transfection of the Fn14-Fc Expression Plasmid

The plasmid pSecTag2/Fn14-Fc was constructed as follows. First, the polymerase chain reaction (PCR) was performed using pBluescript/mFn14 as the template,³ a sense primer containing a 5' AscI restriction site followed by Fn14 nucleotides 112-126 (amino acids 30 to 34), an antisense primer containing a 5' XhoI restriction site followed by Fn14 nucleotides 226-243 (amino acids 68 to 73), and Taq Polymerase (Roche). The DNA product was isolated, ligated into pCR2.1 using a T/A cloning kit (Invitrogen), released by AscI/XhoI digestion, and purified for subcloning. Second, the plasmid pSecTag2/OPG-Fc, which contains a region of the osteoprotegerin (OPG) cDNA fused with the Fc portion of mouse IgG1 heavy chain cDNA and cloned into the expression vector pSecTag2A/Hygro (Invitrogen), was obtained from Dr. M. Tondravi (NIH). The OPG cDNA fragment was released from this plasmid by AscI/XhoI digestion and replaced with the Fn14 AscI/XhoI cDNA fragment encoding the Fn14 extracellular domain. DNA sequence analysis was performed to confirm the identity of the Fn14-Fc plasmid as previously described.³

Human 293T cells were transfected with pSecTag2/Fn14-Fc using the Lipofectamine PLUS reagent (Invitrogen), cultured for 24 hours in standard growth medium, and then split at a 1:10 ratio. At 24 hours post-transfection, cells were cultured in medium containing 200 µg/ml hygromycin B (Calbiochem). One week later, individual hygromycin B-resistant colonies were recovered with glass cloning cylinders. Individual clones were screened for relative levels of Fn14-Fc expression by placing an equivalent number of cells in growth medium supplemented with 10% Ultra-low IgG FBS (Invitrogen) for 24 hours and then collecting conditioned medium. The Fn14-Fc

soluble receptor was isolated from the conditioned medium samples by affinity chromatography using protein A-Sepharose beads (Amersham Pharmacia). Bound protein was analyzed by SDS-PAGE and Western blot analysis as described³ using a 1:500 dilution of anti-myc antiserum (Santa Cruz), a 1:10000 dilution of goat anti-rabbit Ig-HRP (Santa Cruz), and the enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia). Several cell lines expressing the highest level of Fn14-Fc protein were selected and frozen until needed for large scale Fn14-Fc production.

Purification and Characterization of the Fn14-Fc Soluble Receptor

Stably-transfected human 293T cells secreting the Fn14-Fc protein were cultured in growth medium containing 10% Ultra-low IgG FBS (Invitrogen) until they reached confluency and then conditioned medium was collected. Cells were removed by centrifugation and then the conditioned medium was incubated with protein A-Sepharose beads for 30 minutes at room temperature with end-over-end mixing. The beads were collected by a brief centrifugation and washed two times with PBS, once with 0.5 M NaCl in PBS, and once with PBS. The bound Fn14-Fc protein was eluted with 25 mM citrate buffer (pH 2.7) and then the eluate was neutralized with 1.5 M Tris/HCl pH 8.8, dialyzed against PBS, and concentrated in a Centricon-30 (Amicon).

The purity of the Fn14-Fc preparation was assessed by SDS-PAGE using 4-12% Bis-Tris NuPage gels (Invitrogen). Samples were either suspended in 2X gel loading buffer containing 10% 2-mercaptoethanol (ME) and heated at 95°C prior to loading (reducing conditions) or resuspended in 2X gel loading buffer without 2-ME and then loaded directly into the gel lanes (non-reducing conditions). Fn14-Fc was detected by

staining the gel with 0.05% Coomassie Brilliant Blue R-250 (Kodak) and also by Western blot analysis as described³ using a 1:500 dilution of anti-Fn14 antiserum [20153], a 1:10000 dilution of goat anti-rabbit Ig-HRP (Santa Cruz) and the ECL detection system (Amersham Pharmacia).

ELISAs were performed to determine whether human TWEAK could bind to the murine Fn14-Fc protein. Fn14-Fc or OPG-Fc (kind gift from Dr. M. Tondravi) were coated on microtiter wells (Falcon ProBind assay plates) at 1.0 $\mu\text{g/ml}$ in PBS for 2 hours at room temperature with mild shaking. Well contents were decanted and then 5% (v/v) FBS in PBS (incubation buffer) was added for 1 hour at 37°C. The wells were then washed four times with PBS plus 0.05% (v/v) Tween-20 (wash buffer). FLAG-TWEAK (Alexis Biochemicals) at 4 $\mu\text{g/ml}$ in incubation buffer was added to the first set of wells and then a 2-fold dilution series was performed across the plate. Ligand binding was allowed to proceed for 1 hour at 37°C and then the wells were washed four times with wash buffer. Bound TWEAK was detected by incubation with 1.0 $\mu\text{g/ml}$ anti-FLAG Ig-HRP (Upstate Biotechnology) for 1 hour at room temperature. The wells were washed four times with wash buffer and then color development was initiated using Sigma Fast 0-phenylenediamine dihydrochloride tablets (Sigma). The absorbance for each sample was measured at 450 nm using a 96-well plate reader. Data were analyzed by nonlinear regression using SigmaPlot software as described.⁴

Statistical Analysis

Statistical analysis was performed using the Student's t test and differences were considered to be statistically significant at P values <0.05.

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