[CANCER RESEARCH 64, 8968–8972, December 15, 2004]

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 Hybridization. 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 *Bgl*II/*Xba*I fragment of the plasmid phTWEAK (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 *Eco*RI/*Xho*I fragment of pBluescript/hFn14 (21), and human glyceraldehyde 3-phosphate dehydrogenase, ~0.8-kb *Pst*I/*Xba*I fragment of pHcGAP (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

Received 5/28/04; revised 9/8/04; accepted 10/12/04.

Grant support: NIH Grant HL-39727 (J. A. Winkles).

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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′-AGCTGCGGCCGCGCCGCCACCATGG-3′) onto an *Nco*I/*Xba*I fragment of the plasmid phTWEAK (gift of Timothy Zheng, Biogen Idec Inc.) and then by cloning this DNA fragment into *Hind*III and *Xba*I 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′-AAGCCTATTTCTGAAGAAGACTTGAGAGCGATCGCAGCC-3′, and in reaction 2 an antisense T7 primer was used in combination with the sense primer 5′-TTCTTCAGAAATAAGCTTTTGCTCTCGAGCCCGTGTTTT-3′. The two PCR products were isolated, pooled, and used as the template for second stage PCR using the sense primer 5′-AGCTGCGGCCGCGCCGCCAC-3′ and the antisense primer 5′- CGAGATCTGTCAGTGAACCTGGAAGAG-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′-GAAGATCTAACACGGGCTCGAGAGCAA-3′, the antisense primer 5′-CGAGATCTGTCAGTGAACCTGGAAG AG-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 *Bgl*II, and the released DNA fragment was ligated into *Bam*HI-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/TWK-RBD-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 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 $MgCl_2$. 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).

Cell Growth Assays. Cell growth was monitored using the CyQUANT cell proliferation kit (Molecular Probes, Eugene, OR) as described (11). Briefly, the HEK293 cell lines were seeded in triplicate at a density of 5.3×10^2 cells/cm^2 onto gelatin-coated 24-well plates in normal growth medium and allowed to attach for 8 hours. One set of plates was processed and stored at −80°C to provide day 0 cell numbers. The medium was then aspirated from the other plates and replaced with HEK293 cell growth medium containing 0.5% FBS instead of 10% FBS. Cells were refed with fresh medium on days 3 and 5, and cell numbers were calculated for days 2, 4, and 6.

Tumor Growth in Athymic Nude Mice. The HEK293 cell lines were harvested, and 10^6 cells were suspended in 100 μL of growth factor-reduced, phenol red-free Matrigel (BD Biosciences) and injected s.c. into the right flank of 4 to 6-week-old, female athymic NRC-*nu/nu* mice (National Cancer Institute, Frederick, MD). Another group of mice was injected with Matrigel alone. Tumor size was measured every other day using a ruler. Tumor volume was calculated using the formula $V = (W/2)^2 \times L$, where W is the distance across and L is the measurement lengthwise of the tumor. The mice were euthanized at 14 days after injection and photographed. Tumors were excised from each mouse, photographed, and then either weighed and used for measurements of hemoglobin content or directly processed for immunohistochemical analysis of vessel density.

Hemoglobin Assays. Tumors were frozen on dry ice and dried. The dried pellets were minced and dissolved in 0.5 mL of 0.1% Triton X-100 for 2 hours at room temperature with shaking. After centrifugation at 3200 rpm for 60 minutes, 100 μL of the clear supernatant was transferred over to a 96-well plate. The absorbance of each sample was measured at 405 nm using a plate reader. A hemoglobin solution (Sigma) was used to prepare a standard curve, and hemoglobin content was normalized to the weight of each tumor.

Immunohistochemistry. Human colon carcinoma specimens (provided by Chris Haudenschild, American Red Cross, Rockville, MD) were analyzed for TWEAK expression. Briefly, 5-μm paraffin sections were cut, deparaffinized, washed with distilled H_2O, and immersed in 100% methanol/0.3% H_2O_2 for 30 minutes to exhaust endogenous peroxidase activity. Sections were then preincubated with 10% goat serum for 20 minutes at room temperature and then incubated with a 1:20 dilution of rabbit anti-TWEAK IgG (ref. 5; gift of Timothy Zheng, Biogen Idec Inc.) for 1 hour at room temperature. After a wash with PBS, a 1:200 dilution of biotinylated anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA) was added for 30 minutes. The sections were then washed in PBS, incubated with a 1:100 dilution of ABC Elite reagent (Vector Laboratories), developed with 3,3′-diaminobenzidine for 4 minutes, and counterstained with Mayer's hematoxylin for 1 minute.

For analysis of tumor vascularization, tumors were embedded in OCT compound, and 5-μm frozen sections were prepared. The sections were fixed for 10 minutes in 50% methanol/50% acetone, washed with PBS, and immersed in 100% methanol/0.3% H_2O_2 for 30 minutes to exhaust endogenous peroxidase activity. Sections were then preincubated with 10% rabbit serum for 20 minutes at room temperature and then incubated with a 1:50 dilution of rat anti-mouse CD31 antibody clone MEC13.3 (BD Biosciences) for 1 hour at room temperature. After a wash with PBS, a 1:200 dilution of biotinylated anti-rat secondary antibody (Vector Laboratories) was added for 30 minutes. The sections were then washed in PBS, incubated with a 1:100 dilution of ABC Elite reagent (Vector Laboratories), developed with 3,3′-diaminobenzidine for 4 minutes, and counterstained with Mayer's hematoxylin for 1 minute. Angiogenesis was quantitated by direct counting of CD31-positive vascular structures. Representative tumor sections were chosen, and three random microscopic fields were counted per tumor.

Statistics. All of the values are presented as mean ± SD. Statistical significance was evaluated by unpaired Student's *t* test for comparison between two means. Differences were considered statistically significant at $P < 0.05$.

RESULTS AND DISCUSSION

TWEAK Gene Expression in Human Tumor Tissue. The TWEAK cytokine is an angiogenic factor (9); therefore, if expressed by tumor cells it could play a role in tumor angiogenesis. Although previous studies have demonstrated TWEAK mRNA expression in several human tumor cell lines cultured *in vitro* (5, 23, 24), TWEAK

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. 1*A*). 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. 1*B*). 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

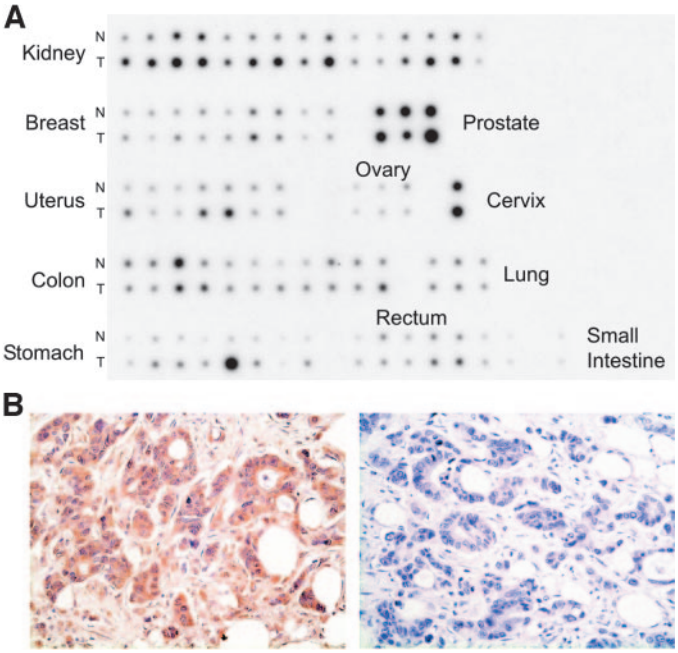

Fig. 1. Analysis of TWEAK expression in human tumor specimens. *A.* Radiolabeled TWEAK cDNA was hybridized to a dot blot array containing normalized loading levels of cDNA prepared from either the tumor (*T*) or adjacent normal (*N*) regions of individual cancer patients diagnosed with the indicated tumor types. The probe did not hybridize to negative control samples [*e.g.,* yeast RNA, poly (A), human genomic DNA] included on the array (this region of the membrane is not shown). *B.* Immunohistochemical analysis was performed on human colon carcinoma tissue using either anti-TWEAK IgG (*left*) or control rabbit IgG (*right*). Magnification, ×40.

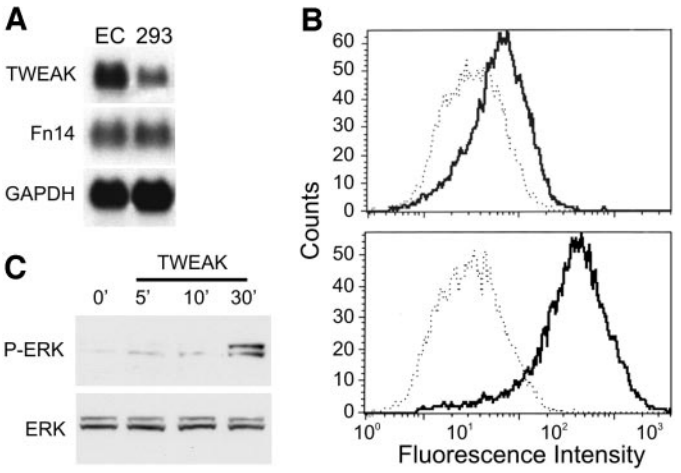

Fig. 2. Analysis of TWEAK expression and Fn14 expression/function in untransfected HEK293 cells. *A.* Total RNA was isolated from human endothelial cells (*EC*; this RNA was used as a positive control) and HEK293 cells (*293*), and equal amounts of each sample were analyzed by Northern blot hybridization using the radiolabeled cDNA probes indicated on the *left*. *B*. HEK293 cells were stained with phycoerythrin-labeled anti-TWEAK antibody (*solid line, top*), anti-Fn14 antibody (*solid line, bottom*), or control IgG (*dotted line, both panels*) and then analyzed by flow cytometry. *C.* Serum-starved HEK293 cells were either left untreated (0′) or treated with 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 (*P*)-ERK or total ERK.

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. 2*A*), relatively low levels of membrane-anchored TWEAK are present on the HEK293 cell surface (Fig. 2*B*), 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. 2*A*) and protein (Fig. 2*B*). 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. 2*C*). 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. 3*A*) 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 NH_2 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 protein with an apparent molecular mass of ~24 kDa was detected in both the cell lysate and conditioned media samples from the pSecTag2/TWEAK-RBD-myc–transfected cell lines (Fig. 3*B*). The TWK1, 2, 3, and 4 lines

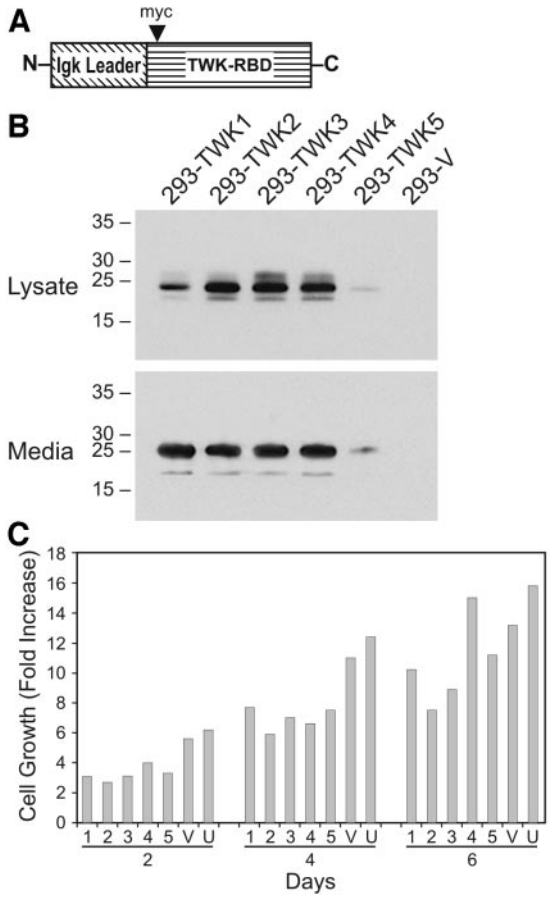

Fig. 3. Effect of TWEAK overexpression on HEK293 cell growth *in vitro*. *A,* schematic representation of the expression cassette encoding soluble TWEAK protein. The immunoglobulin κ chain secretion signal peptide, the TWEAK-receptor binding domain (*TWK-RBD*) region, and the position of the myc epitope 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.

expressed a similar amount of soluble TWEAK, whereas the TWK5 line expressed significantly less. The predicted molecular mass of the TWEAK-RBD–myc protein is ~18 kDa, but it has been reported that soluble TWEAK is *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 blot.

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. 3*C*), although the TWK1, 2, 3, and 4 lines expressed significantly more soluble TWEAK protein than the TWK5 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 overexpression 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 basement 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 calculated. The two TWEAK-overexpressing cell lines showed a significantly increased tumor growth rate compared with the vector-transfected cell line (Fig. 4). Inspection of the tumors harvested from each of the experimental groups revealed that the TWK2 and TWK3 cell line-derived tumors were clearly larger and more vascularized than the vector-transfected cell line-derived tumor (Fig. 5*A*). Enhanced neovascularization of these tumors was confirmed by measuring hemoglobin content (Fig. 5*B*). In addition, immunohistochemical staining of tumor sections using an antibody that specifically recognizes endothelial cells revealed increased microvessel density in the TWK2 (Fig. 5*C*) and TWK3 (data not shown) cell line-derived tumors. Direct counting of vascular structures revealed that microvessel density was ~4-fold higher in the TWK2 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. 5*C, 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 enhances 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.

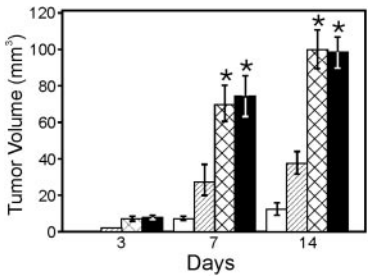

Fig. 4. Effect of TWEAK overexpression on HEK293 cell growth in nude mice. Tumor xenograft volumes at three time points after inoculation of Matrigel alone (□) or Matrigel mixed with either the vector-transfected HEK293 cell line (▨), TWEAK-overexpressing HEK293 cell line 2 (▨), or TWEAK-overexpressing 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.

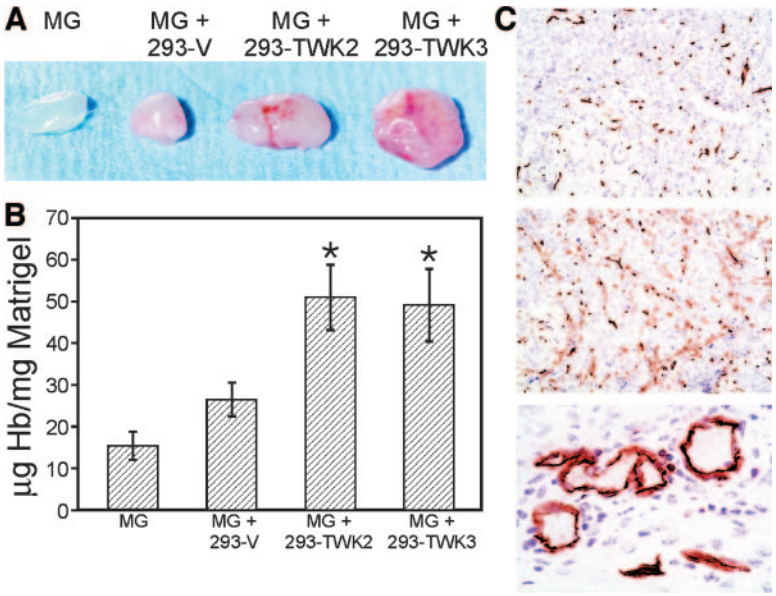

Fig. 5. Effect of TWEAK overexpression on tumor vascularization. *A.* Photographs of representative tumors harvested from each of the four groups on day 14 are shown. *B.* Hemoglobin content was measured in tumors harvested from each of the four groups on day 14. The values shown are mean; *bars,* ±SD ($n = 4$); $*P < 0.05$ compared with vector control. *C.* Immunohistochemical staining for the endothelial cell marker CD-31/PE-CAM-1 was performed on frozen sections prepared from vector-transfected HEK293 cell tumors (*top*) or TWEAK-overexpressing HEK293 cell line 2 tumors (*middle* and *bottom*). Magnification is ×10 in the *top* and *middle* and ×100 in the *bottom*.

ACKNOWLEDGMENTS

We thank Elizabeth Smith and Dr. Mark Williams for technical assistance, Dr. Christian Haudenschild for providing the human colon tumor tissue, Dr. Timothy Zheng for providing the TWEAK plasmid and anti-TWEAK antibody, and Sue Robinson for providing the anti-myc antibody.

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Cancer Res 2004;64:8968-8972.