

Transfection with the Inducible Nitric Oxide Synthase Gene Suppresses Tumorigenicity and Abrogates Metastasis by K-1735 Murine Melanoma Cells

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Summary

Previous studies from our laboratory demonstrated an inverse relationship between the expression level of inducible nitric oxide synthase (iNOS) and the metastatic potential of murine K-1735 melanoma cells. The purpose of this study was to provide direct evidence that the expression of iNOS suppresses metastatic potential of melanoma cells. Highly metastatic K-1735 clone 4 cells (C4.P), which express low levels of iNOS, were transfected with a functional iNOS (C4.L8), inactive-mutated iNOS (C4.S2), or neomycin-resistance (C4.Neo) genes in medium containing 3 mM N^ε-methyl-L-arginine (NMA). Positive transfectants were identified by Southern and Northern blot analyses and homogeneous staining with a specific anti-iNOS monoclonal antibody. Semiconfluent cultures of C4.P (parental), C4.Neo.3 (control transfection), C4.S2.3 (inactive iNOS), and C4.L8.5 (functional iNOS) were harvested, and viable cells were injected intravenously into syngeneic C3H/HeN mice and allogeneic BALB/c nude mice. C4.P, C4.Neo.3, and C4.S2.3 cells were highly metastatic whereas C4.L8.5 cells were not metastatic. Experiments with [¹²⁵I]IdUrd-labeled tumor cells demonstrated that the initial arrest in the lung microvasculature did not differ among the lines, but that C4.L8.5 cells died by 48–72 h after injection. Enhanced survival of all K-1735 C4 cells (including C4.L8.5) was found in mice given twice daily injections of 20 mg NMA. The C4.L8.5 cells produced slow growing subcutaneous tumors in nude mice, whereas the other three lines produced fast growing tumors. In vitro studies confirmed that in the absence of NMA the expression of iNOS in C4.L8.5 cells induced apoptosis. Collectively, these data demonstrate that the expression of recombinant iNOS in melanoma cells is associated with apoptosis, suppression of tumorigenicity, and abrogation of metastasis.

Cancer metastasis is produced by metastatic cells that preexist within a parental neoplasm (1, 2). During hematogenous metastasis, tumor cells are subjected to such extreme selection pressures that the majority of tumor cells entering the circulation rapidly die regardless of their metastatic potential (2–5). Tumor cell features, such as deformability (6), aggregation (7), and cell surface adhesion molecules (7, 8) and host factors, such as blood turbulence (6, 9), platelets (10, 11), T cells (12), NK cells (13, 14), and macrophages (15), all influence the survival of blood-borne tumor emboli. Furthermore, passage of tumor cells through capillaries leads to cell lysis by sheer forces (6) and by nitric oxide (NO)¹ produced by cytokine-activated endothelial cells (16, 17).

NO is derived from the terminal guanido-nitrogen of L-arginine (18, 19), which is catalyzed by constitutive nitric oxide synthases (NOS) or inducible nitric oxide synthase (iNOS) (18–27). NO produces multiple effects that can influence the outcome of metastasis. Specifically, NO regulates vasodilatation (23, 24) and platelet aggregation (25, 26), which affect tumor cell arrest in capillaries (1, 16, 17). NO is also a major cytotoxic mediator secreted by activated macrophages (18–24) and endothelial cells (17) shown to be responsible for the destruction of tumor cells passing through capillary beds. Moreover, we have recently shown that the production of endogenous NO is associated with apoptosis of tumorigenic cells (27). Taken together, these results suggest the possibility that production of endogenous NO may be detrimental to tumor cell survival and production of metastasis.

Previous studies from Fidler's laboratory have shown that

¹ Abbreviations used in this paper: iNOS, inducible nitric oxide synthase; MTT, dimethylthiazole dephenyl tetrazolium bromide; NMA, N^ε-methyl-L-arginine; NO, nitric oxide; NOS, nitric oxide synthase.

nonmetastatic melanoma cells die rapidly after they are introduced into the circulation, whereas many metastatic cells survive (3, 4). Subsequent studies concluded that nonmetastatic cells exhibited high levels of iNOS activity and NO, whereas metastatic cells did not, thus demonstrating an inverse correlation between production of endogenous NO and the ability of K-1735 cells to survive in syngeneic mice to produce lung metastases (28).

The purpose of this study was to provide evidence of a causal relation between enhanced iNOS activity in tumor cells and decreased metastatic potential. Q. W. Xie et al. have recently reported the cloning and characterization of iNOS cDNAs from mouse macrophages (29) that encoded two isoforms of iNOS. The enzymatically active piNOS-L8 has a longer coding region and the other, enzymatically inactive piNOS-S2 has a shorter coding region differing at the COOH terminus (30). The inactivity of piNOS-S2 has been attributed to its inability to bind NADPH (30). In this study, we transfected these iNOS genes into highly metastatic iNOS-deficient K-1735 C4 melanoma cells. Transfection with cDNA encoding active iNOS induces apoptosis, suppresses tumor growth, and abrogates production of metastasis.

Materials and Methods

Reagents. Eagle's MEM, HBSS, PBS, and fetal bovine serum (FBS) were purchased from M. A. Bioproducts (Walkersville, MD). [^3H]dThd (sp act, 1 mCi/ml) was purchased from ICN Biomedicals, Inc. (Costa Mesa, CA). [^{125}I]dUrd (sp act, 1 mCi/ml) was purchased from New England Nuclear Corp. (Boston, MA). N $^{\omega}$ -methyl-L-arginine (NMA) was purchased from Sigma Chemical Co. (St. Louis, MO). All reagents used in tissue culture were free of endotoxin as determined by Limulus amoebocyte lysate assay (sensitivity limit, 0.125 ng/ml) purchased from Associates of Cape Cod (Woods Hole, MA).

K-1735 Line and Culture Conditions. The original K-1735 melanoma, induced in a C3H/HeN mouse by exposure to UV light followed by painting with croton oil, was a gift of Dr. Margaret L. Kripke (The University of Texas M. D. Anderson Cancer Center; 31). The parental tumor was cloned in vitro by a double dilution method. Of the large number of clones thus isolated, we chose clone 4 (C4), a highly metastatic line that produces melanotic lung metastases in syngeneic mice (32). Cell lines were used at low passage numbers (passages 6–10) since the original cloning procedure or isolation from metastases. Cultures were maintained for no longer than 6 wk after recovery from frozen stocks.

Tumor cell lines were maintained in tissue culture in Eagle's MEM supplemented with 5% FBS, sodium pyruvate, nonessential amino acids, L-glutamine, and twofold vitamin solution (Flow Laboratories, Rockville, MD). Cell cultures were maintained on plastic and incubated in 5% CO $_2$ -95% air at 37°C. Cultures were free of *Mycoplasma* and the following murine viruses: reovirus type 3, pneumonia virus, K virus, Theiler's encephalitis virus, Sendai virus, minute virus, mouse adenovirus, mouse hepatitis virus, lymphocytic choriomeningitis virus, ectromelia virus, and lactate dehydrogenase virus (assayed by M. A. Bioproducts).

To prepare tumor cells for inoculation, cells in exponential growth phase were harvested by a brief exposure to 0.25% trypsin-0.02% EDTA solution (wt/vol). The flask was tapped sharply to dislodge the cells, supplemented medium added, and the cell suspension

pipetted again to produce a single-cell suspension. The cells were washed and resuspended in Ca $^{2+}$ - and Mg $^{2+}$ -free HBSS to the desired cell concentration. Cell viability was determined by trypan blue exclusion, and only single-cell suspensions of >90% viability were used.

Transfection Procedures and Selection of Clones. K-1735 C4 parental cells (C4.P) were plated into 100-mm dishes at a density of 10 6 /dish. The monolayers (60% confluence) were washed and refed 12–18 h later in L-arginine-free medium containing 3 mM NMA (MEM-NMA) to increase transfection efficiency. The K-1735 C4.P cells were cotransfected with pcDNA1 plasmids containing the *Neo* resistance gene (pcDNA1/*Neo*) (Invitrogen, San Diego, CA), L8 (enzymatically active iNOS, piNOS-L8), or S2 (enzymatically inactive iNOS, piNOS-S2) genes (29, 30, 33). For each transfection, 0.8 ml of serum-free and L-arginine-free MEM-NMA was mixed with 0.2 ml diluted lipofectin solution (GIBCO BRL, Gaithersburg, MD) containing 2 μg pcDNA1/*Neo*, or 2 μg pcDNA1/*Neo* plus 40 μg piNOS-S2, or 2 μg pcDNA1/*Neo* plus 40 μg piNOS-L8. The K-1735 C4 cell monolayers were washed with MEM-NMA, and 1 ml of a transfection mixture was evenly added to the monolayers. The cultures were placed in a 37°C incubator for 24 h and then washed and fed with MEM-NMA containing 500 $\mu\text{g}/\text{ml}$ G418 (GIBCO BRL). The L-arginine-free MEM-NMA/G418 medium was replaced every 3 d until individual resistant colonies were isolated and established in culture as individual lines. All lines were maintained in L-arginine-free MEM-NMA/G418 and frozen after one to three in vitro passages. For identification of iNOS gene positive cells, 10 5 cells from each clonal line were plated into 38-mm 2 wells of 96-well plates and incubated in MEM-NMA. After 4–6 h, the adherent cells were washed with medium containing 5 mM L-arginine and then incubated in MEM with L-arginine (and without NMA). Culture supernatants were collected for nitrite analysis as described below.

Immunohistochemistry Using Specific Anti-iNOS Antibody. Tumor cells were plated on glass coverslips, and 10–12 h later, the cells were fixed by immersing the coverslips in ice-cold acetone for 10 min. The coverslips were then briefly rinsed in PBS. The endogenous peroxidase was inactivated by incubating the cells with 3% hydrogen peroxide diluted in methanol for 12 min, followed by a rinse with PBS. To minimize nonspecific adsorption of the antibodies to the coverslips, the samples were incubated with a blocking buffer containing 1% BSA and 1% normal goat serum in PBS for 10–20 min, and then mouse anti-iNOS mAb (Transduction Laboratories, Lexington, KY) diluted in the same blocking buffer (4 $\mu\text{g}/\text{ml}$) or preimmune IgG was added. The coverslips were incubated overnight at 4°C and then washed three times in PBS and incubated with blocking buffer solution for 5–10 min, followed by a 30–60-min incubation with peroxidase-conjugated secondary antibodies (rabbit anti-mouse IgG [H+L] antibody, peroxidase conjugated; Boehringer Mannheim, Indianapolis, IN) diluted 1:250 in blocking buffer. The samples were then washed and incubated for 10 min with 3,3'-diaminobenzidine, rinsed with distilled water, counterstained for 10 s with aqueous hematoxylin, washed three times with PBS and once with water, and studied under microscopy.

In Vitro Cytolysis Assay. Cells in their exponential growth phase were incubated for 18 h in medium containing 0.1 $\mu\text{Ci}/\text{ml}$ [^3H]dThd. The cells were washed twice with MEM containing 5 mM L-arginine and harvested by trypsinization, and 1.5–2.0 \times 10 6 viable cells were seeded in triplicate into 30-mm multiple culture dishes for 4–6 h. After removal of nonadherent cells, the monolayers were incubated in MEM alone or MEM-NMA. The dishes were placed in a 37°C incubator, and cultures were termi-

nated at different times. Supernatants were removed and the monolayers washed three times with PBS (containing Ca^{2+} and Mg^{2+}). The adherent cells were lysed by 0.1 N NaOH, and the radioactivity was monitored in a beta counter. The cytotoxicity was calculated according to the formula: cytotoxicity (%) = $(A - B)/A \times 100$ where A is the counts per minute of plated cells and B is the counts per minute of adherent surviving cells.

In Vitro Cytostasis Assay. K-1735 cells were plated at the density of 10^4 cells per 38-mm^2 well of 96-well plates in medium containing MEM-NMA (control) or MEM (test). After 72 h, cell number was determined by the dimethylthiazole dephenyl tetrazolium bromide (MTT, Sigma Chemical Co.) assay. After incubation for 2 h in medium containing MTT at 0.42 mg/mL, the medium was removed, and the cells were lysed in dimethyl sulfoxide. The conversion of MTT to formazan by metabolically viable cells (34) was monitored by a 96-well microtiter plate reader at 570 nm (Dynatech, Inc., Chantilly, VA). Percent cytostasis was calculated by the formula: cytostasis (%) = $[1 - (A/B)] \times 100$, where A is the absorbance of test cells incubated in medium or NMA and B is the absorbance of the control cells incubated in medium.

Nitrite Analysis. Nitrite concentration in culture supernatants was determined by a microplate assay described by Ding et al. (35). Briefly, 50- μl samples were harvested from conditioned medium and allowed to react with an equal volume of Griess reagent (1% sulfanilamide-0.1% naphthylethylene diamine dihydrochloride-2.5% H_3PO_4) at room temperature for 10 min. The absorbance at 540 nm was monitored with a microplate reader. Nitrite concentrations were determined by using sodium nitrite as a standard.

Extraction and Measurement of iNOS Activity. Tumor cells were scraped into a lysis buffer (20 mM Tris/HCl, pH 7.5, 137 mM NaCl, 1 mM PMSF, 10 $\mu\text{g}/\text{ml}$ aprotinin, 10 $\mu\text{g}/\text{ml}$ leupeptin, and 5 $\mu\text{g}/\text{ml}$ pepstatin A) at a density of 4×10^7 cells/ml and disrupted by sonication (Soniprep 150; Curtin Matheson Scientific, Inc., Houston, TX; 60% output, two times for 10 s each); the suspension was centrifuged at 18,000 g for 30 min at 4°C. NOS activity in the supernatants was measured as L-arginine- and NADPH-dependent generation of $\text{NO}_2^-/\text{NO}_3^-$, the stable oxidation products of NO. NOS activity was expressed as nmol/mg protein. Sodium nitrite was used to plot the standard curves (36–38). The protein concentration of iNOS preparations was measured by the Bradford method using a miniprotein assay kit (Bio-Rad Laboratories, Richmond, CA) with BSA as a standard (39).

Southern Blot Analysis. DNA was extracted from different cell lines. Equal amounts of DNA (20 μg) were digested with HindIII and XbaI for 18 h at 37°C. After precipitation, DNA was separated on a 0.8% agarose gel. Equal loading was checked by ethidium bromide staining. DNA in the gel was depurinated by incubation in 0.25 N HCl for 10 min and denatured in 0.5 N NaOH/1.5 M NaCl for 60 min. After neutralizing in 1 M Tris, pH 7.0/1.5 M NaCl for 60 min, DNA was transferred to a Nytran membrane (Schleicher & Schuell, Inc., Keene, NH) in $20\times$ SSC. After being baked at 80°C for 2 h and prehybridized for 4 h, the filter membranes were hybridized for 16–18 h to a ^{32}P -labeled iNOS cDNA probe (see below). Hybridization reactions were performed in 25 mM KPO_4 , pH 7.5, $5\times$ SSC, $5\times$ Denhardt's solution, 50 $\mu\text{g}/\text{ml}$ salmon sperm DNA, 50% formamide, and 10% dextran sulfate. The filters were washed in $2\times$ SSC/0.5% SDS twice for 30 min at room temperature and $0.1\times$ SSC/0.1% SDS twice for 60 min at 65°C. The filter membrane was exposed for 4–24 h to Kodak X-Omat XAR Film (Eastman Kodak, Rochester, NY).

iNOS mRNA Transcript Analysis. Cellular mRNA was prepared by using FastTrack kit (Invitrogen). The mRNA samples (2 μg)

were separated electrophoretically in 1.0% agarose gels containing MOPS buffer with 0.66 M formaldehyde. The RNA was transferred to a GeneScreen membrane (New England Nuclear) in $20\times$ SSC and UV-cross-linked with a UV Stratelinker 1800 (Stratagene, La Jolla, CA). The XbaI- and HindIII-digested 4.0-kb cDNA insert from piNOS-L8 was used as the probe for iNOS (29) and labeled with [^{32}P]CTP using a random labeling kit (Boehringer Mannheim). The membranes were prehybridized for 4 h and then hybridized for 16–18 h in a buffer containing 2% Genius blocking reagent, $5\times$ SSC, 0.1% sodium sarkosyl, 7% SDS, and 50 mM Na PO_4 , pH 6.8, at 42°C. Equal loading of RNA samples was monitored by hybridization with a murine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe. Filters were exposed for 6–48 h to Kodak X-Omat XAR film.

DNA Fragmentation Assay. Quantitative DNA damage was assayed according to previously published procedures with minor modifications (27, 40–43). Briefly, $1.5\text{--}2.0 \times 10^6$ [^3H]dThd-labeled cells were seeded in triplicate into 30-mm dishes in 2 ml of MEM or MEM-NMA. Cells were harvested at different times and centrifuged at 2,500 rpm for 10 min. The supernatant fluids (A) were collected and DNA was precipitated by 5% TCA. The cell pellets were suspended in a lysis buffer consisting of 8 mM EDTA, 20 mM Tris (pH 8.0), and 0.2% (vol/vol) Triton X-100. After 20 min at room temperature, the lysates were centrifuged at 13,000 g for 5 min. The low molecular weight DNA in the postnuclear supernatant (B) was collected and intact chromatin DNA (C) was dissolved in TE buffer consisting of 10 mM Tris (pH 7.5) and 1 mM EDTA. Radioactivity in the three preparations (A, B, and C) was determined in a beta counter. DNA fragmentation expressed as a percentage was calculated by the formula $[(A + B)/(A + B + C)] \times 100$.

Qualitative Analysis of Internucleosomal DNA Fragmentation. Internucleosomal DNA fragmentation was determined qualitatively by agarose gel electrophoresis (27, 40–43). Briefly, the low molecular weight DNA in the postnuclear supernatant (B) and intact chromatin DNA in the pellet (C) were treated with proteinase K and precipitated with ethanol at -70°C overnight after extraction with phenol and chloroform. The DNA was then pelleted and dissolved in TE buffer and treated with RNase I. An equivalent amount of materials from 10^6 cells was loaded and electrophoresed on a 1.5% agarose gel. The gel was stained with 1 $\mu\text{g}/\text{ml}$ ethidium bromide and photographed with Polaroid type 55 film (Sigma Chemical Co.) using a UV transilluminator and an MP-3 Polaroid camera setup.

Mice. Specific pathogen-free female C3H/HeN mice and male athymic BALB/c nude mice were purchased from the Animal Protection Area of the National Cancer Institute Frederick Cancer Research Facility (Frederick, MD). The mice were maintained in laminar air-flow cabinets under specific pathogen-free conditions in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the United States Department of Agriculture, Department of Health and Human Services, and National Institutes of Health. The mice were used between the ages of 8 and 12 wk.

Tumorigenicity and Experimental Metastasis. To produce tumors, 2×10^5 cells suspended in 0.1 ml HBSS were injected subcutaneously into the flank region of nude mice ($n = 5$). Tumor take and size were monitored three times per week. Tumor mass exceeding 3 mm in diameter was recorded as a positive take.

To produce experimental lung metastasis, unanesthetized C3H/HeN mice were injected with viable tumor cells suspended in 0.2 ml

HBSS into the lateral tail vein. The mice were killed 6 wk later and the lungs were removed, washed, and fixed in Bouin's solution to differentiate the neoplastic lesions from the organ parenchyma. The lung nodules were counted with the aid of a dissecting microscope.

In Vitro Labeling of Cells with [¹²⁵I]IdUrd. Tumor cells were seeded into 150-cm² tissue culture flasks at 4 × 10⁶ cells/flask in MEM-NMA. 24 h later, 0.3 μCi/ml [¹²⁵I]IdUrd was added. 24 h after that, the cell monolayers were rinsed twice with excess Ca²⁺- and Mg²⁺-free HBSS to remove nonbound radioiodine (3). Cell monolayers were then overlaid with a thin layer of 0.25% trypsin-0.02% EDTA solution for 1 min. The cells were dislodged from the plastic and suspended in medium. The cell suspension was washed and resuspended in Ca²⁺- and Mg²⁺-free HBSS at a final concentration of 10⁵ cells per 0.2 ml, the inoculum volume per mouse.

Distribution and Fate of [¹²⁵I]IdUrd-labeled Cells after Intravenous Injection. Labeled cells were injected intravenously into unanesthetized mice, and groups of five mice were killed at intervals thereafter. Lung, liver, kidneys, and spleen of each mouse were collected and placed in test tubes containing 70% ethanol. In addition, 0.2 ml of blood from each mouse was collected and placed in a test tube for direct measurement of radioactivity. The ethanol was replaced daily for 3 d to remove all soluble ¹²⁵I released from dead cells (3). The radioactivity in all of the organ samples was determined using a gamma counter (Gamma-Trac model 1185; TM Analytic, Elk Grove, IL). Triplicate input tubes (containing 0.2 ml of inoculum) were retained, and the radioactivity was counted at the same time as the sample organs. The mean counts in organs from each group of five animals per time point were expressed as the percentage of input counts. Measurements were corrected for radioactivity decay in input cells.

We also monitored the survival and fate of [¹²⁵I]IdUrd-labeled cells in mice injected intraperitoneally twice daily with 20 mg/dose NMA dissolved in HBSS. The treatment dose and schedule produced 70% inhibition in levels of nitrate induced in C3H/HeN mice by injection of 50 μg LPS. NMA was administered 1 d before and 3 d after the intravenous injection of radiolabeled cells. Cell survival in vivo was determined as described above.

Statistical Analysis. The in vitro data were analyzed for significance by the Student's *t* test (two-tailed), and the in vivo data were analyzed by the Mann-Whitney U test.

Results

Transfection and Selection of iNOS-expressing Cells. To establish stable transfectants, the K-1735 C4 parental cells (C4.P) were cotransfected with pcDNAI/*Neo* and either piNOS-L8 (active iNOS) or piNOS-S2 (inactive iNOS) genes (29, 32). Cells transfected with only pcDNAI/*Neo* served as a vector control. The MEM was free of L-arginine and contained 3 mM of NMA. Endogenous iNOS DNA was found in C4.P cells growing in MEM and in C4.P cells and C4.*Neo*.3 cells growing in MEM-NMA. Both recombinant and endogenous iNOS DNA was found in C4.S2.3 and C4.L8.5 cells growing in MEM-NMA (data not shown). Gene expression for iNOS was analyzed by Northern blot (Fig. 1) showing highly specific iNOS mRNA transcripts in C4.S2.3 and C4.L8.5. iNOS-positive clones were further characterized by immunocytochemistry using specific anti-iNOS mAb; homogeneously staining clones were identified for in vivo analysis (Fig. 2). The functional activity of iNOS was measured by an enzymatic

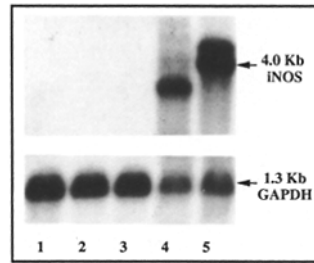


Figure 1. Northern blot analyses for iNOS expression. mRNA was isolated from C4.P cells growing in MEM (lane 1) or MEM-NMA (lane 2), C4.*Neo*.3 (lane 3), C4.S2.3 (lane 4), or C4.L8.5 (lane 5) cells. Samples (2 μg) were separated on 1% agarose gel, transferred to GeneScreen membrane, and probed with [³²P] iNOS cDNA. Note high mRNA expression in C4.S2.3 (lane 4) and C4.L8.5 (lane 5) cells. This is a representative experiment of three.

activity assay. Only the protein preparations from the C4.L8.5 cells had significant enzymatic activity (Fig. 3; *P* < 0.001). These data confirm the introduction into and expression of iNOS genes in K-1735 C4 cells.

Tumorigenicity. Tumor cells were injected subcutaneously into groups of nude mice. C4.P, C4.*Neo*.3, and C4.S2.3 cells produced rapidly growing tumors. By day 21 after injection, no tumors (a mass >3 mm in diameter) were produced by C4.L8.5 cells (data not shown).

Production of Metastasis by Cells Transfected with Functional iNOS Gene. In the next set of experiments, C3H/HeN mice were injected intravenously with 50,000 viable K-1735 C4 parental (C4.P) and several *Neo*-transfected clones. The median number of lung metastases produced by these cells was less than that of parental cells (Table 1). The C4.*Neo*.3 clone produced an average number of metastases and was chosen for all further studies. The incubation of C4 cells in MEM-NMA did not affect their metastatic potential. C4 cells incubated in MEM alone or MEM-NMA produced a median number of 56 and 45 lung metastases, respectively (data not shown). For all experiments, we therefore used cells cultured in MEM-NMA. In the next set of experiments, C3H/HeN mice were injected intravenously with 100,000 viable C4.P cells and several piNOS-S2 (inactive) or piNOS-L8 (active) transfected clones (Table 1). By day 21 after injection, C4.P cells produced a median of >200 lung metastases. C4.S2.3, C4.S2.4, and C4.S2.6 cells produced a median of 94, 72, and 112 lung metastases, respectively. C4.S2.3 cells were chosen for further studies. Mice injected with C4.L8 cells were killed on day 45 after i.v. injection. Even then, C4.L8.5, C4.L8.11, and C4.L8.13 cells were not metastatic. Only a single metastasis in a single mouse was found (Table 1). The single lung metastasis was isolated and a line established in culture. These cells did not produce NO (in the absence of NMA), and the loss of iNOS gene expression was confirmed by a negative Northern blot analysis (data not shown). C4.L8.5 cells were chosen for further studies.

To rule out that the abrogation of metastatic potential by C4.L8 cells was due to immune rejection (1, 13–15), we repeated the studies in athymic nude mice (Table 2). C4.P, C4.*Neo*.3, C4.S2.3, and C4.L8.5 cells were injected intravenously into nude mice at the inoculum of 100,000 (Expt. 1) and 125,000 (Expt. 2) viable cells. The mice were killed after 28 d. C4.P, C4.*Neo*.3, and C4.S2.3 cells were all highly

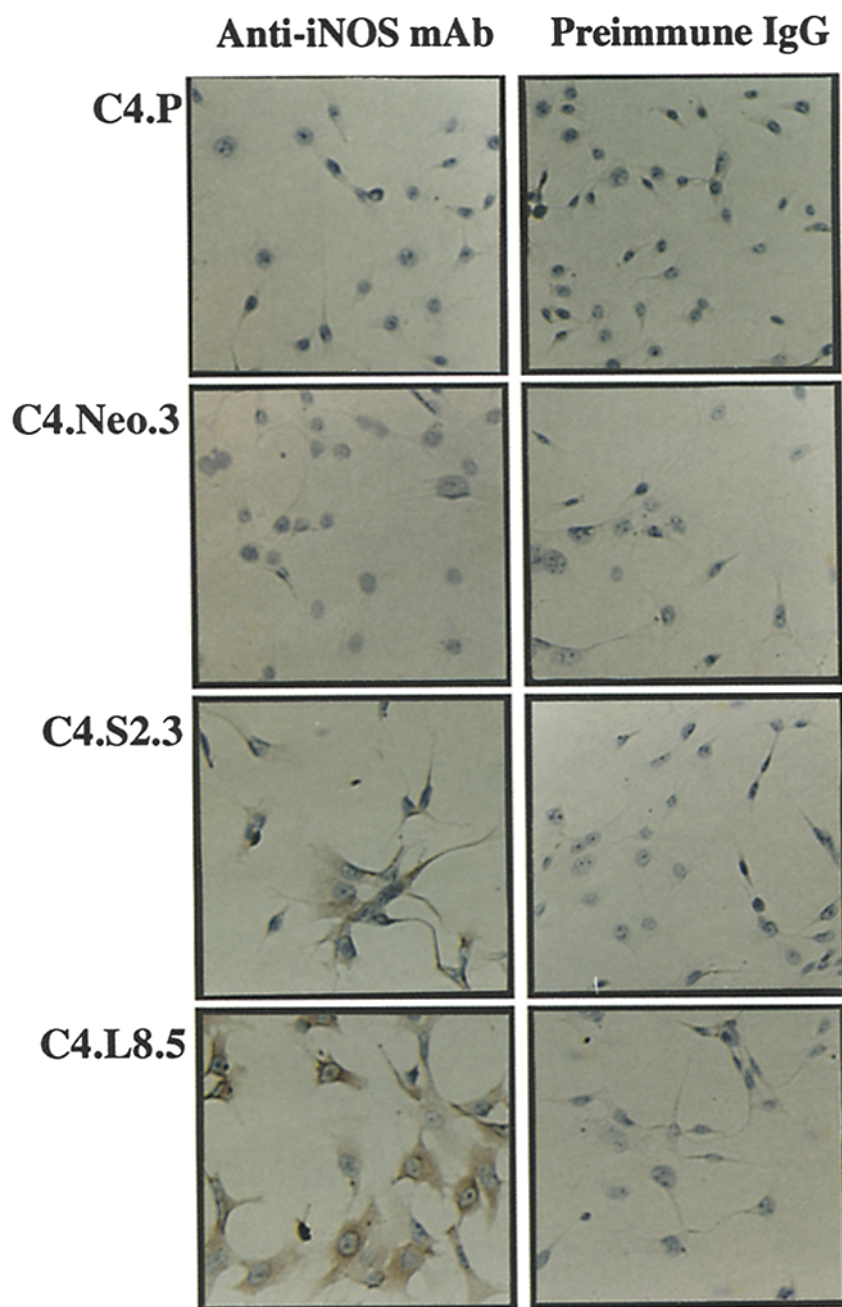


Figure 2. Immunohistochemistry for iNOS protein. Cells were seeded into chamber slides, incubated for 4–6 h, and then fixed and stained with either anti-iNOS mAb or control IgG. Note positive reaction in cells transfected with piNOS-S2 or piNOS-L8 gene. The reactivity in the C4.L8.5 cells is more intense and uniform.

metastatic. In contrast, C4.L8.5 cells were not metastatic. (Only a single metastasis in a single mouse was found).

Distribution and Fate of [¹²⁵I]IdUrd-labeled Tumor Cells. The organ distribution and fate of [¹²⁵I]IdUrd-labeled C4.P, C4.Neo.3, C4.S2.3, and C4.L8.5 cells were followed for from 10 min to 72 h after i.v. injection (Fig. 4 A). The only organ in which significant proportions of labeled cells were detected was the lung. At 10 min and 24 h after injection, there were no discernible differences among the lines in the percentage of input cells remaining in the lungs. By 72 h, however, significant differences had emerged in the number of cells surviving in the lungs, making it possible to separate the lines. The percentage of viable C4.P, C4.Neo.3, and C4.S2.3 cells

surviving in the lungs was 4, 4, and 2%, respectively. In contrast, the percentage of C4.L8.5 cells surviving in the lung was <0.2%. These data clearly agreed with a previous report on survival of metastatic and nonmetastatic K-1735 clones in lungs of syngeneic mice (28, 44).

In the next set of experiments, we injected [¹²⁵I]IdUrd-labeled C4.P, C4.Neo.3, C4.S2.3, and C4.L8.5 cells intravenously into normal mice or mice injected intraperitoneally with 20 mg NMA twice a day for 4 d (1 d before and 3 d after tumor injection). The percentage of surviving cells in the lungs of mice was determined on day 3 after i.v. injection (Fig. 4 B). Regardless of the tumor cells injected, enhanced survival of tumor cells was found in the lungs of mice treated

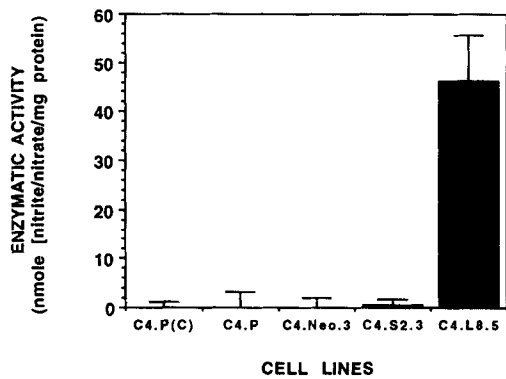


Figure 3. iNOS enzymatic activity. Cells were cultured in MEM-NMA to semiconfluence, at which point protein was extracted and NOS activity was measured and expressed as nmole of (nitrite/nitrate)/mg protein. The values are mean \pm SD of triplicate cultures. This is one representative experiment of three.

with NMA, suggesting that one major factor that contributes to the death and clearing of tumor cells from the lung (28) is the production of NO.

Expression of Recombinant iNOS and Apoptosis. Previous studies from our laboratory demonstrated that synthesis of recombinant NO induces apoptosis in murine-transformed fibroblasts (27). We next determined whether expression of

Table 1. Experimental Lung Metastasis Produced by K-1735 C4 Cells Transfected with the iNOS Gene

Cell line*	Lung metastasis		
	Incidence	Median	Range
Expt. 1			
C4.P	5/5	50	21-70
C4.Neo.1	5/5	28	12-35
C4.Neo.2	5/5	39	31-56
C4.Neo.3	5/5	33	23-50
C4.Neo.4	5/5	27	16-31
C4.Neo.5	5/5	48	43-68
Expt. 2			
C4.P	10/10	>200	All >200
C4.S2.3	10/10	94	34-123
C4.S2.4	10/10	72	39-156
C4.S2.6	10/10	112	51-182
C4.L8.5	0/10	0 [†]	All 0
C4.L8.11	1/10	0 [†]	0-1
C4.L8.13	0/10	0 [†]	All 0

* Syngeneic mice were injected intravenously with 50,000 (Expt. 1) and 100,000 (Expt. 2) viable tumor cells and killed after 21 or 45 d (C4.L8) when the number of experimental lung metastases was determined using a dissecting microscope.

[†] $p < 0.001$, Mann-Whitney test.

Table 2. Abrogation of Metastatic Potential in K-1735 C4 Cells Transfected with the iNOS Gene and Injected into Nude Mice

Cell line*	Lung metastasis		
	Incidence	Median	Range
Expt. 1			
C4.P	10/10	174	114->200
C4.Neo.3	10/10	146	77->200
C4.S2.3	10/10	110	41-184
C4.L8.5	0/10	0 [†]	All 0
Expt. 2			
C4.P	10/10	>200	All >200
C4.Neo.3	10/10	>200	All >200
C4.S2.3	6/6	180	102->200
C4.L8.5	1/10	0 [†]	0-1

* BALB/c nude mice were injected intravenously with 100,000 viable cells (Expt. 1) or 125,000 viable cells (Expt. 2) and killed 28 d later. The number of experimental lung metastases was determined using a dissecting microscope.

[†] $p < 0.001$, Mann-Whitney test.

recombinant iNOS in the K-1735 C4 cells was associated with apoptosis. The cells were cultured in MEM or MEM-NMA. Nitrite accumulation was only detected in the cultures of C4.L8.5 cells growing in MEM (Fig. 5 A). The peak level of nitrite was reached by 48 h of incubation in MEM (Fig. 5 B). In parallel studies, we determined whether the expression of recombinant iNOS correlates with inhibition of cell growth or cell lysis. Tumor cells were cultured in MEM or MEM-NMA. Significant cytostasis (Fig. 6 A) and cytolysis (Fig. 6 B) were found only in C4.L8.5 cells cultured in MEM. The inhibition of NO by NMA prevented death of the C4.L8.5 cells.

The death of the C4.L8.5 cells (cultured in MEM) was associated with genomic DNA damage. The data shown in Fig. 7 A reveal that by 48 h, significant DNA fragmentation occurred in C4.L8.5 cells incubated in medium without NMA. This DNA fragmentation could be inhibited by 3 mM NMA. To characterize the genomic DNA damage, we electrophoresed genomic DNA fragments (Fig. 7 B, lanes 1-6) and the corresponding intact chromatin DNA (Fig. 7 B, lanes 7-12) from the various cultures (after 48 h incubation) on a 1.5% agarose gel. DNA laddering of C4.L8.5 cells was found (lane 5), indicating that cell death occurred by apoptosis.

Discussion

The process of metastasis consists of multiple sequential steps that include motility, invasion, survival in the circulation, adhesion, extravasation, and growth (1-4). During metastasis, tumor cells are subjected to such extreme selection pressures that the majority of tumor cells that enter the

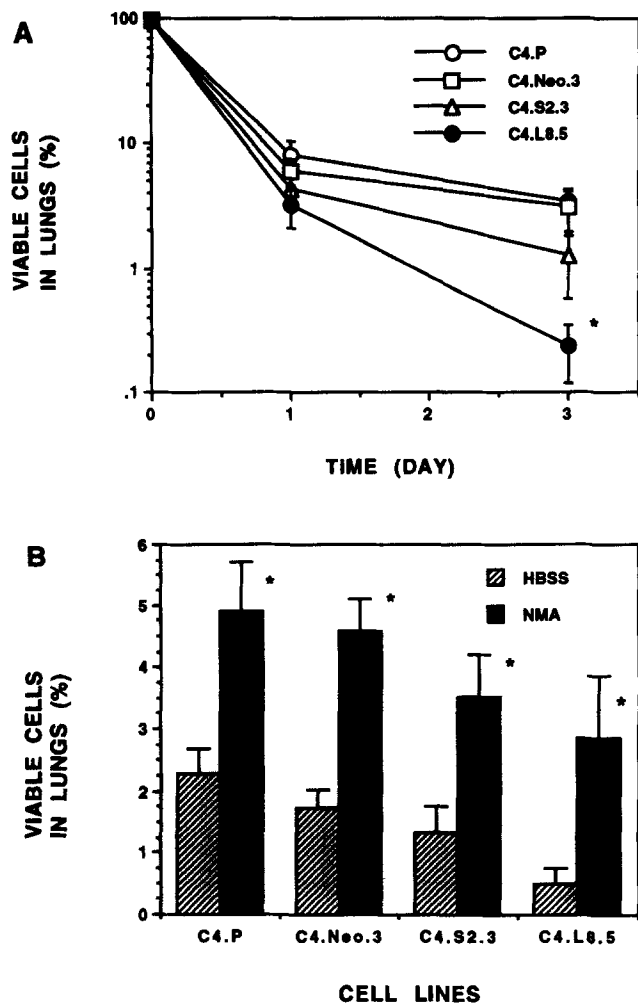


Figure 4. Arrest and survival of [¹²⁵I]IdUrd-labeled tumor cells in the lungs of syngeneic mice. (A) Radiolabeled cells (10^5) were injected intravenously into mice. At the indicated time points, groups of mice ($n = 5$) were killed and visceral organs were removed and processed as described in Materials and Methods. Percent surviving cells in the lungs was calculated by comparison to initial inoculum. This is one representative experiment of two. * $p < 0.01$ Mann-Whitney U test. (B) Mice were injected intraperitoneally with either HBSS or 20 mg NMA twice daily for 4 d. Radiolabeled tumor cells were injected intravenously after 1 d of treatment and the mice were killed on day 3 after tumor cell injection. The enhanced survival of tumor cells in NMA-treated mice was significant. * $p < 0.01$.

circulation rapidly die (7–11). This selection pressure may explain the finding that metastases are clonal in origin (45) and can develop from single progenitor cells (31). Neoplasms exhibit heterogeneity for invasion and metastasis (1, 4), which is expressed in differences in survival between nonmetastatic cells and metastatic cells (46). Studies using the well-characterized murine K-1735 melanoma system of clones, cell lines, and somatic cell hybrids (between nonmetastatic and metastatic cells) concluded that nonmetastatic cells exhibited high levels of iNOS, whereas metastatic cells did not (28). The in vitro treatment of tumor cells with cytokines, such as TNF- α , IL-1, and IFN- γ , induced production of NO and then apop-

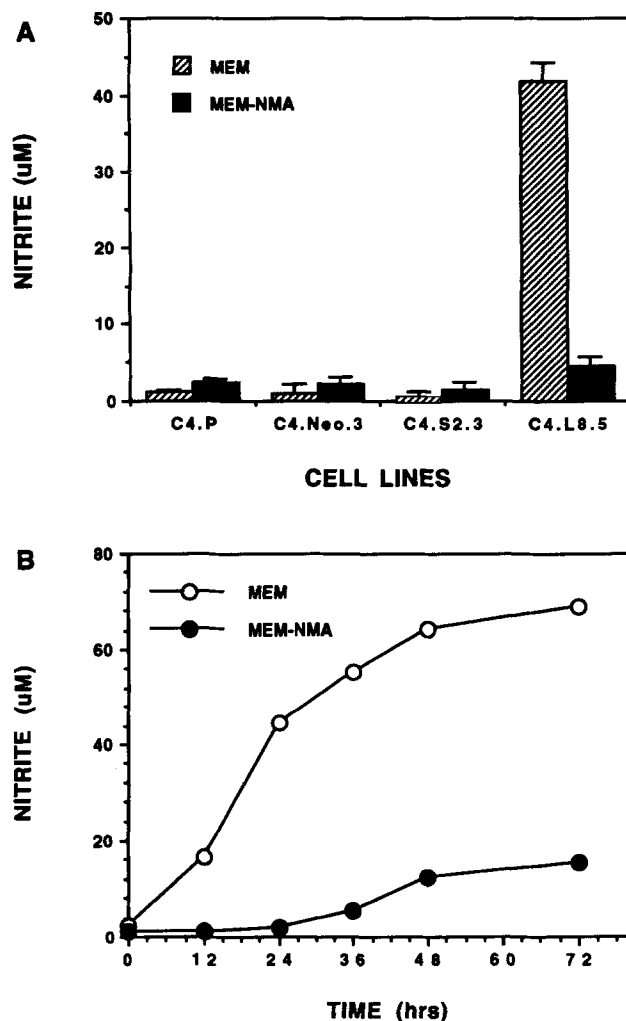


Figure 5. NO production by K-1735 C4 cells. (A) Cells ($1.5\text{--}2 \times 10^6$) were plated into 30-mm dishes in triplicate in MEM or MEM-NMA. After 48 h, the amount of nitrite in the culture supernatants was determined as described in Materials and Methods. This is one representative experiment of five. * $p < 0.001$. (B) Kinetics of NO production by C4.L8.5 cells. C4.L8.5 cells ($1.5\text{--}2 \times 10^6$) were plated into 30-mm dishes in triplicate in MEM or MEM-NMA. After 12, 24, 36, 48, and 72 h, the amount of nitrite in the culture supernatants was determined as described in Materials and Methods. The differences in nitrite were significant ($p < 0.001$). This is one representative experiment of five.

toxis and both sequela could be blocked by NMA (28). These data suggest that one factor contributing to the death of circulating tumor cells is the production of NO, but its causal relationship to metastasis remained unclear.

The present results demonstrate that the transfection of highly metastatic K-1735 C4 cells (which express low levels of iNOS) with an enzymatically active iNOS expression vector renders the cells nonmetastatic by inducing high levels of NO production. In the presence of NMA, the cells' proliferation in vitro and survival in vivo was very similar to that of parental cells transfected with a control vector (neomycin resistance) or cells transfected with inactive iNOS cDNA. The

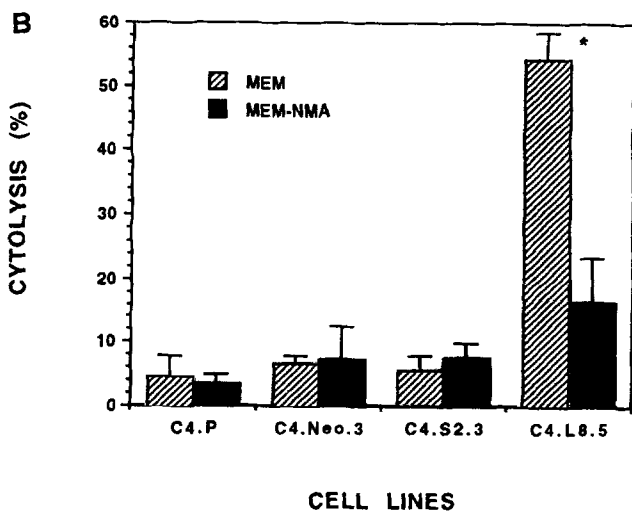
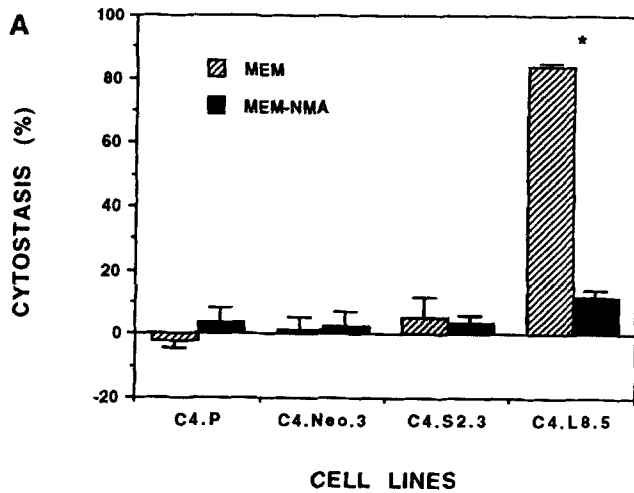


Figure 6. In vitro survival of K-1735 C4 cells transfected with the iNOS gene. (A) In vitro cytotostasis assay. Cells were plated into culture dishes in MEM or MEM-NMA. After 72 h, the number of viable cells in triplicate cultures was determined by the MTT assay as described in Materials and Methods. The difference in proliferation between C4.L8.5 cells incubated in the presence or absence of NMA was highly significant ($p < 0.001$). This is one representative experiment of three. (B) In vitro cytolysis assay. Tumor cells prelabeled with $[^3\text{H}]$ thymidine were plated into culture dishes in MEM or MEM-NMA. After 48 h, triplicate cultures were washed, the adherent cells lysed, and radioactivity monitored as described in Materials and Methods. The difference in survival between C4.L8.5 cells incubated in the presence or absence of NMA was highly significant ($p < 0.001$). This is one representative experiment of three.

growth (tumorigenicity) of the active iNOS cDNA transfected cells was inhibited and production of metastasis was abrogated, providing direct evidence for the role of iNOS/NO in the prevention of cancer metastasis.

The successful transfection of the K-1735 C4 cells with the enzymatically active iNOS gene L8 (29, 30, 33) required the use of L-arginine-free medium and the continuous presence of 3 mM NMA. In medium containing L-arginine and devoid of NMA, the transfected C4.L8 cells produced high levels of NO and underwent apoptosis. To examine the growth

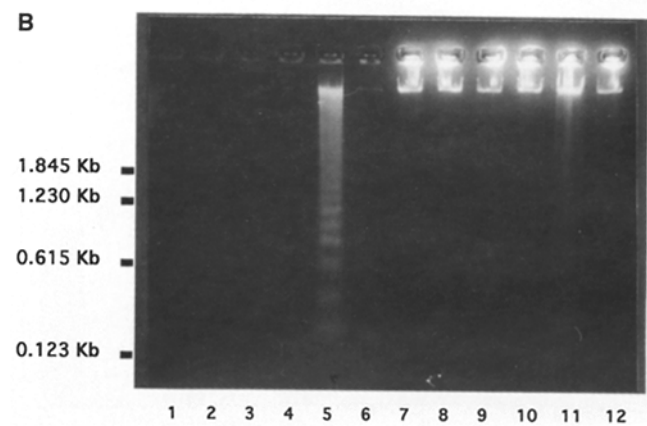
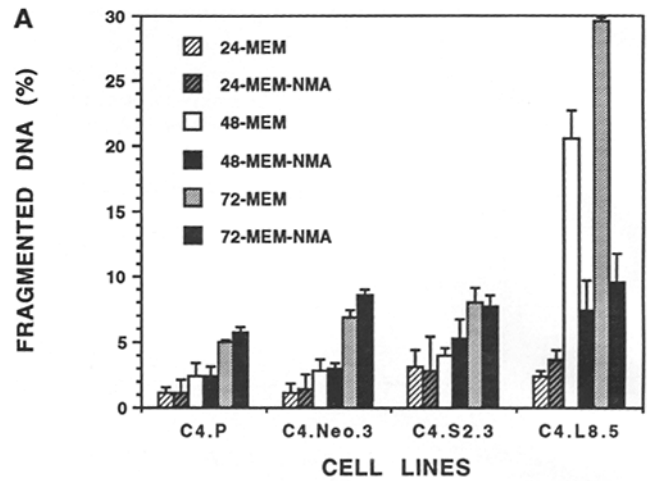


Figure 7. Internucleosomal DNA fragmentation. (A) $[^3\text{H}]$ thymidine-labeled tumor cells ($1.5\text{--}2 \times 10^6$) were plated into 30-mm dishes in MEM or MEM-NMA. After different times, fragmented and intact chromatin DNA samples were collected from triplicate cultures. After different incubation periods, the percentage of fragmented DNA was determined as described in Materials and Methods. This is one representative experiment of two. (B) Induction of internucleosomal DNA fragmentation in K-1735 C4 cells. Cells were incubated in MEM or MEM-NMA for 48 h when fragmented and intact chromatin DNA from 10^6 cells was collected. The DNA was treated with proteinase K, extracted, precipitated, pelleted, and dissolved in TE buffer and then treated with RNase I. An equivalent amount of material was loaded on a 1.5% agarose gel, electrophoresed, stained, and photographed (lanes 1–6, fragmented DNA, and lanes 7–12, chromatin DNA). Lanes 1 and 7, C4.Neo.3, MEM; lanes 2 and 8, C4.Neo.3, MEM-NMA; lanes 3 and 9, C4.S2.3, MEM; lanes 4 and 10, C4.S2.3, MEM-NMA; lanes 5 and 11, C4.L8.5, MEM; lanes 6 and 12, C4.L8.5, MEM-NMA. This is a representative experiment of three.

of the various K-1735 C4 cells under in vivo conditions, we injected them into the subcutis of nude mice. All the control cells produced rapidly growing tumors, whereas the C4.L8.5 cells did not. Because the kinetics of subcutaneous growth are difficult to assess, we used the cells in a well-established experimental metastasis assay.

Experimental metastases are tumor colonies produced after i.v. injection of cells. Although these tumor cells bypass the initial steps of metastasis (separation from primary neoplasm and invasion and release into blood vessels or lymphatics),

all of the subsequent steps in the metastatic process must occur for metastases to be formed. In this study, we equate experimental metastasis with the blood-borne spread of tumor cells. The transfection of *neo* resistance gene or enzymatically inactive iNOS gene into the highly metastatic K-1735 C4 cells reduced the number of lung metastases. In contrast, the transfection of enzymatically active iNOS gene into the highly metastatic C4 cells abrogated production of lung metastasis after i.v. injection.

To rule out that the abrogation of metastasis by C4.L8 cells was due to immune rejection (1, 13, 15), we repeated the experiment using athymic nude mice. Even in the immune-deficient mice, C4.L8.5 cells were not metastatic, whereas C4.P, C4.Neo.3, and C4.S2.3 cells were still highly metastatic. Collectively, these data show that although transfection per se can somewhat reduce metastatic potential of K-1735 C4 cells, only transfection with piNOS-L8 gene renders the cells (C4.L8.5, C4.L8.11, C4.L8.13) nonmetastatic.

To determine whether the abrogation of metastasis by C4.L8.5 cells (active iNOS gene) was due to initial cell arrest or to later events, we studied the fate of [¹²⁵I]IdUrd-labeled tumor cells in normal and NMA-treated mice. The initial arrest and survival of [¹²⁵I]IdUrd-labeled K-1735 C4 cells (transfected with enzymatically active iNOS gene) in the lungs of syngeneic mice was very similar to that of all control cells. Within 24–72 h however, only 0.1% of the iNOS-transfected cells survived, whereas >2% of control cells survived. The low survival rate of C4.L8.5 cells was very similar to that found for nonmetastatic clones of K-1735 (28).

The death of K-1735 C4.L8.5 cells could have been due to factors unrelated to transfection with the enzymatically active iNOS gene. To examine this possibility, we studied the fate and survival of [¹²⁵I]IdUrd-labeled cells in mice treated twice daily with i.p. injections of 20 mg NMA (a

dose sufficient to inhibit 70% of production in mice injected with 50 µg LPS). Enhanced survival of all K-1735 cells was found in NMA-treated mice with significant increase in survival for C4.L8.5 cells. These in vivo data confirm that NO is a major factor that contributes to the death of circulating tumor cells. Since lung metastases produced by K-1735 melanoma cells can originate from single surviving cells (31, 44), the survival of cells in the lung at 72 h after injection predicts the development of visible metastases (4, 46).

The production of NO by tumor cells may reduce their capacity to produce metastasis by at least two nonmutually exclusive mechanisms. The aggregation of platelets around tumor cells enhanced arrest in capillary beds (10, 11) and NO has been shown to inhibit platelet aggregation (25, 26, 47). More likely, however, the production of NO can induce cell death in normal, transformed, and tumorigenic cells by an apoptotic process (27). The K-1735 C4 cells transfected with the enzymatically active iNOS gene (C4.L8.5) but not the enzymatically inactive iNOS gene (C4.S2.3) or the *Neo* resistance gene (C4.Neo.3) produced high levels of NO followed by genomic DNA damage and cell death, as monitored by both cytostasis and cytolysis analyses. These data provide direct evidence that NO can induce DNA fragmentation and apoptosis and suggest the usefulness of this cell system to study apoptosis.

In summary, the introduction of an enzymatically active iNOS gene into highly metastatic murine melanoma cells induces apoptosis, suppresses growth, and abrogates metastasis. These data provide direct evidence that expression of iNOS and production of NO can induce apoptosis and is responsible for inhibiting the malignant potential of tumor cells. The use of the iNOS gene for treatment of malignant melanoma metastases is now under investigation.

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