TNF-α downregulates vascular endothelial Flk-1 expression in human melanoma xenograft model

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Menon, Chandrakala, Malini Iyer, Indira Prabakaran, Robert J. Canter, Shannon C. Lehr, and Douglas L. Fraker. TNF-α downregulates vascular endothelial Flk-1 expression in human melanoma xenograft model. Am J Physiol Heart Circ Physiol 284: H317–H329, 2003. First published September 19, 2002; 10.1152/ajpheart.00971.2001.—High-dose TNF with melphalan has significant antitumor activity in regional perfusion of the limbs and liver in human malignancies. TNF is believed to target tumor vasculature, but the precise molecular mechanism is unknown. The present study demonstrates that TNF downregulates the VEGF receptor, fetal liver kinase-1 (Flk-1), on tumor endothelium in a human melanoma xenograft model. NIH1286 human melanoma cells were transduced with a 720-bp fragment of the human VEGF121 gene to develop well-vascularized tumors that served as an amplified system for measuring Flk-1 expression changes. We injected 5 × 10⁶ cells subcutaneously into each of two distinct single cell clones (NIH1286/3 and NIH1286/15), into athymic nude mice to produce tumors ∼10 mm in size. Each animal then received either BSA or TNF in BSA by tail vein. Tumors harvested at different time points post-TNF were analyzed for Flk-1 mRNA and protein expression. Data obtained showed that intravascular TNF downregulated Flk-1 expression in tumor endothelial cells. This effect could contribute to the antitumor activity of TNF known to target tumor vasculature.

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after ILP, TNF has been shown to selectively shutdown the tumor vasculature by causing increased tumor vascular leakage and erythrocytosis while sparing normal vasculature in the limb (7, 16). Vascular shutdown has been shown to increase hypoxia and, therefore, to increase VEGF production within the tumor (30). VEGF is a known endothelial mitogen and a potent inducer of angiogenesis that works via its two receptors, fetal liver kinase-1 (Flk-1) and fms-like tyrosine kinase-1 (Flt-1). Therefore, to prevent tumor recovery after ILP with TNF and melphalan, it would be important to not only shutdown existing vasculature in the tumor but to also prevent angiogenesis induced by the resulting hypoxic milieu.

There appears to be a direct correlation between Flk-1 expression levels and angiogenic activity because the highest levels of Flk-1 expression are observed during vasculogenesis and angiogenesis and pathological processes associated with neovascularization, such as tumor angiogenesis (22, 24, 26). Also, suppression of VEGF activity by inhibiting its receptors has been shown to suppress retinal neovascularization in a murine model of ischemic retinopathy (1), and deletion of the Flk-1 gene by homologous recombination has been shown to abolish vasculogenesis in mice (29). Several studies have also shown a direct correlation between Flk-1 inhibition and tumor regression/cell kill (3, 17, 21, 27, 32). These findings, taken together, indicate that any factor that can decrease the levels of expression of Flk-1 in tumors is likely to directly affect the process of tumor angiogenesis and, therefore, tumor growth.

Earlier work has shown that TNF downregulates VEGF-specific receptors Flk-1 and Flt-1 on endothelial cells in vitro. This TNF effect is transcriptionally mediated and is accompanied by a decrease in immunoprecipitable Flk-1 protein (25). No data are available on the effect of TNF on VEGF receptors Flk-1 or Flt-1 in an in vivo model. One obstacle in evaluating the effect of TNF on VEGF receptor expression in vivo is the relatively low baseline level of these receptors in many tissues, including tumors. To circumvent this problem, two high VEGF-expressing human melanoma xenografts were developed in nude mice for the present study. These tumors showed increased vascular density and also higher baseline levels of Flk-1 expression compared with their parent or vector only-containing tumors and were used to show, for the first time, that high-dose TNF downregulated Flk-1 mRNA and protein in tumors in an in vivo setting.

**MATERIALS AND METHODS**

**Cell Lines and Culture Conditions**

NIH1286 human melanoma cell line, a generous gift from Dr. Steven Rosenberg (National Institutes of Health, Bethesda, MD) was grown in RPMI medium supplemented with 10% fetal calf serum, 2 mM l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, all obtained from LTI/GIBCO-BRL (Gaithersburg, MD). PA317 retroviral packaging cell line (American Type Culture Collection; Manassas, VA) was cultured in DMEM (LTI/GIBCO) supplemented with the above mentioned additives.

**Cloning of VEGF Gene**

PL301 vector containing a 720-bp fragment of human VEGF121 (a generous gift from Dr. Meenhard Herlyn, Wistar Institute, Philadelphia, PA) was digested with Not1 and cloned into the Not1 site of the retroviral vector, PG1EN obtained from Dr. Patrick Hwu (National Cancer Institute, Bethesda, MD). PG1EN VEGF or PG1EN DNA was transfected using lipofectamine (GIBCO-BRL) into the packaging line PA317. A heterogeneous population of PA317 containing PG1EN-VEGF (PLVh121EN) or PG1EN vector alone was selected with G418 (200 μg/ml) (LTI/GIBCO-BRL). Virus was harvested from these stable packaging cell lines and used to transduce the melanoma cell line NIH1286.

**Screening of VEGF Clones by PCR**

Single cell clones were selected, and DNA was extracted and screened for the VEGF insert by PCR.

**DNA extraction.** Cells (500,000) were washed in PBS, pelleted, and resuspended in 200 μl of proteinase K buffer containing 1 ml of 10× PCR buffer (Boehringer-Mannheim), 9 ml deionized water, 50 μl Tween 20 (Sigma), and 20 μg of proteinase K (Sigma). The solution was incubated at 55°C for 2 h, boiled for 5 min, placed on ice for 2 min, and centrifuged at 300 g. Supernatant (2 μl) was used for the PCR reaction.

**PCR for VEGF.** PCR was carried out in a 100-μl volume containing 200 μM 2-deoxynucleotide 5′-triphosphate (dNTP), 1 unit Taq polymerase and PCR buffer (all purchased from Roche Molecular Biochemicals). We used 0.25 μM each of the upstream and downstream primers. The reaction was allowed to run for 35 cycles. Each cycle had the following settings: 94°C for 1 min, 53°C for 1 min, and 72°C for 1 min. The upstream primer sequence was 5′-AGGAGGAGGCGAATCCTACAC-3′, and the downstream primer sequence was 5′-TTTGGGCGAGGAGGAAAGAC-3′. The downstream primer chosen was in the vector region to avoid expression of endogenous VEGF. PCR products were electrophoresed on a 1.2% agarose gel in 1× TAE buffer and stained with ethidium bromide.

**Selection of High VEGF Protein-Producing Clones by ELISA**

Clones positive by PCR for the VEGF transgene were assayed for protein production using a Quantikine human VEGF ELISA kit (R&D Systems; Minneapolis, MN). Briefly, 100,000 cells of each selected clone were plated on 24-well plates and incubated for 24 h in RPMI medium. Supernatants were harvested and assayed for human VEGF.

**Tumor Production in Nude Mice**

Athymic nude mice (Harlan Sprague Dawley; Indianapolis, IN) were housed in the Animal Care Facility at the University of Pennsylvania according to National Institutes of Health and institutional guidelines. At 6–8 wk of age, the mice were subcutaneously injected with 5 × 10⁶ of NIH1286 (parent), NIH1286/18 (vector alone), NIH1286/15, or NIH1286/15 cells in 0.1 ml of RPMI medium. The latter two clones were high VEGF-expressing cell lines selected on the basis of in vitro cell supernatant ELISA assays for VEGF. Tumors were allowed to grow over the next 3–6 wk until they reached 10 mm in diameter.

**TNF Treatment**

Forty milligrams of recombinant TNF (Knoll Pharmaceuticals; Whippany, NJ) suspended in 0.5 ml of sterile 0.5%
PBS-BSA were administered via tail vein injection to mice bearing NIH1286/3 or NIH1286/15 tumors. Control mice were injected with 0.5 ml of sterile 0.5% PBS-BSA alone. Tumors were harvested under anesthesia (intramuscular injection of 0.035 ml bacteriostatic water, 0.015 ml ketamine, and 0.05 ml xylazine) at 0, 0.5, 1, 2, 4, 6, 8, 12, 24, and 48 h after TNF administration. A total of 60 mice (6 mice per time point) were used for each tumor type for treatment with TNF (4 mice per time point) or BSA (2 mice per time point). Mice were euthanized by CO2 inhalation. Half of the TNF-treated and half of the BSA-treated tumors were harvested and frozen immediately in liquid nitrogen for total RNA isolation, and half of the BSA-treated tumors were harvested and frozen immediately in liquid nitrogen for total RNA isolation, and the other half were frozen in OCT (Tissue-Tek) for cryosectioning. Tissue sections were cryosectioned and stored at −80°C until use for in situ hybridization and immunohistochemistry.

**Platelet Endothelial Cell Adhesion Molecule Immunohistochemistry of Tumor Sections**

Cryopreserved tumor tissue sections of NIH1286, NIH1286/18, NIH1286/3, and NIH1286/15 tumors and NIH1286/15 tumors that were TNF or BSA treated were fixed in cold acetone on ice for 5 min and blocked with PBS containing 5% BSA and rabbit serum. The sections were immunostained using a rat antiserum platelet endothelial cell adhesion molecule (PECAM) antibody (a generous gift from Dr. Steven Albelda, University of Pennsylvania, Philadelphia, PA), an anti-rat secondary antibody (Vector Labs; Burlingame, CA), the ABC Elite kit, and the Vector VIP Peroxidase Substrate kit (Vector Labs). A tumor tissue section of each type that was not exposed to primary antibody was used as negative control for PECAM immunostaining. The slides were viewed and photographed under a light microscope at a total magnification of ×200.

**Microvessel Density Measurements**

Microvessel density (MVD) was assessed by light microscopy in NIH1286, NIH1286/18, NIH1286/3, and NIH1286/15 tumor tissue sections. Areas of most intense vascularization were picked out by scanning PECAM-immunostained tumor sections under a light microscope using a ×100 objective. Individual microvessels were then counted within a ×200 field equal to a 0.95-mm² area. Branches arising from vessels were counted as individual vessels. Any brown-staining endothelial cell or endothelial cell cluster, clearly separate from adjacent microvessels and tumor cells, was considered a single countable microvessel. Vessel lumens, although sometimes present, were not necessary for a structure to be defined as a microvessel. All counts were performed separately by two investigators. The mean of five fields was computed for each tumor section, and standard deviations were computed.

**In Situ Hybridization for Flk-1 and Flt-1**

**Hybridization protocol.** Frozen sections of TNF-treated NIH1286/15 tumors were mounted on poly-l-lysine-coated slides, baked at 50°C for 0.5 h, and fixed in cold 4% paraformaldehyde for 10 min. Sections were treated with 0.2 M HCl in DEPC-H₂O at room temperature (RT) to solubilize cross-linked proteins and then were equilibrated in buffer containing 10 mM Tris-HCl and 1 mM EDTA in DEPC-H₂O. Sections were permeabilized with proteinase K (1 µg/ml in TE) at 37°C for 15 min and were rinsed sequentially in 0.2% glycine in DEPC-PBS, DEPC-PBS, and 4% paraformaldehyde at RT to neutralize proteinase K. Sections were acetylated with TEA solution containing 0.1 M triethanolamine and 1:200 vol/vol acetic anhydride. Sections were rinsed with 2 × SSC for 10 min at RT. Hybridization was then carried out in a humidified chamber at 50°C overnight with sense and antisense cRNA probes to Flk-1 or Flt-1 with 50 µl of hybridization buffer containing 50% deionized formamide, 10% dextran sulfate, 0.3 M NaCl, 10 mM Tris-HCl (pH 7.6), 5 mM EDTA, 10 mM DTT, 0.1 µg/µl yeast tRNA, cold S-UTP (17.9 µ/800 µl of hybridization fluid; NEN LifeSciences, Boston, MA), and 600,000 counts/min of labeled probe. Two posthybridization washes were carried out in a solution containing 50% formamide, 2× SSC, and 10 mM DTT for 30 min each at 50°C followed by a rinse in a solution containing 4× SSC, 10 mM Tris-HCl, and 1 mM EDTA for 15 min at 37°C. Sections were then treated with 10 µg/ml RNase A in 4× SSC, 10 mM Tris-HCl, and 1 mM EDTA at 37°C for 30 min to degrade unbound single-stranded probe. A high stringency wash in 50% formamide was then done at 65°C for 1 h followed by dehydration of the slides through graded alcohol.

**Autoradiography and development of slides.** After completion of posthybridization washes, the tissue sections were allowed to dry in a fume hood and stored at 4°C until they were developed. RNase-resistant hybrids indicative of areas of mouse Flk or mouse Flt probe binding were detected by autoradiography using Kodak NTB-2 emulsion. After being dipped in emulsion, slides were stored in a dark container at 4°C for 3 wk. Slides were then developed using Kodak D19 developer and Kodak Fixer and then lightly counterstained with hematoxylin. Finally, sections were rinsed with xylene, dried, and then mounted in Permunt (Fisher, Pittsburgh, PA). Slides were viewed and photographed using a dark-field microscope at ×200 magnification and Image Pro Plus Software (Media Cybernetics, Silver Spring, MD). White silver grains indicate sites of accumulation of Flk-1 or Flt-1 mRNA under study. Parallel sections hybridized with the sense probe served as negative controls.

**Semiquantitative RT-PCR for Flk-1**

NIH1286/15 tumors that were treated with TNF were harvested at 0, 0.5, 1, 2, 6, 12, and 24 h after TNF treatment, frozen immediately in liquid nitrogen, and stored at −80°C. Total RNA was extracted from these tumors using the Stratagene micro RNA extraction kit (La Jolla, CA) and quantitated by spectroscopy at 260 nm. RNA (3.5 µg) was reverse transcribed in a 20-µl volume using 0.5 µg of oligo(dT) (Promega; Madison, WI), 0.5 mmol dNTPs, 10 mmol DTT, 4 µl of first-strand buffer, and 10 U/µl M-MLV reverse transcriptase.
(GIBCO-BRL) for 1 h at 37°C. After activation of the reverse transcriptase at 95°C for 5 min, 2 μl of the reaction mixture were PCR amplified with murine Flk-1- and β-actin-specific primers. Murine Flk-1 upstream and downstream primers were 5'-GCT TGG CCC GCC ACA TTT AT-3' and 5'-CAG TGG CCG CTT CTC TGG CTT ACT-3', respectively. β-Actin upstream and downstream primers were 5'-TGA GCC CAT CCA TCG GCC CAT CTA-3' and 5'-CTA GAA GCA TTT GTG GAC GAT GGA GGG-3', respectively. PCR for Flk-1 (35 cycles, 94°C for 1 min, 53°C for 1 min, and 72°C for 1 min) was carried out in a 100-μl volume with 0.25 μM primers, 200 μM dNTPs, 1 unit Taq polymerase and PCR buffer. Taq PCR buffer, and dNTPs were obtained from Roche Biochemicals (Indianapolis, IN). PCR for β-actin was performed in a similar manner except that the annealing temperature for the reaction was changed to 51°C and only 0.1 μM β-actin primers were included. PCR products were then electrophoresed on a 1.2% agarose gel in 1× TAE buffer and stained with ethidium bromide. The bands were quantitated by densitometry using the NIH Image software after normalizing for β-actin levels in each lane. Care was taken to ensure that the PCR reaction was quantified in the linear range of the reaction.

Protein Extraction and Western Blot Analysis for Flk-1

Protein from TNF- and BSA-treated NIH1286/15 tumors at 0, 2, 4, 6, 8, 12, and 24 h posttreatment was extracted as described previously (8). Briefly, frozen tumor tissue was ground using a mortar and pestle and then homogenized in ice-cold buffer containing 20 mM Tris⋅HCl (pH 7.2), 1 mM EDTA, 1 mM EGTA, 0.1 mM NaCl, 1 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 2 μg/ml pepstatin. After 1 h on ice, lysates were microcentrifuged at 14,000 rpm for 15 min. The supernatant containing solubilized cellular protein was decanted, and the pellet was resuspended in ice-cold buffer containing 50 mM Tris⋅HCl (pH 7.4), 5 mM EDTA, 150 mM NaCl, 0.5% NP-40, 0.1% SDS, 1 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 2 μg/ml pepstatin. This solution was kept on ice for 1 h with frequent vortexing and then microcentrifuged at 14,000 rpm for 15 min. Supernatants from this centrifugation were then utilized for subsequent Western blot analysis. Quantitation of protein in the supernatants was carried out using a BCA protein assay kit (Pierce; Rockford, IL). Thirty micrograms of protein from TNF and BSA-treated tumors from each time point were electrophoresed on a 7.5% SDS-PAGE gel and transferred to polyvinylidene difluoride membranes. Membranes were then blocked for 1 h at room temperature with 5% nonfat milk in TBS containing 0.1% Tween 20. A polyclonal rabbit antimurine Flk-1 antibody (Santa Cruz Biotechnology; Santa Cruz, CA) was added at 1:2,500 dilution in 5% nonfat milk in TBS containing 0.1% Tween 20. An enhanced chemiluminescence kit (Amersham; Piscataway, NJ) was used to detect immunoreactive bands. After detection of Flk-1 protein bands, blots were washed in TBS containing 0.1% Tween 20 and reprobed with a polyclonal rabbit anti-β-actin antibody (1:1,000 dilution) to confirm equal loading of protein. The protein bands were detected by scanning densitometry after normalization for β-actin. Although β-actin is a soluble cytosolic protein and much of it was discarded in the supernatant, enough of the protein shows up in the microsomal fraction as a contaminant and was used to normalize for protein loading in the SDS-PAGE gel. Densitometric data from three independent Western blot analyses were used to compile means ± SE.

Immunohistochemistry for Flk-1

Cryopreserved tumor sections from NIH1286/3 and NIH1286/15 treated with TNF or BSA were fixed in 3.7% PBS-buffered formalin for 10 min. Slides were rinsed well in PBS and permeabilized in 0.1% Triton in PBS for 2 min. After being rinsed in PBS, the slides were incubated in 0.5% H2O2 for 10 min to quench endogenous hydroperoxides. Sections were blocked for 1 h in a 5% PBS-BSA solution containing 5% goat serum. The sections were incubated overnight at 4°C in blocking serum with an anti-mouse Flk-1 rabbit polyclonal IgG (Santa Cruz Biotechnology) at a 1:100 dilution. Slides were then rinsed well in PBS and incubated for 2 h in blocking serum containing an anti-rabbit horseradish peroxidase-conjugated IgG (Santa Cruz Biotechnology) at a 1:100 dilution. Slides were rinsed well in PBS and developed using the Elite Standard Vectastain ABC kit and the Vector VIP peroxidase substrate kit (Vector Labs). The slides were mounted using glycerol, and bright-field pictures were taken using a light microscope at a magnification of ×200.

RESULTS

TNF and melphalan administered by ILP are effective for the treatment of extremity melanoma and soft tissue sarcoma in a clinical setting. It is the most optimal treatment in the above stated instances. Figure 1 shows the leg of a human melanoma patient who failed melphalan alone (by ILP) and later responded to TNF plus melphalan (by ILP preop, Fig. 1A; and postop, Fig. 1B).

Human melanoma xenografts were developed in nude mice to study the effect of TNF on Flk-1 expression. Human melanoma xenografts not transfected with the VEGF gene had blood vessels that were only faintly visible by Flk-1 immunohistochemistry in situ hybridization. RT-PCR analysis of RNA isolated from these tumors yielded only faint baseline bands representing Flk-1 expression in DNA gels that disappeared between 30 min and 12 h post-TNF. Thus the first attempts to use TNF-responsive parent cell line (NIH1286)-derived human melanoma xenografts for studies on the effect of TNF on Flk-1 expression changes produced positive but inconclusive data about the effect of TNF on the expression of Flk-1. An amplified system was therefore developed in the form of two VEGF-overexpressing tumors derived from two melanoma cell lines, NIH1286/15 and NIH1286/3. These cell lines were derived from the parent NIH1286 cell line and contained the PG1EN vector carrying a 720-bp fragment of the human VEGF121 gene. NIH1286/clone 3 and NIH1286/clone 15 were selected for further study on the basis of PCR analysis and VEGF ELISA assays carried out on cell supernatants. NIH1286/3 cell supernatants expressed a 80-fold increase and NIH1286/15 expressed a 100-fold increase in VEGF expression when compared with the parent NIH1286 or the vector only (NIH1286/18-containing line (data not shown)). Grossly, the high VEGF-expressing tumors showed a prominent blush when compared with the pale pink tumors produced by either the parent cell line or vector.
only-containing cell line. These observations can be explained by increased vascularity in the NIH1286/3 and NIH1286/15 tumors when compared with the parent or vector only tumors as seen by MVD analysis of PECAM-immunostained tumor tissue sections (Fig. 2A). MVDs of the different tumor types were as follows: NIH1286/H11005 41, NIH1286/H11006 6, NIH1286/18/H11005 39, NIH1286/18/H11006 4, NIH1286/3/H11005 72, NIH1286/3/H11006 15, and NIH1286/15/H11005 74, NIH1286/15/H11006 11. Also, the high VEGF-expressing tumors had a three- to fourfold higher baseline level of Flk-1 than the nontransfected NIH1286 parent tumor (Fig. 2B). This increase in Flk-1 expression is probably the result of the increased vascularity seen in the transfected tumors. It is relevant to point out here that only the tumor cells and not the vascular endothelial cells were genetically manipulated for high VEGF expression in the tumors. Therefore, any changes in the tumor endothelial cells after TNF treatment would be physiologically relevant.

TNF, when used alone, shows varying extents of anti-tumor effect on a number of human melanoma mouse xenografts including NIH1286/3 and NIH1286/15. These two tumor types were therefore used to demonstrate the effect of TNF on Flk-1 expression changes independent of the melphalan effects.

Mice with subcutaneous NIH1286/3 or NIH1286/15 tumors were given PBS-BSA alone or 40 μg of recombinant human TNF in PBS-BSA by intravenous injection, and tumors were harvested at 0 (pretreatment), 0.5, 1, 2, 4, 6, 12, 24, and 48 h post-TNF. Hematoxylin and eosin-stained tumor tissue sections from PBS-BSA-treated tumors showed little to no necroses (Fig. 3, A and B). TNF-treated tumors, however, showed onset of tumor necroses between 24 and 72 h posttreatment (Fig. 3, C and D).

In situ hybridization with the antisense cRNA probe showed that Flk-1 expression was downregulated by 2 h post-TNF treatment and remained downregulated until 6 h posttreatment. Flk-1 mRNA expression started recovering by ~12 h and returned to control levels by 24–48 h post-TNF treatment (Fig. 4). The control sense probe did not produce any signal, as expected. Flt-1 mRNA, however, did not show decreased expression after TNF treatment (data not shown).

To confirm the time course of Flk-1 mRNA expression in response to TNF, RT-PCR analysis of Flk-1 expression was carried out in the BSA- and TNF-treated NIH1286/15 tumors (Fig. 5A) using Flk-1 primers that yielded a 560-bp fragment as expected. These bands were standardized against β-actin expression (661-bp fragment) by densitometry. The results showed a similar time course of Flk-1 mRNA expression as seen with in situ hybridization. Flk-1 mRNA expression was downregulated to 40% of baseline levels as early as 0.5 h post-TNF, was at its lowest level of expression (20% of baseline levels) at 6 h post-TNF, and recovered to near-baseline levels by 12–24 h post-TNF treatment. There was no change in Flk-1 mRNA expression in the BSA-treated control tumors (Fig. 5B). The quantification of the RT-PCR products was carried out in the linear range of the reaction (Fig. 5C).

Western blot analysis was used to quantify changes in NIH1286/15 xenograft Flk-1 protein expression before and after TNF treatment (Fig. 6). Scanning densitometric measurements of protein Western blot bands showed a 60% decrease in Flk-1 protein expression by 2 h post-TNF treatment compared with pretreatment baseline levels. A near-complete loss of protein expression occurred at 8 and 12 h following TNF treatment (8 ± 2% and 5 ± 1% respectively). By 24 h, Flk-1 protein expression recovered to 23 ± 3% of baseline levels (Fig. 6, A and C). In contrast, the BSA-treated xenografts demonstrated a modest increase in

Fig. 1. Photograph of the leg of a melanoma patient who initially failed a melphalan-alone isolated limb perfusion (ILP) and later responded to a TNF plus melphalan ILP. A: preop; B: 4-mo postop.
Flk-1 expression relative to baseline levels (Fig. 6, B and C).

Immunohistochemical analysis of NIH1286/3 and NIH1286/15 tumor tissue sections clearly showed Flk-1 protein expression on blood vessels (Fig. 7). Flk-1 protein expression decreased as early as 2 h post-TNF treatment in both NIH1286/15 (Fig. 7D) and NIH1286/3 (data not shown) tumors, was at its lowest levels of expression at 6 h posttreatment (Fig. 7F and Fig. 10D), and recovered to control levels by 24–48 h.
No change in Flk-1 protein expression was seen in the BSA-treated controls of both tumor types (Fig. 8, Fig. 10, A and B). Although limited in scope, PECAM immunohistochemical analysis of the above tumor tissue sections showed no downregulation of this vascular endothelial antigen and no apparent injury to the vascular endothelium at any time point after TNF treatment, indicating that the downregulation of Flk-1 protein was not a nonspecific response to TNF (Figs. 9, and 10, E and F).

Taken together, the data indicate that high-dose TNF negatively affects Flk-1 expression at the mRNA and protein levels in vivo in the preclinical human melanoma tumor model studied.

Fig. 4. In situ hybridization of cryopreserved NIH1286/15 tumor tissue sections with sense (left) and antisense (right) Flk-1 cRNA probes at different time points of post-TNF treatment. A and B: pretreatment control; C and D: 2 h post-TNF; E and F: 6 h post-TNF; G and H: 12 h post-TNF; I and J: 24 h post-TNF. The sections showed a decrease in Flk-1 expression by 2 h post-TNF that returned to near-baseline levels by 24 h.
DISCUSSION

The ability of TNF to cause hemorrhagic necrosis in transplanted tumors in vivo was first demonstrated by Carswell et al. (5) over 25 years ago. Since then, clinical trials using ILP for advanced extremity melanoma (11, 18, 20) and sarcoma (7, 13, 16) and isolated hepatic perfusion for adenocarcinoma (2) with a single treatment of TNF and melphalan have reported remarkable response rates in human patients with bulky and heavily pretreated disease. Information gained from these clinical responses via the results of radiological studies and pathological biopsies show that TNF targets the tumor vasculature and not the tumor cells directly. The changes seen in pre- and postoperative angiograms show complete disappearance of the vascular blush within the tumor shortly after the treatment (16, 18). Pathological studies show early tissue edema followed by erythrostatosis and complete obliteration of the lumen of tumor vessels (23). An antivascular mechanism could explain why this treatment is widely effective against a variety of solid tumors including adenocarcinoma, melanoma, and sarcoma in such diverse locations as the skin, subcutaneous adipose tissue, muscle, and liver. This wide range of activity against several tumor histologies in a variety of locations may stem from the fact that tumor vascular characteristics and the process of tumor angiogenesis is common to multiple solid tumor types. During regional perfusion of the limb and liver, all the normal tissues, i.e., skin, muscle, bone, and liver, are exposed to the same intravascular concentration of TNF, yet the antivascular effect is not seen in these normal tissues. However, the precise molecular events that limit these antivascular effects to the tumor while sparing treated normal tissue have not been fully elucidated.

TNF is a molecule known to act in a pleiotropic manner, and prior studies have shown that its antivascular effect is mediated by more than one mechanism (14, 33, 34). Although the effect of TNF on Flk-1 and Flt-1 expression has been shown to be a downregulation of the protein in an in vitro setting, no in vivo studies have been conducted that look directly at the effect of TNF on Flk-1 expression at the protein and cellular levels.
Fig. 6. Western blot analysis showing decrease in Flk-1 protein expression post-TNF (A) or BSA (B). Lane 1, pretreatment control; lane 2, 2-h post-TNF/BSA; lane 3, 4-h post-TNF/BSA; lane 4, 6-h post-TNF/BSA; lane 5, 6-h post-TNF/BSA; lane 6, 12-h post-TNF/BSA; lane 7, 24-h post-TNF/BSA. C: densitometric measurements for individual Flk-1 bands after normalization to β-actin.

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Fig. 7. Photomicrographs of cryosections of NIH1286/15 tumors immunostained for FLK-1 protein showing a time-dependent expression after TNF treatment. A, pretreatment control; B, 0.5-h post-TNF; C, 1-h post-TNF; D, 2-h post-TNF; E, 4-h post-TNF; F, 6-h post-TNF; G, 12-h post-TNF; H, 24-h post-TNF; I, 48-h post-TNF; J, negative control. After TNF treatment, FLK-1 protein expression followed its mRNA expression pattern and showed a downregulation by 2 h that returned to baseline levels by 24–48 h.
mRNA levels. The finding presented in this work that TNF decreases Flk-1 in vivo is novel, is previously undescribed, and has relevance to therapy. The tumor is more acidic, more hypoxic, and is exposed to a very different milieu than the in vitro system that was used in the earlier work. Moreover, TNF is known to be antivascular in its effect in tumors, which could create a hypoxic milieu conducive to angiogenesis and Flk-1 upregulation. Therefore, the finding that TNF actually downregulates Flk-1 in vivo is a significant one.

VEGF receptors are expressed at relatively low baseline levels in many tissues, including tumors. The VEGF-overexpressing melanoma tumors NIH1286/15 and NIH1286/3, which develop larger, more promi-

Fig. 8. Photomicrographs of cryosections of NIH1286/15 BSA-treated tumors immunostained for Flk-1. A, pretreatment control; B, 2-h post-TNF; C, 4-h post-TNF; D, 6-h post-TNF; E, 8-h post-TNF; F, 12-h post-TNF. After BSA treatment, there was no change in Flk-1 protein expression in NIH1286/15 tumors.

Fig. 9. Photomicrographs of cryosections of NIH1286/15 TNF-treated tumors immunostained for PECAM. A, pretreatment control; B, 1-h post-TNF; C, 2-h post-TNF; D, 4-h post-TNF; E, 6-h post-TNF; F, 12-h post-TNF; G, 24-h post-TNF; H, negative control. After TNF treatment, there was no apparent damage to the vascular endothelium of the tumor.
... provided an amplified system to carry out this study. With the use of these tumors, it was been established in this study that TNF downregulates Flk-1 expression at the mRNA and protein levels in vivo in a time-dependent manner. Specifically, a reduction in Flk-1 mRNA expression was seen as early as 0.5 h post-TNF treatment: expression was 20% of baseline levels at 6 h and returned to baseline levels by 12–24 h. Protein levels were reduced as early as 2 h post-TNF and were barely detectable at 6–12 h posttreatment. Onset of tumor necrosis in the TNF-treated tumors was seen between 24 and 72 h posttreatment. Downregulation of Flk-1 occurred without gross damage to tumor blood vessels or decrease in expression of PECAM, another endothelial cell-specific antigen. The posttreatment Flk-1 mRNA and protein expression pattern correlates with the half-life of TNF (between 11 and 30 min) (31). It appears that the transient decrease in Flk-1 expression after TNF treatment works in conjunction with other established tumor-selective antiangiogenic effects of TNF, such as increased vascular leakiness and blood vessel occlusion, to produce tumor necrosis. The time course of Flk-1 expression changes between 30 min and 12 h after TNF administration appears to support this hypothesis because it precedes the onset of tumor necrosis. To demonstrate that a downregulation of Flk-1 by TNF independently contributes to the therapeutic effect of TNF, its effect on Flk-1 will have to be separated from its effect on vascular permeability and blood vessel occlusion. Such a separation of TNF effects is almost impossible to achieve with currently available techniques and resources. However, there is some indirect evidence in the literature to show that a transient decrease in Flk-1 expression impacts neovascularization. Cyclic angiogenic processes in the ovarian corpus luteum of monovulatory species are characterized by distinct phases of blood vessel growth, vessel maturation, and vessel regression. The VEGF/VEGF receptor system is expressed through most of the ovarian cycle and is only transiently downregulated during luteolysis, which has been shown to directly lead to a regression of the neovasculature before the 21-day cycle repeats itself (15). Conversely, in a rat infarct model, an initial rapid rise in VEGF and its receptors (275–400%) was observed ~1 h after an acute myocardial infarction and was sustained above baseline levels until ~6 h postinfarct. New vessels were found infiltrating the infarct at 3–7 days after the infarction. Increase in Flk-1 was due to tissue hypoxia (19). If it is assumed, for the sake of argument, that TNF causes erythrostasis without decreasing Flk-1 in the human melanoma xenograft used in this study, then increased tumor hypoxia that will follow erythrostasis could upregulate Flk-1 and allow for tumor recovery. It is therefore significant that TNF downregulates Flk-1 rather than upregulates it. Ample evidence in the literature suggests that Flk-1 upregulation and activation is an absolute requirement for tumor angiogenesis. There is also abundant evidence in the literature to show that inhibition or downregulation of Flk-1 can, by itself, significantly inhibit tumor growth by inhibiting tumor angiogenesis.
(3, 17, 21, 27, 32). One can infer from this information that a transient decrease in Flk-1 expression, coincident with vascular shutdown caused by TNF, will delay the process of angiogenesis and tumor recovery. Since tumor necrosis begins in the time after this transient Flk-1 decrease, it could be rationalized that a delay in tumor recovery due to Flk-1 downregulation could be therapeutically beneficial. Taken together, evidence in the literature and our present work shows that TNF can be both antivascular and antiangiogenic in tumors.

Significant upregulation of VEGF receptor expression is seen in the neovascularature of tumors compared with established normal tissue (21, 26). This could be one explanation for the specificity of intravascular TNF targeting tumor vessels while having minimal effects on adjacent normal vessels during isolated perfusion procedures. Also, VEGF as a growth factor is present more in the microenvironment of the tumor than in quiescent normal tissue. It has been shown that VEGF protein diffusion does not extend beyond 50 μm from the tumor border (6). If the downregulation of Flk-1 by TNF is important in its antitumor response, this localization of VEGF in the tumor microenvironment would provide a mechanism by which the response would be seen only in the tumor vasculature. An area that has remained largely unexplored due to the complexity of the process involved, is the regulation of VEGF receptor expression by TNF. There is some evidence to show that AP-1 and NF-κB, the common regulators of TNF action, are not involved in this regulation (25) and that some novel transacting factors are involved. It is also not known whether the TNF receptors TNFR-I and/or TNFR-II and the TNF receptor-associated factors are involved or whether these effects are mediated in a TNF receptor-independent pathway. It is also not clear as to what other factors within the tumor, if any, interact with TNF in producing the antivasacular effect seen in tumors. Moreover, the basis of the synergy between TNF and melphalan in ILP has not been elucidated. Clearly, these are areas of research that need to be pursued and can further our understanding of the effect of TNF in tumors and their treatment with TNF and melphalan. Understanding the mechanism of the specificity of TNF action against tumor vasculature may contribute to planning systemic antivascular/antiangiogenic treatment strategies that may destroy tumors and leave normal tissue unharmed.

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REFERENCES


19. Li J, Brown LF, Hibberd MG, Grossman JD, Morgan JP, and Simons M. VEGF, Flk-1, and Flt-1 expression in a rat...


