Soluble Tumor Necrosis Factor-Like Weak Inducer of Apoptosis Overexpression in HEK293 Cells Promotes Tumor Growth and Angiogenesis in Athymic Nude Mice

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ABSTRACT

Tumor necrosis factor-like weak inducer of apoptosis (TWEAK) is a member of the tumor necrosis factor superfamily of structurally related cytokines. TWEAK acts on responsive cells via binding to a cell surface receptor named Fn14. Recent studies have demonstrated that TWEAK can stimulate numerous cellular responses including cell proliferation, migration, and proinflammatory molecule production. It has also been reported that TWEAK can stimulate blood vessel formation in the rat cornea angiogenesis assay, but it is presently unknown whether this cytokine could play a role in the pathological angiogenesis associated with human diseases such as cancer, rheumatoid arthritis, and diabetic retinopathy. In the present study we investigated whether TWEAK was expressed in human tumors and whether it could promote tumor growth and angiogenesis in vivo. TWEAK mRNA expression was detected in many tumor types by cDNA array hybridization analysis, and TWEAK protein expression was confirmed in human colon cancer tissue by immunohistochemistry. As an initial approach to address whether TWEAK might act as a tumor angiogenesis factor, we established several human embryonic kidney cell lines that constitutively secrete a soluble TWEAK protein and examined their growth properties in vitro and in vivo. We found that although TWEAK-overexpressing cells do not have a growth advantage in vitro, they form larger and more highly vascularized tumors in athymic mice when compared with control, vector-transfected cells. This result suggests that the TWEAK-Fn14 signaling system may be a potential regulator of human tumorigenesis.

INTRODUCTION

Angiogenesis, the formation of new blood vessels from preexisting vasculature, is crucial for primary tumor growth and metastasis (1–3). Tumor cells stimulate angiogenesis by producing polypeptide factors that act in a paracrine manner to promote endothelial cell proliferation, migration, and ultimately, vessel assembly and stabilization (1–3). One of these proangiogenic polypeptides, vascular endothelial cell growth factor, is now recognized as an important regulator of both normal vessel growth and tumor neovascularization, and indeed a humanized anti-vascular endothelial growth factor monoclonal antibody has been approved for use in the treatment of metastatic colorectal cancer patients (4). Nevertheless, it is likely that other tumor-derived angiogenic factors also function as key regulators of tumor neovascularization, and the identification and characterization of these factors, their cell surface receptors, and their intracellular signaling pathways may result in the development of additional cancer drugs.

Tumor necrosis factor-like weak inducer of apoptosis (TWEAK) was first described in 1997 (5) as a member of the tumor necrosis factor superfamily of cytokines (6, 7). TWEAK is initially synthesized as a type II transmembrane protein but can be cleaved to generate a ~17-kDa soluble factor with biological activity (5). Soluble TWEAK induces various biological effects when it is added to cells in culture (8). For example, TWEAK has been reported to stimulate cell proliferation (9–12), migration (10, 11, 13), and differentiation (14). Also, TWEAK treatment of several different cell types induces the expression of proinflammatory molecules (5, 10, 15–18). TWEAK activity is mediated via binding to Fn14 (19), a member of the tumor necrosis factor receptor superfamily (6, 7). The TWEAK and Fn14 genes are coexpressed in a variety of cell and tissue types (8). In addition, Fn14 gene expression is up-regulated after growth factor stimulation of quiescent cell cultures (19–21) and after injury to the vessel wall (19) or liver (21). It has also been shown that Fn14 is overexpressed in advanced liver (8, 21) and brain (8, 13) tumors, but the functional significance of these findings is presently unknown.

In 1999 Lynch et al. (9) reported that TWEAK could stimulate blood vessel formation when delivered onto the rat cornea, but the role of this cytokine in either physiologic or pathological angiogenesis has not been described. We report here that TWEAK expression can be detected in many human tumor types and that a soluble form of TWEAK promotes tumor growth and angiogenesis in athymic nude mice. These results suggest that the TWEAK cytokine may play a role in tumor progression.

MATERIALS AND METHODS

Cell Culture. Human umbilical vein endothelial cells (Cambrex, Walkersville, MD) and human embryonic kidney (HEK) 293 cells (American Type Culture Collection, Manassas, VA) were grown as described previously (11).

cDNA Dot Blot Hyridization. The Matched Tumor/Normal Expression Array blot was obtained from BD Biosciences (San Diego, CA), and hybridization analysis was conducted as described previously for Northern blots (20). The cDNA hybridization probe was a ~1.0-kb BglII/XhoI fragment of the plasmid pTWEAK (gift of Timothy Zheng, Biogen Idec Inc., Boston, MA).

RNA Isolation and Northern Blot Hybridization. Total RNA was isolated from human umbilical vein endothelial cells and HEK293 cells using RNA Stat-60 (Tel-Test, Friendswood, TX) according to the manufacturer’s instructions. Northern blot hybridization analysis was conducted as described (20). The cDNA hybridization probes were human TWEAK (see above), human Fn14, ~1.0-kb EcoRI/XhoI fragment of pBluescript/Bf14 (21), and human glyceraldehyde-3-phosphate dehydrogenase, ~0.8-kb PstI/XbaI fragment of pH GAP (American Type Culture Collection).

Flow Cytometric Analysis. HEK293 cells were harvested using an enzyme-free cell dissociation solution (Quality Biological, Gaithersburg, MD) and ~10^6 cells were incubated with 0.5 μg of phycoerythrin-labeled anti-human TWEAK monoclonal antibody (clone CARL-1), mouse IgG3 isotype control, anti-human Fn14 monoclonal antibody (clone ITEM-4), or mouse IgG2b isotype control for 30 minutes at 4°C. These antibodies and control IgG proteins were obtained from eBioscience (San Diego, CA). The cells were washed with cold fluorescence-activated cell sorter buffer (1× Hank’s Balanced Salt Solution containing 0.5% bovine serum albumin and 0.1% sodium azide) and then resuspended in 500 μL fluorescence-activated cell sorter buffer containing 0.5 μg/mL propidium iodide (Sigma, St. Louis, MO). The stained cells (live gated on the basis of forward and side scatter profiles and propidium iodide exclusion) were analyzed on a FACSCaliber (BD Biosciences) and the data processed using the CellQuest program (BD Biosciences).

Construction of the TWEAK Expression Plasmid. The TWEAK expression plasmid pSecTag/TWEAK-Receptor Binding Domain (TWK-RBD)-myc encoding human TWEAK amino acid residues 101 to 249 (5) with a myc

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epitope tag was constructed as follows. First, a plasmid containing the full-length human TWEAK coding region was made by ligating a double-stranded linker containing an ATG start codon surrounded by an optimal Kozak motif (5'-AGCTGTGACCCCGGCGGACGAC-3') onto a NcoI/XhoI fragment of the plasmid pcTWEAK (gift of Timothy Zheng, Biogen Idec Inc.) and then by cloning this DNA fragment into HindIII and XhoI digested pBluescript (Stratagene, La Jolla, CA). PCR overlap extension (22) using Vent Polymerase (New England Biolabs, Beverly, MA) and 5% DMSO in all of the reactions was then used to insert a myc epitope tag between TWEAK amino acids 104 and 105 and to subclone the DNA into the plasmid pCMVScript (Stratagene). For first-stage PCR pBluescript/TWEAK was used as the template. In reaction 1 a sense T3 primer was used in combination with antisense primer 5'-AAGCTTATAGAAGACTGGAGGCCGTCGACCC-3' and in reaction 2 an antisense T7 primer was used in combination with the sense primer 5'-TCTTGTACGAAATAGGTTTTGCTGCAAGCCGTGT-3'. The two PCR products were isolated, pooled, and used as the template for second stage PCR using the sense primer 5'-AGCTGTGACCCCGGCGGACGAC-3' and the antisense primer 5'-GAGGATCTGTCAGCTGACCGACGACCC-3'. The PCR product was isolated and then cloned into pCMVScript (Stratagene) according to the manufacturer's instructions. The construct was verified by DNA sequence analysis. Second, PCR was performed using the plasmid pCMVScript/TWEAK as the template, the sense primer 5'-GAAGATCTAACAACGGGCGTCGACGACCA-3', the antisense primer 5'-GAGGATCTGTCAGCTGACCGACGACGACCC-3', and Vent Polymerase. The PCR product was isolated and then cloned into pCRScript CAM Sk+ (Stratagene) according to the manufacturer's instructions. This plasmid was digested with BglIII, and the released DNA fragment was ligated into BamHI-digested pSecTag2A/Hygro (Invitrogen, Carlsbad, CA). The final construct was verified by DNA sequence analysis.

Cell Transfection. HEK293 cells were transfected with 8 μg of either the pSecTag2A/Hygro vector or the pSecTag/TWKR-BRD-myc plasmid using Lipofectamine Plus (Invitrogen) according to the manufacturer's instructions. At 24 hours after transfection the cells were split 1:2 and incubated in growth medium containing 200 μg/mL hygromycin B (Invitrogen). Cells were refed every other day, and individual drug-resistant colonies were recovered using disposable sterile loops. The stably transfected cell lines were screened for TWEAK expression levels by Western blot analysis, and several cell lines were selected for further characterization.

Western Blot Analysis. For the TWEAK time course experiments using untransfected HEK293 cells, the cells were first cultured for 24 hours in normal growth medium containing 0.2% fetal bovine serum (FBS) instead of 10% FBS and then treated with 100 ng/mL recombinant human TWEAK (PeproTech, Rocky Hill, NJ) for various time periods. Cells were harvested, cell lysates were prepared, and Western blot analysis was performed using an anti-phospho-p44/p42 extracellular signal-regulated kinase (ERK) and anti-total p44/p42 ERK antibodies (Cell Signaling Technology, Beverly, MA) as described (11). For characterization of the transfected HEK293 cell lines, cells were cultured in normal growth medium until they reached confluency, and then the conditioned medium was collected and centrifuged to remove any contaminating cells. Cells were harvested from each dish by scraping with a rubber policeman and then lysed in 50 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 10% glycerol, and 1.5 mmol/L MgCl2. Protein concentrations were determined using the BCA protein assay kit (Pierce, Rockford, IL). Equivalent amounts of each cell lysate and the proportional amount of conditioned medium were then subjected to SDS-PAGE using a 4% to 12% NuPage gradient gel (Invitrogen). Proteins were transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH), and then visualized using Ponceau S Stain (Sigma). The membranes were blocked for 1 hour at 37°C in Tris-Buffered Saline-Tween 20 (TBST) (25 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, and 0.1% Tween 20) containing 5% nonfat dry milk and then incubated for 1 hour at room temperature in TBST containing 5% bovine serum albumin and a 1:500 dilution of anti-myc monoclonal antibody 9E10 (gift of Sue Robinson, University of Maryland School of Medicine). The membranes were then washed three times with TBST and incubated for 1 hour in TBST containing 5% nonfat dry milk and a 1:10,000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were washed three times in TBST, and bound secondary antibodies were detected using the Supersignal West Pico kit (Pierce).
gene expression in human tumor specimens has not been reported. Therefore, we examined TWEAK mRNA expression levels in human tumor biopsies and corresponding adjacent normal tissue specimens from 68 cancer patients representing 11 tumor types. TWEAK transcripts were present at variable levels in all of the tumor and normal tissue specimens surveyed (Fig. 1A). In some tumor/normal tissue pairs, the TWEAK mRNA expression level was higher in the tumor tissue. This was most apparent in the kidney tumor/normal tissue panel, where TWEAK gene expression was elevated in 14 of the 15 tumor specimens.

Because tumor tissue specimens contain multiple cell types, including tumor cells, endothelial cells, pericytes, and immune cells, the cellular source(s) of the TWEAK transcripts detected in the hybridization analysis described above is unknown. Therefore, TWEAK gene expression in tumor tissue was also evaluated by immunohistochemistry to determine whether TWEAK protein expression could be detected in tumors and if so, whether tumor cells were a source of this cytokine. Human colon carcinoma was chosen as a representative tumor type for this study, and the staining was performed using a previously characterized anti-TWEAK polyclonal antibody (5, 25). We were able to detect TWEAK expression in the colon cancer cells within the tumor specimen (Fig. 1B). These results provide the first demonstration that TWEAK is expressed in human primary tumors; in addition, because vascular endothelial cells express the Fn14 receptor (refs. 8, 10, 11, 19; also see below), they support the possibility that TWEAK could act as a paracrine regulator of tumor angiogenesis.

**TWEAK Overexpression Does Not Stimulate HEK293 Cell Proliferation In Vitro.** As an initial experimental approach to investigate whether the TWEAK produced by tumor cells could contribute to tumor growth and angiogenesis we established several HEK293 cell lines that constitutively express the TWEAK protein and examined their growth properties in vitro and in vivo. Two distinct TWEAK species have been identified: a 249-amino acid species representing full-length, plasma membrane-anchored TWEAK and a 156-amino acid proteolytic product that is released into the extracellular environment (5, 8). This proteolytic product, referred to as soluble TWEAK, is essentially the COOH-terminal receptor-binding domain of transmembrane TWEAK and is biologically active in vitro and in vivo (8). We chose to examine the effects of soluble TWEAK overexpression in the present study. HEK293 cells were used for these experiments because they are efficiently transfected, can readily be isolated as stable cell lines by drug selection, and are weakly tumorigenic in nude mice (26). Also, although TWEAK mRNA expression can be detected in these cells (Fig. 2A), relatively low levels of membrane-anchored TWEAK are present on the HEK293 cell surface (Fig. 2B), and we have been unable to detect soluble TWEAK in HEK293 cell-conditioned medium samples using either Western blot analysis or a sensitive ELISA developed in our laboratory (data not shown). HEK293 cells were also chosen for this study because they express Fn14 mRNA (Fig. 2A) and protein (Fig. 2B). We reported previously that TWEAK treatment of Fn14-positive endothelial cells activates the ERK signal transduction pathway (11). We found that TWEAK treatment of HEK293 cells also activates this pathway (Fig. 2C). This result demonstrates that the Fn14 receptors detected on the HEK293 cell surface are functional receptors.

An expression plasmid encoding myc-tagged soluble TWEAK (Fig. 3A) was constructed using the vector pSecTag2/Hygro. This vector is ideal for high-level constitutive expression and efficient secretion of recombinant proteins and contains a hygromycin B resistance gene for selection of stable cell lines. A myc epitope tag was inserted near the NH2 terminus of the secreted TWEAK protein to facilitate its detection by Western blot analysis. HEK293 cells were transfected with either the pSecTag2/Hygro vector or the pSecTag2/TWEAK-RBD–myc expression plasmid, and individual drug-resistant colonies were recovered and expanded. TWEAK expression and secretion was examined in six of the selected cell lines by Western blot analysis. A major immunoreactive band of ~24 kDa was detected in both the cell lysate and conditioned media samples from the pSecTag2/TWEAK-RBD–myc–transfected cell lines (Fig. 3B). The TWK1, 2, 3, and 4 lines...
immunoglobulin

matic representation of the expression cassette encoding soluble TWEAK protein. The
region, and the position of the myc epitope are indicated.

TWK-RBD

fold-increase in cell growth for each cell line was calculated using the day 0 mean cell
examined in cell culture medium containing 0.5% serum over a period of 6 days. The
was

V

vector-transfected HEK293 cell line (V),

U

vector-transfected HEK293 cell line (U) was

C

lines (1–5) and a vector-transfected HEK293 cell line (V). Equal amounts of protein were
analyzed by SDS-PAGE and Western blot analysis using an anti-myc antibody. C. The
growth potential of the five TWEAK-expressing HEK293 cell lines (1–5), the
were analyzed by SDS-PAGE and Western blot analysis using an anti-myc antibody.

C

soluble TWEAK is

TWEAK-RBD–myc protein is

18 kDa, but it has been reported that

N-glycosylated at a single site (27), and this is
likely to explain the size discrepancy that we observe as well as the
presence of additional, weakly immunoreactive species in the Western

HEK293 cells express functional Fn14 receptors; therefore, we first
tested whether soluble TWEAK overexpression altered HEK293 growth potential in vitro. To increase the likelihood of detecting a
TWEAK effect, the cells were cultured in suboptimal, reduced serum
conditions. We found that all five of the TWEAK-overexpressing cell
lines had similar proliferation capacities (Fig. 3C), although the
TKW1, 2, 3, and 4 lines expressed significantly more soluble
TWEAK protein than the TKW5 line. Furthermore, the five TWEAK-
overexpressing cell lines did not display enhanced proliferative
capacity when compared with either the vector-transfected cell line or
untransfected cells. Taken together, these results demonstrate that
TWEAK-overexpressing HEK293 cell lines can be generated and that
soluble TWEAK production is not promoting HEK293 cell growth or
death under these experimental conditions. This latter finding is
consistent with additional assays performed under similar culture
conditions demonstrating that recombinant soluble TWEAK does not
stimulate HEK293 cell proliferation or apoptosis (data not shown).

TWEAK Overexpression Potentiates Tumor Growth and Angiogenesis in Nude Mice. The effect of soluble TWEAK overexpres-
sion on HEK293 cell growth in vivo was then investigated. For these
experiments, two of the TWEAK-overexpressing cell lines and one of
the vector-transfected cell lines were suspended in Matrigel, a base-
ment membrane protein mixture, and then injected into the flank of
athymic nude mice. Another group of mice received Matrigel alone.
Serial tumor measurements were obtained and tumor volumes cal-
culated. The two TWEAK-overexpressing cell lines showed a signifi-
cantly increased tumor growth rate compared with the vector-trans-
fected cell line (Fig. 4). Inspection of the tumors harvested from each
of the experimental groups revealed that the TKW2 and TKW3 cell
line-derived tumors were clearly larger and more vascularized than
the vector-transfected cell line-derived tumor (Fig. 5A). Enhanced
neovascularization of these tumors was confirmed by measuring he-
moglobin content (Fig. 5B). In addition, immunohistochemical stain-
ing of tumor sections using an antibody that specifically recognizes
endothelial cells revealed increased microvessel density in the TKW2
(Fig. 5C) and TKW3 (data not shown) cell line-derived tumors. Direct
counting of vascular structures revealed that microvessel density was
4-fold higher in the TKW2 cell line-derived tumors compared with
the vector-transfected cell line-derived tumors [171 ± 31 versus
40 ± 4 vessels per microscopic field; mean ± SD (n = 3),
P = 0.002]. Red blood cells were detected within the lumens of the
tumor vessels, indicating that the vessels were functional conduits
(Fig. 5C, bottom). Similar results were obtained when these in vivo
experiments were performed using a pooled population of TWEAK-
overexpressing cells instead of isolated clonal cell lines (data not
shown). These results demonstrate that TWEAK overexpression en-
hances tumor growth and angiogenesis in athymic mice.

In summary, we report here that the tumor necrosis factor-related
 cytokine TWEAK is expressed in various types of human tumors. To
examine the potential relevance of this observation, we generated
HEK293 cell lines that constitutively secrete TWEAK and examined
the growth properties of these cells. We found that although soluble
TWEAK overexpression does not confer a growth advantage on
HEK293 cells cultured in vitro, it does potentiate HEK293 tumor
growth in vivo. Also, the TWEAK-overexpressing HEK293 tumors
had significantly more blood vessel penetration when compared with
the control HEK293 tumors. Additional studies are necessary to
ascertain whether the TWEAK-Fn14 signaling system contributes to
solid tumor growth and metastasis in humans.

Fig. 3. Effect of TWEAK overexpression on HEK293 cell growth in vitro. A. Schematic representation of the expression cassette encoding soluble TWEAK protein. The immunoglobulin chain secretion signal peptide, the TWEAK-receptor binding domain (TWEAK-RBD) region, and the position of the myc epitope are indicated. B. Cell lysates and conditioned medium samples were prepared from five TWEAK-expressing HEK293 cell lines (1–5) and a vector-transfected HEK293 cell line (V), and equal amounts of protein were analyzed by SDS-PAGE and Western blot analysis using an anti-myc antibody. C. The growth potential of the five TWEAK-expressing HEK293 cell lines (1–5), the vector-transfected HEK293 cell line (V), and untransfected HEK293 cells (U) was examined in cell culture medium containing 0.5% serum over a period of 6 days. The fold-increase in cell growth for each cell line was calculated using the day 0 mean cell numbers as the baseline values. Another independent experiment gave similar results.

Fig. 4. Effect of TWEAK overexpression on HEK293 cell growth in nude mice. Tumor xenograft volumes at three time points after inoculation of Matrigel alone (◇) or Matrigel mixed with either the vector-transfected HEK293 cell line (●), TWEAK-overexpressing HEK293 cell line 2 (◆), or TWEAK-overexpressing HEK293 cell line 3 (■) are shown. The values shown are mean; bars, ± SD (n = 5); *P < 0.05 compared with vector control. Another independent experiment gave similar results.
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