

Inducible Nitric Oxide Synthase and Nitrotyrosine in Human Metastatic Melanoma Tumors Correlate with Poor Survival¹

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ABSTRACT

Despite recognition of the malignant potential of human melanomas, the mechanisms responsible for the pathobiological characteristics contributing to tumor growth, vascular invasiveness, and distant organ metastasis remain undefined. Recent studies have shown that various human tumors express an inducible form of nitric oxide synthase (iNOS) and nitrotyrosine (NT), which suggests a mechanistic role of tumor-associated nitric oxide (NO) in tumorigenesis. We investigated iNOS and NT expression by immunohistochemistry in 20 human metastatic melanoma tissue specimens specifically with respect to iNOS-expressing cell types in the tumor area, pathological and clinical response to systemic therapy, potential role as a prognostic indicator, and NT formation. Our results showed that melanoma cells from 12 of 20 tumors express iNOS, yet the expression of this molecule in the tumor did not correlate with pathological or clinical response to therapy. More importantly, iNOS and NT expression by the melanoma cells strongly correlated with poor survival in patients with stage 3 disease ($P < 0.001$ and $P = 0.020$, respectively), suggesting a pathway whereby iNOS might contribute to enhanced tumor progression. In conclusion, our findings strongly suggest that iNOS expression has potential to be considered as a prognostic factor and NO as a critical mediator of an aggressive tumor phenotype in human metastatic melanomas.

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INTRODUCTION

Malignant melanoma is currently the leading cause of death from cutaneous malignancies, and at this time, it is estimated that 1 in 75 persons in the United States will develop malignant melanoma during their lifetime (1). Therefore, improvement of melanoma prevention and treatment is now becoming a priority. Although the clinical biology and pathogenesis of melanoma are well studied and the relationship between cytokine production and progression of human melanoma has been examined, it remains to be determined which of the many biological characteristics of the tumor or patient are most important for tumor development and progression.

Nitric oxide (NO) is an important bioactive agent and signaling molecule that mediates a variety of actions such as vasodilatation, neurotransmission, and host defense and has been proposed to contribute to the pathogenesis of cancer (2–5). NO is synthesized from arginine by NOSs.³ The genes for three isoforms of NOS have been cloned; two of these isoforms are calcium dependent and constitutively expressed: endothelial NOS (type III) and neuronal NOS (type I). The iNOS (type II) is calcium independent and cytokine inducible. It is also now known that NOS type I and NOS type III can be induced and that low levels of NOS type II are expressed in some tissues constitutively (6–8). Only the inducible form, iNOS, once activated can produce higher levels of NO (in the micromolar range) for a long duration (days; Refs. 9 and 10). Recent studies have shown that NO may be involved in inhibiting cell proliferation, differentiation (11), and apoptosis (12). Damaging effects of NO may be attributable to reaction with superoxide anions to yield peroxynitrite, which is a potent nitrating and nitrosylating agent. Peroxynitrite can oxidize nuclear DNA and membrane phospholipids and also nitrate either free or protein-associated tyrosines to form NTs (13). Thus, the occurrence of NT in tissues has been measured as a marker of peroxynitrite formation (14). Some human tumors are reported to express iNOS, and the product of its enzymatic action, NO, is proposed to affect the clinical features of these tumors (3, 4, 8, 15–18). Although iNOS expression by tumors in animal models has been studied for years, human tumor studies are recent.

The treatment of advanced melanoma patients with a 5-drug regimen consisting of IL-2, IFN- α 2a, dacarbazine (DTIC), cisplatin, and vinblastine, referred to as “biochemotherapy,” has shown encouraging results (19, 20). Although the mechanism of biochemotherapy is not well understood, some biological responses that may be related to immune effector cell activity have been investigated during and after therapy. In the original report of a clinical trial using biochemotherapy in the neoadjuvant setting for stage III patients, Anderson *et al.* (21)

³ The abbreviations used are: NOS, nitric oxide synthase; iNOS, inducible NOS; NT, nitrotyrosine; IL, interleukin.

found that serum nitrite levels were higher in patients who showed major responses to therapy, relative to those who did not, although the statistical significance was marginal. Levels of serum neopterin, a product of activated macrophages, correlated weakly with nitrite levels; however, there was no correlation of neopterin levels with response to treatment. It was therefore concluded that the elevated serum nitrite in responding patients was not of macrophage origin and perhaps derived from tumor cells or endothelial cells.

In the present study, we have attempted to further explore the source of elevated nitrite levels in patients responding to biochemotherapy, using the same patient population studied by Anderson *et al.* (21). The working hypothesis was that melanoma tumor cells produce iNOS and provide the source of nitrite seen in the sera of responding patients. A corollary to this would be the expectation that patients with tumor iNOS would have a higher rate of response to therapy and potentially a longer survival. We designed our study to determine whether: (a) melanoma cells express iNOS and NT; (b) tumor expression of these molecules correlates with serum nitrite levels; and (c) tumor expression of these molecules correlates with patient outcomes. We report our findings that some melanoma tumors do express iNOS and NT, but there is no correlation between tumor expression and serum nitrite levels or response to therapy. We also report the unexpected finding that tumor expression of iNOS and NT is associated with poor survival of melanoma patients.

MATERIALS AND METHODS

Tumor Samples. The melanoma tumor samples used in this study consist of lymph node or in-transit metastases surgically removed from patients with stage III disease who were enrolled in an institutionally approved neoadjuvant biochemotherapy trial. The details of this regimen and of the clinical trial are described by Legha *et al.* (19) and Buzaid *et al.* (20). Briefly, the biochemotherapy regimen consisted of cisplatin, 20 mg/m² i.v. on days 1–4; vinblastine, 1.5 mg/m² i.v. on days 1–4; dacarbazine, 800 mg/m² i.v. on day 1; IL-2, 9 M IU/m²/day i.v. by continuous infusion on days 1–4 (total, 96 h); and IFN- α 2a, 5 M units/m² s.c. on days 1–5. One cycle of therapy was 28 days. All patients received a minimum of two cycles of therapy prior to surgery. Surgery was usually performed within 2–4 weeks after the last biochemotherapy cycle. For the purpose of the present report, patients were categorized by the presence or absence of a major response to neoadjuvant therapy. Major response is defined as a 50% or greater reduction in clinical or radiographic bidimensional tumor measurements (clinical complete or partial response), or the pathological finding of <50% residual viable tumor in the surgical specimen, regardless of clinical measurements.

Specimen Collection and Storage. Blood was collected at ~10 a.m. on 4 separate days: before biochemotherapy (baseline), and on days 5, 6, and 7 of treatment. The day 5 collection took place at the termination of the IL-2 infusion, corresponding to 24 h after the last cisplatin dose. Serum was separated from the blood within 2 h of collection and frozen at –80°C for later analysis. Formalin-fixed and paraffin-embedded tissue sections were obtained from the Melanoma and Skin Cancer Core Lab-

oratory of the University of Texas M. D. Anderson Cancer Center for use in immunohistochemical staining of tumor tissues, as approved in the research arm of the protocol.

Reagents. Anti-iNOS mouse monoclonal antibody (Transduction Laboratories, Lexington, KY) was used for iNOS immunohistochemistry, and anti-NT mouse monoclonal antibody (Upstate Biotechnology, Lake Placid, NY) was used for NT staining; both antibodies were confirmed as being cross-reactive between species. Preimmune normal mouse IgG (Vector Laboratories, Burlingame, CA) was used as a negative control. Anti-vimentin antibody (BioGenex Laboratories, San Ramon, CA) was used as a positive control for all melanoma staining. Other reagents including sulfanilamide, *N*-1-naphthylethylenediamine, ammonium formate, zinc sulfate, sodium nitrate, and sodium nitrite was purchased from Sigma Chemical Co., Inc. (St. Louis, MO).

Nitrite Determination in Serum. NO was measured as the total of its oxidation products (nitrite and nitrate) after enzymatic reduction by *Escherichia coli* nitrate reductase prepared according to published methods (22). The serum assay for NO oxidation products (nitrite plus nitrate) was performed on freshly thawed serum samples as described previously (21, 22). Briefly, frozen serum was thawed at room temperature, and 100 μ l were aliquoted into a microcentrifuge tube; 5 μ l of 30% zinc sulfate were added with vortexing to allow protein precipitation, the tubes were centrifuged for 12 min at 14,000 rpm. Then 56 μ l of supernatant were removed and placed in a new microcentrifuge tube to which 62 μ l of nitrate reductase mix [1 part enzyme suspension to 10 parts 2.4 M HEPES (pH 7.2) and 10 parts 1 M ammonium formate (pH 7.2)] were added; the tubes were vortexed and incubated for 30 min at 37°C, followed by centrifugation for 5 min. The enzyme suspension used was *E. coli* nitrate reductase, used as a crude preparation of *E. coli* according to published methods (23). Eighty μ l of supernatant were pipetted into a 96-well plate with 80 μ l of Griess reagent (1% sulfanilamide, 0.1% *N*-1-naphthyl-ethylenediamine, and 2.5% phosphoric acid in distilled water) and incubated at room temperature for 10 min, and the absorbance was read at 540 nm against a reference of 650 nm in a microplate spectrophotometer. A Dynatech MR700 spectrophotometer plate reader (Chantilly, VA) was used to read the sample plates for NO determination. Standards of known concentrations of sodium nitrate and sodium nitrite in serial dilutions were used as positive controls to create a standard curve. Standards and samples were subjected to identical treatment. The final nitrite concentration, which was the sum of the serum nitrite plus the reduced nitrate in the Griess reaction, was reported in μ M.

Immunohistochemical Staining. Immunohistochemical staining was performed with 10% formalin-fixed, paraffin-embedded melanoma tissue, cut 4–6 μ m thick. Sections were placed on silanized slides (Histology Control Systems, Glen Head, NY), deparaffinized in xylene, and rehydrated in descending grades (from 100 to 85%) of ethanol. To enhance the immunostaining and restore the maximal antigenicity of cytokines, sections then were placed in antigen unmasking solution (Vector Laboratories, Burlingame, CA) and microwaved intermittently for up to 10 min to maintain a boiling temperature. After the slides were cooled at room temperature for 30 min, they were washed in distilled water and PBS. After this initial

Table 1 Patient response, survival time, and tumor staining characteristics in biochemotherapy protocol

Patient no.	Patient characteristics				Immunohistochemistry		
	Response	Survival time (mo) ^a		Status	iNOS		
		First diagnosis ^b	First treatment day ^c		Pretreatment	Posttreatment	NT
1	R ^d	17	9	DOD	++	+++	-
2	NR	20	14	DOD	N/A	++	++
3	R	33	32	DOD	N/A	++	++
4	NR	21	17	DOD	-	+	-
5	NR	59	18	DOD	++	+++	++
6	R	?	19	DOD	N/A	++	++
7	NR	40	22	DOD	N/A	++	+
8	NR	29	28	DOD	N/A	+	-
9	NR	26	25	DOD	++	+	++
10	NR	30	29	DOD	++	+++	+++
11	NR	67	44	DOD	N/A	++	++
12	NR	?	48	DOD	N/A	-	-
15	R	54+	53+	ANED	N/A	-	-
16	NR	74+	56+	AWD	-	-	-
13	NR	72+	61+	AWD	-	-	-
14	NR	74+	61+	ANED	N/A	-	-
20	R	69+	60+	ANED	-	-	-
17	R	99+	65+	ANED	N/A	-	-
19	NR	?	65+	ANED	-	-	-
18	R	92+	66+	ANED	+++	++	+++

^{a-c} Survival measured from: ^b the date of primary tumor diagnosis, and ^c the initiation of treatment.

^d R, major response; NR, absence of major response; DOD, dead of disease; ANED, alive with no evidence of disease; AWD, alive with disease; N/A, not available; ?, not known.

preparation, the slides were removed from PBS and covered with 3% H₂O₂ (Sigma Chemical Co.) in methanol to block endogenous peroxidase activity. All incubations were carried out at room temperature in a humidified covered slide chamber. The slides were washed in PBS before incubation in Tris-buffered saline (TBS) containing 0.05% Triton X-100 (Sigma Chemical Co.) for 15 min to permeabilize the cells. An avidin-biotin-peroxidase complex (ABC) kit (Vectastain; Vector Laboratories) was then used to detect the primary antibody staining. These kits are specific for the species of primary antibody used and contain a blocking serum, a secondary biotinylated antibody, and the ABC reagent. After the slides were incubated for 30 min with the blocking serum, the primary antibody at various dilutions (1:100 to 1:200 for iNOS and 1:50 for NT) was added, and the slides were incubated for 60 min at room temperature. The slides were then washed, incubated for 30 min with secondary biotinylated antibody, washed again, and then incubated for 30 min with the ABC reagent. After the slides were washed in PBS, the immunostaining was developed with the use of 3-amino-9-ethylcarbazole as a chromogen for 15 min. Slides were counterstained with hematoxylin (Vector Laboratories) and mounted with Aqua-Mount (Lerner Laboratories, Pittsburgh, PA). Control tissues used were the samples of formalin-fixed and paraffin-embedded normal human nevi obtained as incidental material from our Pathology Department. The following scores were assigned to each specimen according to the percentage of positively stained cells in the tumor: 4+, >75% cells were positive; 3+, 51–75% cells positive; 2+, 26–50% cells positive; 1+, 5–25% cells positive; +/-, <5% cells positive; and -, no positive staining.

Statistical Analysis. Kaplan-Meier estimates of survival were calculated from the original date of melanoma diagnosis

and also from the first day of treatment for stage III disease. Comparisons of survival between groups of patients who tested positive and those who tested negative for iNOS and NT were assessed with the log-rank test. An original date of melanoma diagnosis could not be ascertained for two patients who were negative for iNOS and NT and one patient who was positive for iNOS and NT. These three patients were excluded from the analysis of survival performed from date of diagnosis. McNemar's test of symmetry was used to evaluate the consistency of testing positive for iNOS and for NT (24). The null hypothesis for the McNemar's test is that there is an agreement between two parameters. Fisher's exact test was used to compare the proportion of treatment responders between those patients who tested iNOS positive and those who tested negative.

RESULTS

Patient Population. Postbiochemotherapy tumor material was available for 20 patients; for 10 of these patients, pretreatment tumor samples were available as well. This group represents a subset of the population reported by Anderson *et al.* (21) and includes all patients who had posttreatment tumor tissue available for immunohistochemistry. The study population consisted of 14 males and 6 females, with a median age of 45.5 years (range, 18–65 years). All patients had stage III melanoma, as specified by the clinical protocol. Other details are as listed in Table 1.

NO Production by Tumor Cell. Evidence for possible NO production by tumor cells was addressed by performing two immunohistochemical tests: one for the enzyme known to generate NO in such tissues (iNOS); and the other for NT, which is an intracellular reaction product from NO (Table 1). Posttreat-

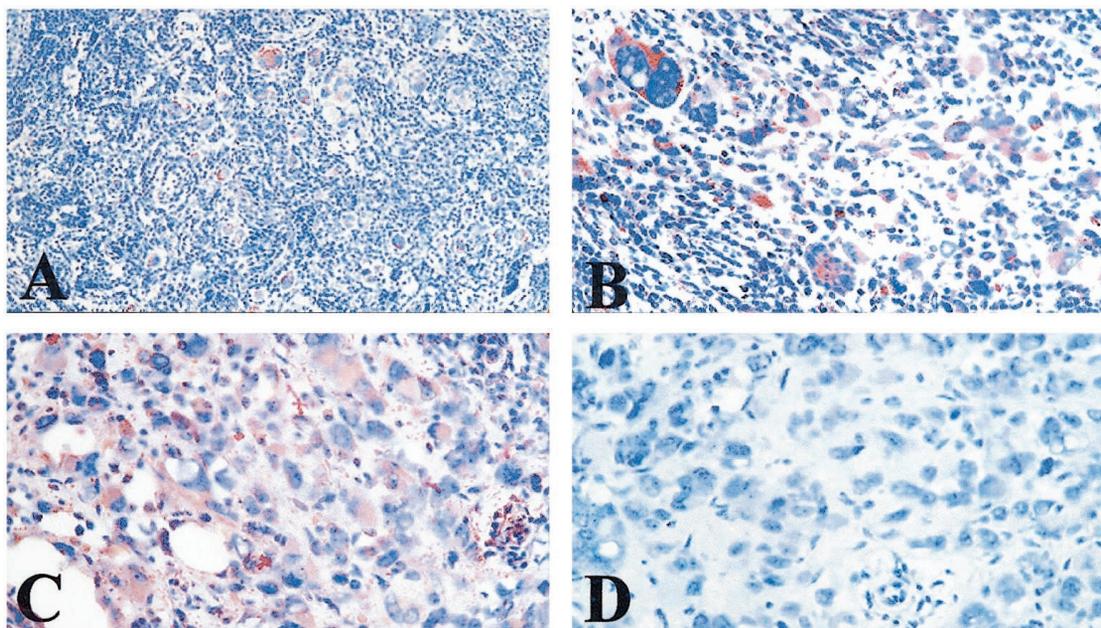


Fig. 1 Expression of iNOS in formalin-fixed, paraffin-embedded sections of human metastatic melanoma lesions. Notice the strong labeling in the melanoma cells (A and B). C, expression of vimentin showing preserved immunoreactivity. D, negative control. For anti-iNOS staining, 3-amino-9-ethylcarbazole and hematoxylin were used. A, $\times 100$; B–D, $\times 400$.

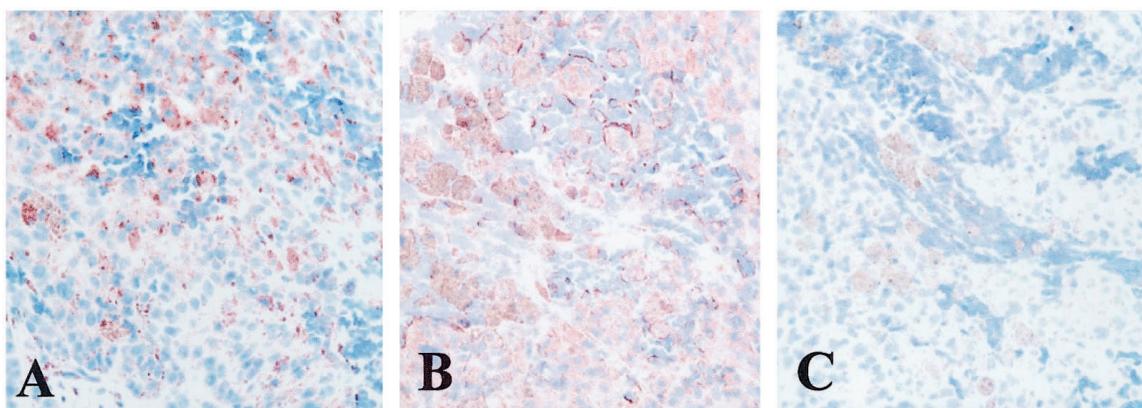


Fig. 2 Expression of NT in formalin-fixed, paraffin-embedded sections of human metastatic melanoma lesions. Notice the strong labeling in the melanoma cells (A). B, expression of vimentin showing preserved immunoreactivity. C, negative control. For anti-NT staining, 3-amino-9-ethylcarbazole and hematoxylin were used. $\times 400$.

ment tumors from 12 of the 20 subjects were positive for iNOS (Fig. 1), and nine of these tumors were positive for NT (Fig. 2). An interesting observation was related to the correlation between iNOS and NT expression within the same tumor ($P = 0.248$ by McNemar's test), suggesting that there may be agreement between the two markers (24). All iNOS-negative tumors were also negative for NT by immunohistochemistry. Only 3 of 12 iNOS-positive tumors did not express NT.

For 10 of the 20 patients, we were able to study tumor specimens obtained before treatment started. Nine of 10 tumors expressed the same result for iNOS and/or NT as after therapy (data not shown). These data suggest that biochemotherapy treatment did not change iNOS and/or NT expression

in the tumor. However, the small numbers of pretherapy patient samples precludes formal statistical analysis and conclusions.

Serum Nitrite Levels. As a surrogate measurement of NO production, serum nitrite levels for the 20 patients during the first biochemotherapy treatment were measured (Fig. 3). For all patients, serum nitrite rose higher than baseline on days 5 and 6 and declined on day 7. The levels of nitrite in the serum did not show a correlation with tumor expression of iNOS. Therefore, tumor iNOS does not appear to be the source of serum nitrite in these patients.

Correlation of Tumor iNOS with Outcome. A final goal of this study was to test the hypothesis that those patients

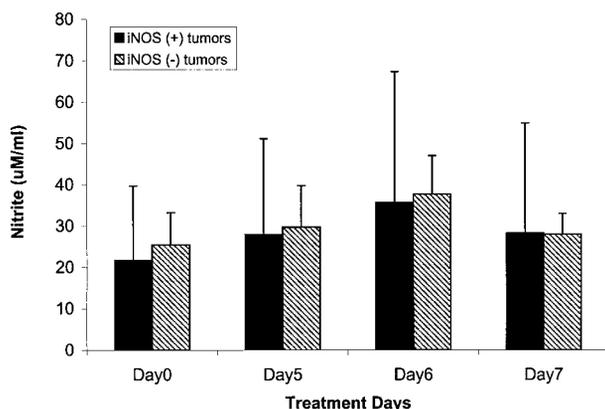


Fig. 3 Lack of correlation of serum nitrite levels with tumor iNOS expression. Serum nitrite levels measured by Griess reaction, for patients with advanced locoregional melanoma during treatment with a biochemotherapy regimen are shown. Serum nitrite was measured at four time points during the first course of therapy, whereas iNOS status was determined using tumor tissue obtained at surgery several months later. ■, presence of iNOS in the patient's tumor; ▨, absence of iNOS in the tumor. Columns, means of patients' serum nitrite levels; bars, SD.

with melanoma metastases producing iNOS, and hence NO, would have a superior response to therapy and an improved survival. In the study population, major responses were seen in 4 of 12 patients (33.3%) with iNOS-positive tumors and in 3 of 8 patients (37.5%) with iNOS-negative tumors ($P = 1.00$ by Fisher's exact test; Ref. 24). Thus, the presence of tumor iNOS does not appear to influence response to biochemotherapy in this setting.

To address the impact of tumor iNOS and NT on survival, information on survival times from both the original diagnosis of melanoma and from the initiation of therapy was gathered for all patients. Two patients who presented with stage III disease had an unknown site of primary tumor; for another patient, records regarding the primary tumor could not be obtained. These three patients are excluded from analysis of survival from the date of original diagnosis but are included in the analysis from the initiation of therapy. In contrast to the lack of correlation of iNOS expression with response to therapy, survival appeared to be significantly impacted (Fig. 4). The median survival from diagnosis of the primary tumor (Fig. 4A) and from initiation of therapy for stage III disease (Fig. 4B) was 30.3 and 22.1 months, respectively, for the patients who had iNOS-expressing tumors; the median survival times were not reached in either case for iNOS-negative patients ($P = 0.001$ and $P < 0.001$, respectively). A similar pattern was found when analyzing the association of tumor NT expression with survival (Fig. 5). A significantly shorter survival was found from initiation of treatment for patients with NT-positive tumors (median survival, 24.4 months *versus* not reached for patients with NT negative tumors, $P = 0.020$; Fig. 5B). There was a trend toward inferior survival from diagnosis for patients with NT-positive tumors, but the difference did not reach significance (Fig. 5A).

DISCUSSION

The initial goal of our study was to determine whether NO products in the sera of melanoma patients receiving biochemo-

therapy might be a reflection of the presence of iNOS in tumors from the same patients. In addition, we anticipated that patients with iNOS-producing tumors would have improved outcomes in the form of higher rates of response to therapy and/or improved survival. Although we found that 12 of 20 posttreatment tumors expressed iNOS, we were unable to demonstrate a correlation of tumor iNOS expression with an elevated serum nitrite level or with response to therapy. The nitrosylation of tyrosines on intracellular proteins is a stable and direct reflection of tumor NO production and is superior to serum nitrite levels, which could be increased because of endothelial cell production of NO in response to systemic inflammatory factors such as IL-1, TNF- α , or IL-6. Thus, tumor does not appear to be the source of elevated serum nitrite in responding patients reported by Anderson *et al.* (21). Finally, contrary to our expectations, we found that the presence of iNOS and NT in posttreatment tumors predicted a poor survival.

There are several limitations to our study that must be considered when interpreting these data. The first limitation is the fact that our analysis is based on posttreatment tumor samples. These samples, which were obtained as part of the clinical trial, provided the advantage of surgically excised material of adequate quantity and preserved histology, as opposed to the scant fine-needle aspirates used in the pretreatment, diagnostic setting. However, if we are to suggest that melanoma tumors producing iNOS display an aggressive phenotype, it would be more appropriate to make this observation on pretreatment material, as one could postulate that the cytokines included in biochemotherapy might indirectly induce tumor iNOS expression. We were able to analyze pretreatment tumors in half of the patients and found good correlation between pre- and posttreatment samples; however, future studies should focus on material from untreated patients. The second limitation also derives from our use of posttreatment samples in that it forced us to exclude from analysis four patients who achieved complete resolution of their disease, thus leaving no tumor to study. This may be less important, as we were unable to demonstrate a correlation of tumor iNOS with response to treatment. Finally, the number of patients in this study was small. Although the survival data are provoking, they must be confirmed in an appropriately designed trial with a larger number of patients before strong conclusions can be drawn.

Bearing in mind the above limitations, two observations are worthy of discussion, those being the findings that some melanoma metastases produce iNOS and NT, and that stage III patients bearing these iNOS/NT-positive melanomas have a shortened survival compared with those with iNOS/NT-negative tumors. This suggests that the presence of tumor iNOS, and thus NO, provides an advantage for enhanced tumor growth and survival. Potential mechanisms include vasodilatation with improved tumor blood flow and damaging oxidative effects directed toward lymphocytes, inducing membrane and DNA damage. In agreement with our findings are reports of increased iNOS expression by primary human tumors compared with normal tissue, including colon carcinomas (18), colon adenomas (8), pancreatic adenocarcinomas (15), and gastric cancers (17). To our knowledge, only one other study has examined expression in human melanoma (25). Those investigators studied lymph nodes and subcuta-

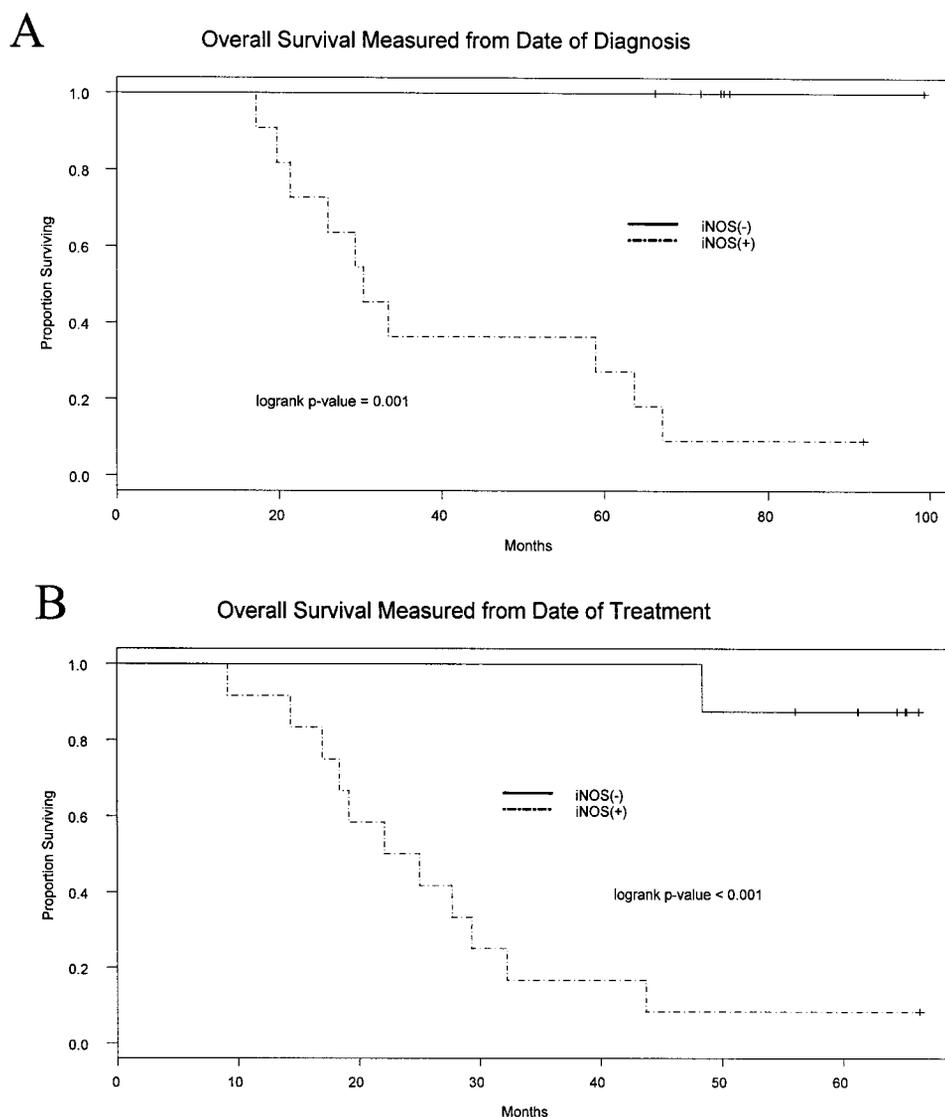


Fig. 4 Survival of patients relative to iNOS status. Overall survival measured from the date of diagnosis of primary tumors (A) and overall survival measured from the initiation of treatment (B) are shown. *Solid line*, absence of iNOS expression; *dotted lines*, iNOS positivity in the tumor.

neous metastases and found that patients with a lower percentage of iNOS-expressing tumor cells had a greater risk of developing distant metastases. Treatment details and specific survival times were not provided, and a significant impact on survival could not be demonstrated. The apparent discrepancy between these data and ours may be explained in part by different methodologies used to quantitate iNOS expression. However, the two studies cannot be directly compared with respect to influence on survival, based on the information provided.

Similar examples of conflicting reports of iNOS expression exist for other tumor types as well (26). One explanation for the inconsistent findings may be a differential effect of NO on tumor behavior, depending on the quantity of this molecule produced. Recent studies have shown that transfecting metastatic murine melanoma cells with the full-length *iNOS* gene leads to the production of micromolar quantities of NO and suppression of metastatic potential (27). Melanoma cells simi-

larly transfected with a truncated *iNOS* gene did not produce NO and retained their metastatic potential, suggesting that high levels of NO caused self-destruction of these tumor cells (27, 28). Conversely, transient or low levels of NO are suggested to benefit tumor growth by increasing blood supply via its well-known, vascular-relaxing property (29). Another factor that may contribute to the conflicting observations is the cytokine inducibility of iNOS expression. One recent study showed that treatment of murine melanoma cells with IFN- γ plus lipopolysaccharide results in the up-regulation of iNOS transcription in nonmetastatic cells but not in a metastatic cell line, suggesting a variable transcriptional basis for the differences in iNOS (30). Taken together, these observations indicate that the level of iNOS expression and NO production by the tumor may be associated with tumor viability, with the very high NO levels leading to tumor destruction but moderate levels promoting growth. Such alterations in the levels of iNOS and NO could be influenced by numerous patient factors, including medical and

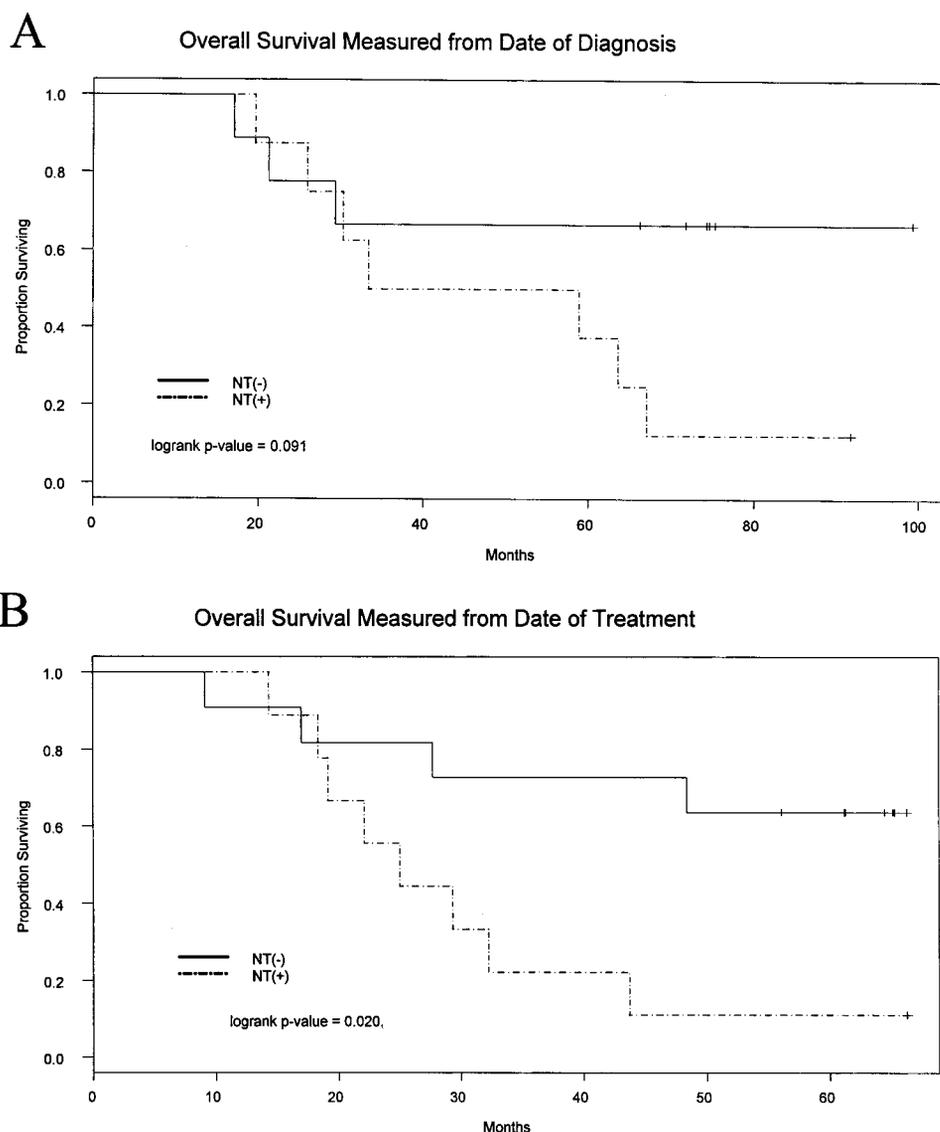


Fig. 5 Survival of patients relative to NT status. Overall survival measured from the date of diagnosis of primary tumors (A) and overall survival measured from the initiation of treatment (B) are shown. Solid line, absence of NT expression; dotted line, NT positivity in the tumor.

surgical treatment received, and other cancer-related physiological stresses such as pain, dehydration, cachexia, and infection.

In conclusion, our data suggest that iNOS and NT expression by melanoma metastases may be predictive of a poor outcome, information that may be of clinical value when making therapeutic decisions for individual patients. However, we view our findings as preliminary. Our current efforts focus on confirming these data with a larger number of patients, studying untreated samples to eliminate the impact of exogenous cytokines, and analyzing early-stage tumors for prognostic information.

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