Induction of Endothelial Nitric Oxide Synthase Expression by Melanoma Sensitizes Endothelial Cells to Tumor Necrosis Factor-Driven Cytotoxicity

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ABSTRACT

Purpose: The cascade of molecular events leading to tumor necrosis factor (TNF)-mediated tumor regression is still incompletely elucidated. We investigated the role of endothelial nitric oxide synthase in determining the tumor-selective activity of TNF.

Experimental Design: Using quantitative real-time PCR, endothelial nitric oxide synthase gene levels were measured in melanoma metastases of the skin and normal skin biopsies obtained from 12 patients before undergoing TNF-based therapy. In vitro, the ability of melanoma cells supernatant to affect endothelial nitric oxide synthase transcription by endothelial cells and the influence of nitric oxide synthase inhibition on TNF cytotoxicity toward endothelial cells was evaluated.

Results: Endothelial nitric oxide synthase transcript abundance resulted significantly greater in tumor samples rather than in normal skin samples and in patients showing complete response to TNF-based treatment rather than in those showing partial/minimal response. In vitro, melanoma cells' supernatant induced endothelial nitric oxide synthase gene expression by endothelial cells. Nitric oxide synthase inhibition slowed endothelial cells proliferation and, if induced before TNF administration, decreased the cytokine-mediated cytotoxicity on endothelial cells.

Conclusions: Taken together, these findings support the hypothesis that high expression of endothelial nitric oxide synthase in the tumor microenvironment might increase or be a marker for endothelial cells sensitivity to TNF. These observations may have important prognostic and/or therapeutic implications in the clinical setting.

Received 4/22/04; revised 7/12/04; accepted 7/15/04. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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INTRODUCTION

Tumor necrosis factor (TNF, formerly known as TNF-α) was originally characterized as an antitumor protein causing necrosis of methylcholanthrene-induced sarcomas in animal models (1). Combined with other cytotoxic agents (e.g., doxorubicin, melphalan), TNF is currently used to treat patients affected with locally advanced soft tissue sarcoma and melanoma of the extremities, as well as unresectable liver tumors (2–4). Several mechanisms underlying the anticancer properties of TNF have been proposed. On susceptible tumor cells, TNF exerts a direct cytotoxic effect mediated by the apoptosis pathway (5, 6). Moreover, in animal models, TNF stimulates the antitumor activity of innate immunity (7, 8) and increases tumor concentrations of coadministered antineoplastic drugs (9, 10). However, most investigators currently believe that the main mechanism responsible for the antitumor activity of TNF is the damage of tumor vessels (11–13), which in turn is responsible for an ischemic insult to malignant cells. TNF can directly cause endothelium apoptosis after the engagement with type I TNF receptor (14), supposedly through a reduced activation of integrin-αvβ3 (15). The apoptotic pathway driven by TNF is known quite in detail and involves the cascade of caspases activation (16, 17). However, caspase-independent cell death is caused by this cytokine as well (18, 19). A complex relationship exists between TNF-induced cell cytotoxicity and nitric oxide, a gaseous messenger involved in various biological processes and mainly produced by two nitric oxide synthase isoenzymes. Although the inducible nitric oxide synthase is expressed by different cell types (e.g., macrophages, endothelial cells, tumor cells) under certain circumstances, endothelial nitric oxide synthase is selectively transcribed by endothelial cells (20, 21). Although nitric oxide can contribute to later stages of TNF-induced apoptosis (22–24), low levels of nitric oxide can inhibit central mediators of the apoptotic process such as caspases (25–27). On the other side, some investigators have recently reported that nitric oxide production after the activation of TNF receptors could mediate a built-in negative feedback signal, with which the cytokine would control its own apoptotic program (28).

Despite the large body of experimental and clinical data regarding the antineoplastic effect of TNF, the cascade of molecular events leading to the selective activity of this cytokine against the tumor tissue (as compared with normal tissues such as skin) has not yet been characterized.

In the present study, we investigated on the potential role of nitric oxide synthase in determining the sensitivity of tumor tissue to TNF cytotoxic effects.

PATIENTS AND METHODS

In vivo Study. Skin melanoma metastases (n = 12) and normal skin biopsies (n = 12) were obtained from 12 patients.
before they underwent TNF-based isolated limb perfusion (29), according to a protocol approved by the local ethics committee. Fully informed consent was obtained from each patient participating in this study. Before entering this study, no patient had been treated with biochemotherapy or radiation therapy.

Using quantitative real-time PCR, endothelial nitric oxide synthase and inducible nitric oxide synthase transcriptional levels expressed by tumor specimens were compared with those found in normal skin biopsies.

To document vessel destruction after TNF-based treatment, pre- and posttreatment tumor and normal skin samples were also used for the immunohistochemical evaluation of CD31 protein expression. The immunohistochemical staining was performed using CD31-specific monoclonal antibodies (Dako, Copenhagen, Denmark). Immunoperoxidase staining was performed using an Avidin-Biotin kit (Vector Laboratories, Burlingame, CA). The chromogen consisted of 3,3′-diaminobenzidine tetra-chloride and counterstained with Mayer’s hematoxylin. CD31 expression was assessed by image analysis as performed by the Zeiss-Cires workstation (Zeiss, Jena, Germany). Slides were viewed through an Axioskop microscope with a ×40/0.75 objective (Zeiss). Images were captured by a couple-charged device color videocamera and analyzed with a 66-MHz personal computer and digitized by a frame grabber (Kontron, Eching, Germany). The number of nuclei identified by image analysis on the basis of hematoxylin counterstaining represented the total number of cells. CD31 expression measured in 10 randomly selected ×40 fields was expressed as the mean percentage of positive cells.

In vitro Study. To dissect the results of the in vivo study, we assessed whether the supernatant obtained from the culture of different cell types potentially present in the tumor microenvironment [i.e., melanoma cells, fibroblasts and peripheral blood mononuclear cells (PBMCs)] could affect endothelial nitric oxide synthase gene expression by human umbilical vein endothelial cells (HUVECs). To this aim, 2-day-old supernatant from adherent cells (grown to 75% confluence) or from PBMCs (5 × 10⁵ cells/mL) was used to condition HUVECs for 4 hours. Furthermore, the effect of nitric oxide synthase inhibition on the sensitivity of endothelial cells to TNF-induced cytotoxicity was evaluated. In particular, the nitric oxide synthase inhibitor N-omega-nitro-L-arginine methyl ester [l-NAME (500 μmol/L); Sigma, St. Louis, MO] was added both before and at the same time of TNF administration to assess the influence of nitric oxide before and after the cytokine interaction with its receptor. l-NAME (500 μmol/L; Sigma), the inactive enantiomer of l-NAME, was used as a control.

Cell Cultures. Second passage HUVECs (Clonetics/Bio-Whittaker, Walkersville, MD) were used for most experiments. These cells were cultured in endothelium growth medium-1 according to Clonetics instructions. In some experiments (nitric oxide synthase expression and TNF-induced cytotoxicity on endothelial cells), the supernatant obtained from the culture of three melanoma cell lines [i.e., SK23, Mel-888, and Mel-624, kindly provided by Francesca M. Marincola (NIH, Bethesda, MD)], fibroblasts, or PBMCs was used to condition endothelial cells for 3 hours.

Three melanoma cell lines (i.e., SK23, Mel-888, and Mel-624) were grown in complete Iscove’s medium containing 10% heat-inactivated FCS (Biofluids, Rockville, MD).

For fibroblasts (CCL-210; American Type Culture Collection, Manassas, VA), the DMEM containing 10% heat-inactivated FCS was used.

PBMCs were collected before treatment from the patients enrolled in this study, pooled together, and cultured in complete RPMI medium supplemented with heat-inactivated human antibody serum (Gemini Bioproducts, Calabasas, CA).

Endothelial Cell Viability, Apoptosis, and Proliferation Assays. HUVECs were incubated with and without recombinant human TNF (100 ng/mL; Boehringer, Ingelheim, Germany) and cycloheximide (1 μg/mL; Calbiochem, La Jolla, CA) for 6 hours. In some wells, the nitric oxide synthase inhibitor L-NAME (500 μmol/L) was added to the endothelial cell culture either 2 hours before or together with TNF addition. Cell viability was measured using the 2.3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt-based colorimetric assay (Roche Diagnostics, Indianapolis, IN), following the manufacturer’s guidelines. Briefly, 10⁵ cells/well were seeded in 100 μL of culture medium, and then 50 μL of 2.3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt labeling mixture were added per well and cells incubated for 6 hours at 37°C and 6.5% CO₂. Finally, spectrophotometrical absorbance was measured by an ELISA reader (test wavelength of 450 nm, reference wavelength of 690 nm).

To additionally characterize the effect of TNF on HUVECs treated with nitric oxide synthase inhibitor, we measured the activation of caspase-3, a central mediator of TNF-α–driven caspase-dependent apoptosis, by detection of the chromophore p-nitroanilide (pNA) after cleavage from the labeled substrate DEVD-pNA (ApoAlert Caspase-3 Colorimetric Assay; Clontech, Palo Alto, CA), following the manufacturer’s instructions. Briefly, HUVECs (5 × 10⁵) were treated for 6 hours with TNF ± l-NAME (500 μmol/L), the nitric oxide synthase inhibitor being added 2 hours before or at the same time of the cytokine addition. Then, cells were washed with PBS, resuspended in ice-cold lysis buffer, and incubated on ice for 10 minutes. Cell lysate supernatants were prepared after centrifugation (10,000 × g for 3 minutes). The supernatants were incubated with caspase-3 substrate DEVD-pNA for 1 h at 37°C, and then absorbance (corresponding to substrate cleavage and thus caspase activity) was measured at 405 nm.

To assess the effect of nitric oxide synthase inhibition on endothelial cell proliferation rate, HUVECs (10⁴ cells/well in 100 μL of culture medium) were grown under basal conditions, as well as in the presence of l-NAME (500 μmol/L). The above mentioned 2.3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt-based colorimetric assay (Roche Diagnostics) was used to quantify cell proliferation.

Nitric Oxide Synthase Activity Assay. Nitric oxide synthase activity was assayed in intact endothelial cells by measuring the conversion of l-[3H]arginine (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) into l-[3H]citrulline. Endothelial cell monolayers were washed and then incubated for 20 minutes at 37°C in a reaction buffer containing 145 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgSO₄, 10 mmol/L glucose, 1 mmol/L CaCl₂, and 10 mmol/L HEPES (pH 7.4), with or without l-
NAME (500 μmol/L). A total of 2.5 μCi/ml L-[3H]arginine was added 5 minutes before conditioning endothelial cells with l-NAME or melanoma supernatant. Control cells (endothelial cells conditioned with the inactive enantiomer d-NAME) were run in parallel. Fifteen minutes later, the monolayers were washed with 2 mL of ice-cold PBS supplemented with l-arginine (5 mmol/L) and EDTA (4 mmol/L). A total of 0.5 mL of 100% cold etomidate was added to the dishes and left to evaporate before a final addition of 2 mL of 20 mmol/L HEPES. Separation of L-[3H]citrulline from L-[3H]arginine was obtained by Dowex 50 × 8 to 400 chromatography (Sigma; ref. 30).

**cDNA Synthesis and Quantitative Real-Time PCR.** After RNA extraction using RNeasy Mini kits (Qiagen, Santa Clarita, CA), 1 μg of total RNA was reverse transcribed into cDNA using 1 μL of random hexamers, 5 μL of first-strand buffer, 2 μL of 0.1 mol/l DTT, 2 μL of magnesium sulfate, 1 μL of 10 mmol/L deoxynucleotide triphosphate, and 1 μL of Superscript II. The reaction mixture was heated to 65°C for 10 minutes before adding Superscript II; synthesis was then continued with 1-hour incubation at 42°C. Levels of gene expression were assessed by quantitative real-time PCR as performed by the ABI Prism 7700 Sequence Detection System (Perkin-Elmer, Foster City, CA), as already described by us (31, 32).

To validate the amount and quality of source RNA, transcript levels of two housekeeping genes (i.e., β-actin and CD31) were also measured. As endothelial nitric oxide synthase is selectively produced by endothelial cells, we could use it as an endothelial cell-specific housekeeping gene (i.e., CD31; refs. 33, 34). Instead, we used β-actin to analyze inducible nitric oxide synthase mRNA abundance because this isoenzyme can be produced by different cell types (e.g., melanoma cells, endothelial cells, and macrophages). For each sample, endothelial nitric oxide synthase and inducible nitric oxide synthase transcript values were corrected by the copies of the respective housekeeping gene (CD31 and β-actin, respectively) to obtain normalized values independent of variations in the starting material. In particular, endothelial nitric oxide synthase gene levels were reported as the number of gene copies per 106 copies of CD31. Probes and primers were the following: endothelial nitric oxide synthase (fwd) 5'-CCGGGACTTCAT-CAACCAGTC-3', (rev) 5'-TTGAAAGCCGCTTGTCTG-3', (probe) 5'-TCCAATAGAGAAGGTCGCCCCAGG-3'; inducible nitric oxide synthase (fwd) 5'-GGGAGTACGTTG- GTCCA-3', (rev) 5'-AACATTTTCCCCGCCAGTG-3', (probe) 5'-GTCAGGTCTCTGATGCCGCAGCA-3'; β-actin (fwd) 5'-GGCACCCAGACACAAATGAAG-3', (rev) 5'-GGCGATCCACACAGTCTAC-3', (probe) 5'-TCAAGATGACTGCTCCTCC-TGAGGACC-3'; and CD31 (fwd) 5'-GTCTGCTGTACCCCTTCTGCTT-3', (rev) 5'-TGTACTCTCGGGACGCCT-3', (probe) 5'-CAAGCCTTGAGGGTCGACAAGAACACTCT-3'. They were designed to span intron-exon junctions (to avoid amplification of genomic DNA) and to obtain ampiclons < 150 bp in length, thus enhancing PCR efficiency. TaqMan probes were labeled at the 5'-end with the reporter dye 6-carboxyfluorescein (emission λmax = 518 nm) and at the 3'-end with the quencher dye 6-carboxytetramethylrhodamine (emission λmax = 582 nm). Reactions were conducted with 1 μL of cDNA in a 25-μL final volume mixture containing primers and probe at optimized concentrations (400 and 200 nmol/L, respectively) and 1× TaqMan Master Mix (Perkin-Elmer). Thermal cycler parameters included 2 minutes at 50°C, 10 minutes at 95°C, and 40 cycles involving denaturation at 95°C for 15 seconds and annealing/extension at 60°C.

**Statistical Analysis.** In vitro experiments were repeated four times. All experiments were performed in triplicate and the results were expressed as mean values ± SD.

The unpaired two tails Student’s t test was used to compare gene expression between tumor samples. The paired version of the same test was performed to compare gene levels of tumor samples with those observed in skin biopsies, as well as to analyze the results of the in vitro study. P values < 0.05 were considered significant.

**RESULTS**

**In vivo Study.** Tumor response was evaluated 1 month after treatment by physical examination. Complete (disappearance of all clinically detectable tumor nodules), partial (regression of tumor burden > 50%), and minimal (25 to 50% regression of tumor burden) tumor responses were observed in 6 (50%), 4 (33%), and 2 (17%) patient, respectively. The overall (complete + partial) tumor response rate was 83% (10 of 12), which is in line with the results reported worldwide (2).

Skin toxicity was recorded in five patients (42%) and was limited to mild erythema [grade 1 according to the Wieberdink scale for the evaluation of locoregional toxicity after isolated limb perfusion (35)].

Image analysis data showed vessel destruction within tumor samples after TNF-based treatment: the mean percentage of CD31-positive cells was significantly lower 3 days after treatment rather than in those obtained before therapy (2 ± 0.4 and 11 ± 1.9%, respectively, P = 0.015). No such difference was found in normal skin samples (mean percentage of CD31-positive cells: 9 ± 2.1 and 12 ± 1.1%, respectively, P = 0.52).

Endothelial nitric oxide synthase gene expression was different in tumor as compared with normal skin biopsies (Fig. 1, top panel). Its transcriptional levels in skin melanoma metastases were significantly higher than those found in normal skin (endothelial nitric oxide synthase mean copy number = 397 ± 196 and 118 ± 44 per 105 copies of CD31, respectively, P = 0.005). In addition, endothelial nitric oxide synthase mRNA abundance was higher in complete responders rather than in partial/minimal responders (endothelial nitric oxide synthase mean copy number = 548 ± 192 and 236 ± 90 per 105 copies of CD31, respectively, P = 0.014; Fig. 1, bottom panel).

No differences in inducible nitric oxide synthase transcriptional levels were found between normal and tumor tissues or between complete responders and partial/minimal responders.

**CD31 and Nitric Oxide Synthase In vitro Gene Expression.** To assess whether CD31 could be used as an endothelial cell-specific housekeeping gene, CD31 mRNA expression by endothelial cells (HUVECs) was measured under different conditions (supernatant from melanoma cells/fibroblasts/PBMCs). As shown in Fig. 2, top panel, CD31 mRNA levels were uniformly highly expressed by HUVECs, no matter what culture conditions were applied. No significant mRNA levels of CD31 were expressed by other cell types such as fibroblasts, PBMCs, and melanoma cell lines (data not shown).

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Endothelial nitric oxide synthase (eNOS) and inducible (iNOS) nitric oxide synthase (NOS) are key factors in the expression and function of endothelial cells. The expression of eNOS and iNOS is regulated by various factors, including cytokines and growth factors. The presence of eNOS and iNOS in tumor and normal skin biopsies obtained from 12 patients before TNF-based treatment was measured by quantitative real-time PCR and normalized by either a nonspecific housekeeping gene (β-actin) or an endothelial cell-specific reference gene (CD31). eNOS expression was found to be higher in tumor rather than in skin samples using both housekeeping genes.

Transcriptional levels of eNOS and iNOS in tumor (skin melanoma metastases) biopsies obtained from 12 patients before treatment (TNF-based isolated limb perfusion) were measured under different conditions (basal medium or supernatant from melanoma cells/fibroblasts/PBMCs) using quantitative real-time PCR. CD31 mRNA levels were uniformly highly expressed by endothelial cells (HUVECs) measured under different culture conditions. eNOS transcriptional abundance was significantly higher in the presence of the supernatants collected from three melanoma cell lines as compared with basal conditions (i.e., endothelial cell basal medium; P = 0.001, 0.003, 0.017; Fig. 2, bottom panel). No such effect was observed when HUVECs were grown with the supernatant obtained from fibroblasts or PBMC cell cultures. No variations in inducible nitric oxide synthase mRNA expression by HUVECs were observed under any of the above-mentioned culture conditions.

Endothelial Cell Viability, Apoptosis, and Proliferation Assays. As demonstrated by the cell viability and apoptosis assays, inhibition of nitric oxide synthase activity with 1-NAME significantly decreased TNF-mediated cytotoxicity against endothelial cells when the nitric oxide synthase inhibitor was added 2 hours before TNF addition (Fig. 4; at time 6 hours, t test, P = 0.01 and 0.02, respectively). By contrast, nitric oxide synthase inhibition at the same time of TNF administration had no significant effect on the cytotoxic effect.

The proliferation of endothelial cells was significantly diminished by adding the nitric oxide synthase inhibitor to the culture medium (Fig. 5; at time 6 days, t test, P = 0.004).

Nitric Oxide Synthase Activity Assay. The correlation between endothelial nitric oxide synthase gene expression and enzymatic activity under different experimental conditions is illustrated in Table 1.
DISCUSSION

The selectivity of TNF cytotoxic effect for tumor as compared with normal tissues is well known to clinicians (2–4). The identification of the mechanisms underlying this tumor-specific activity of TNF could be of paramount importance to open new avenues of research in the field of molecularly targeted anticancer therapy.

In the in vivo study, we found that endothelial nitric oxide synthase mRNA levels were significantly higher in tumor rather than in skin samples (Fig. 1). As endothelial nitric oxide synthase is selectively expressed by endothelial cells (Fig. 3), it can be postulated that only endothelial cells could be responsible for this differential gene display. We ruled out that this difference was due to a different number of endothelial cells present in the tumor/skin specimens by normalizing endothelial nitric oxide synthase transcriptional levels by the mRNA levels of an endothelial-specific housekeeping gene (i.e., CD31; refs. 33, 34), according to a method already validated (31, 36). Using a nonspecific housekeeping gene such as β-actin, differences in endothelial nitric oxide synthase expression between tumor and normal tissue were still significant. This was supported by the fact that no difference in endothelial cell density was found between diseased and normal tissue samples, as determined by the ratio CD31/β-actin gene expression (Fig. 1). However, because differences in vessel density cannot be ruled out a priori, we chose to normalize endothelial nitric oxide synthase mRNA levels by CD31 to minimize the risk of false findings due to dissimilar degree of tissue vascularization.

Tumor endothelial nitric oxide synthase levels resulted higher in complete rather than in partial/minimal responders (Fig. 1). Considering the minimal skin toxicity and the different tumor responses (complete versus partial/minimal) recorded after the TNF-based treatment, these in vivo findings led us to hypothesize that endothelial nitric oxide synthase overexpression might underlie both the tumor specific activity of TNF and the variation of tumor response observed in the clinical setting. In vitro experiments did support this hypothesis. We first verified that endothelial nitric oxide synthase expression by endothelial cells was quite constant under different experimental conditions, except when melanoma supernatant was used (Fig. 2). In this case, endothelial nitric oxide synthase transcription significantly increased, showing that—just like the so-called inducible nitric oxide synthase—the endothelial isoenzyme is also inducible. A good correlation between endothelial nitric oxide synthase gene expression and nitric oxide synthase enzymatic activity (Table 1) strengthened these results. By demonstrating that melanoma cells can up-regulate endothelial nitric oxide synthase expression, we provided additional evidence that melanoma supernatant could be responsible for the increased endothelial nitric oxide synthase activity observed in tumor endothelial cells.

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oxidase synthase expression by endothelial cells, in vitro data supported the above discussed in vivo findings (i.e., different endothelial nitric oxide synthase expression by tumor as compared with skin samples).

Nitric oxide is believed to play a significant role in angiogenesis (as supported by the inhibition of endothelial cell proliferation by nitric oxide synthase inhibition; see Fig. 5) and immune suppression during tumor progression (37–44). From a teleological viewpoint, endothelial nitric oxide synthase overexpression by endothelial cells might be a mechanism of malignant cells to promote the angiogenic process and inhibit the immunologic surveillance. Some investigators have postulated that malignant cells themselves produce nitric oxide by increasing their own expression of inducible nitric oxide synthase (45–48). Others have reported that stromal cells (e.g., tumor-infiltrating macrophages) might be responsible for the production of nitric oxide and consequently favor tumor progression-related processes such as neoangiogenesis (49). In the present study, we found that melanoma cell lines did not overexpress inducible nitric oxide synthase as compared with normal cell types (Fig. 3), whereas their supernatant did cause endothelial cells to transcribe endothelial nitric oxide synthase by a not yet identified soluble factor. A proteomics-based study (50) aimed at identifying this/these molecule(s) is currently under way in our laboratory.

Inhibition of nitric oxide synthase activity was accompanied in vitro by a reduction of TNF-driven cytotoxicity of endothelial cells when nitric oxide synthase suppression was achieved before TNF addition to the culture medium. This effect most likely depended on the inhibition of the endothelial isoenzyme because no significant levels of inducible nitric oxide synthase were found to be expressed by HUVECs (Fig. 3). These data indicate that high nitric oxide synthase expression (as obtained conditioning endothelial cells with melanoma cell line supernatant) favors the cytotoxicity of TNF toward human endothelial cells, thus providing a potential link between clinical tumor response to TNF-based treatment and endothelial nitric oxide synthase expression levels in tumor samples (Fig. 1). Endothelial cells are relatively resistant to the cytotoxic effect of TNF, as demonstrated by the need of coadministering cycloheximide to kill endothelial cells in vitro (51, 52); however, high pretreatment levels of nitric oxide might lower the threshold of endothelial cell sensitivity to the cytokine, as supported by the inhibition of TNF cytotoxicity obtained with nitric oxide synthase inhibitors (Fig. 4). In particular, it might be hypothesized that the production of cytotoxic-mixed free radicals (e.g., –ONO) is favored by high levels of nitric oxide in the presence of reactive oxygen species, whose production is notoriously increased by TNF and involved in cytokine-induced cytotoxicity (53). On the other hand, caspase-dependent apoptotic death can be inhibited by nitric oxide (25–27), which might theoretically reduce TNF-mediated cytotoxicity toward endothelial cells. However, some investigators have reported that caspase inhibition can favor reactive oxygen species-mediated cytotoxicity (54, 55), which in turn might favor TNF cytotoxic activity. Finally, in our model, suppression of nitric oxide synthase activity (and thus nitric oxide production) soon after the interaction between the cytokine and its receptor did not affect TNF cytotoxicity, which confirms the complex and still unclear relationship between nitric oxide and TNF effects and calls for additional studies aimed at elucidating the molecular basis of their interaction.

Another hypothesis might be formulated to help interpret our findings. Nitric oxide has been reported to mediate vascular endothelial growth factor-proliferative effects on endothelial cells (37), and melanoma cells are known to produce vascular endothelial growth factor (56, 57). Furthermore, we found that in the presence of melanoma supernatant, endothelial cells ex-

<table>
<thead>
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<th>EC treatments</th>
<th>l-citrulline (nmol/L/mg/min)</th>
<th>eNOS (gene copy number/10^5 actin)</th>
</tr>
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<tbody>
<tr>
<td>d-NAME</td>
<td>62 ± 13.31</td>
<td>310 ± 40.05</td>
</tr>
<tr>
<td>l-NAME</td>
<td>11 ± 4.02</td>
<td>350 ± 62.01</td>
</tr>
<tr>
<td>Melanoma supernatant</td>
<td>194 ± 54.03</td>
<td>634 ± 58.37</td>
</tr>
<tr>
<td>Melanoma supernatant + l-NAME</td>
<td>76 ± 10.76</td>
<td>711 ± 69.03</td>
</tr>
</tbody>
</table>

NOTE. Values are shown as mean ± SD. Melanoma supernatant significantly increased eNOS gene expression and enzymatic activity (t test, P = 0.006).

Abbreviations: NOS, nitric oxide synthase; eNOS, endothelial NOS; iNOS, inducible NOS; EC, endothelial cell.
press more endothelial nitric oxide synthase, which correlates with a higher rate of cycling endothelial cells (Fig. 6). Accordingly, the overexpression of this gene in tumor tissue samples (as compared with normal skin samples) might represent just a marker of endothelial cell cycle status. If high-endothelial nitric oxide synthase/cycling (rather than low endothelial nitric oxide synthase/resting) endothelial cells are more sensitive to TNF activity, the blockage of endothelial nitric oxide synthase-proliferative effect (by L-NAME) before TNF administration likely enables us to appreciate the inhibitory effect of L-NAME on TNF cytotoxicity. By contrast, in the case of synchronous administration of L-NAME and TNF, the cytokine cytotoxic effects might occur before cell cycle is shut down, and endothelial cells become resistant to TNF.

Overall, this study suggests that the induction of endothelial nitric oxide synthase expression by melanoma, which might provide the tumor with a survival advantage by promoting angiogenesis, increases the sensitivity of endothelial cells to TNF, providing a new hypothesis to explain both the tumor selective cytotoxic activity of this cytokine and the variable tumor responses observed in the clinical setting. According to these considerations, the measurement of endothelial nitric oxide synthase gene expression in pretreatment tumor samples might be used as a prognostic factor for tumor response and guide physicians during the selection of patients candidate for TNF-based anticancer therapy. Moreover, the antineoplastic activity of this cytokine might be augmented by adding nitric oxide synthase inhibitors to the drug regimen. In fact, because nitric oxide synthase inhibitors do not inhibit TNF-driven cytotoxicity when they are added together with this cytokine, these agents could be coadministered to add an antiproliferative effect to the cytotoxic activity of TNF. This way, an improved tumor response rate is expected, as recently observed in an animal model of soft tissue sarcoma (58).

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