Nitric oxide synthase II suppresses the growth and metastasis of human cancer regardless of its up-regulation of protumor factors

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Inducible nitric oxide (NO) synthase (NOS) II protein was initially identified in activated murine macrophages (1, 2). The human NOS II gene was first cloned from hepatocytes in 1993 and has high homology among animal species (3, 4). Once stimulated, NOS II generates large amounts of NO throughout the life of the active enzyme, plays an important role in host defense against pathogens, and participates in tumoricidal activity against many types of tumors (2, 4–8). Although NOS II is easily induced and expressed in macrophages during host defense mechanisms, NOS II expression and/or activity is also observed in many other cell types, including endothelial and epithelial cells and a variety of human cancer cells (2, 4, 5).

Induction of NOS II expression in tumor and/or stromal cells and the subsequent biological impact of NO production on tumor biology are complex and remain unclear. Over the past decade, many reports have presented positive and negative aspects of NO production in tumor biology. Whereas some studies found that NO was cytostatic or cytotoxic, others provided evidence that NO was protective (5–9). NO appears to be either beneficial or deleterious in cell culture. The net effect of NO is thought to depend on its available concentration, target cell type, and interactions with reactive oxygen species, metal ions, and proteins (5–7). Other factors must also be considered. It also has recently become clear that the specific locations of NO synthesis can dramatically influence its actions (10); thus, studies with donors or inhibitors are highly likely to have global effects that may not mimic the physiological/pathophysiological environment. For example, the influence of NO donors on molecular and cellular aspects of tumor biology must be interpreted with caution, because the use of NO in cell cultures may have only limited physiological and pathological relevance in vivo (5, 11). Moreover, the results of numerous studies with animal models and human tumor specimens and interpretation of these results further complicates the understanding of the biological impact of NOS II on tumor biology (5). For example, the impact of NOS II expression on human tumor development and progression has been inferred primarily from descriptive observations of protein staining of NOS II and measurement of NOS II activity in human specimens (6, 11–14). Data generated from the use of NO activators and/or inhibitors in animal models is much less convincing because of the existence of multiple NOS isoforms and other known and unknown pharmacological effects of various NOS activators/inhibitors (5).

In addition, gene transfection experiments designed to analyze the impact of NO on tumor biology have been less straightforward than initially thought. For instance, transfection of the NOS II gene inhibited growth of tumors and metastasis of various murine and human tumor cell lines (5, 15), whereas several other reports indicated that transfection and expression of NOS II promoted human tumor growth in animal models (16, 17). Besides the clear differences in transfection procedure, cell type, and animal model, the relative levels of NOS II activities were also apparently considerably different. Because in vivo NO concentrations can vary considerably depending on the location and conditions of its production, the biological role of NOS II expression in human tumor growth and metastasis is far from clear and must be investigated in proper in vivo model systems (5, 7).

In the present study, we sought to determine the influence of cell type and NO concentration on tumor growth and metastasis. Whereas forced production of NO significantly up-regulated multiple angiogenic factors, NO-producing tumor cells did not form tumors or metastasize in ectopic or orthotopic xenograft nude mouse models. This dramatic loss of malignancy was due to NO-mediated apoptosis. More significantly, we also generated a series of adenoviral vectors harboring mutant NOS II genes, which expressed mutant NOS II proteins with defined levels of enzymatic activity. Tumor cells transfected with these NOS II genes produced NO at different levels, which directly correlated with the antitumor activity in vitro and in vivo. This demonstration using a relevant biological system shows that NO produces dose-dependent antitumor activity in vitro and in vivo, regardless of its up-regulation of protumor factors.

Materials and Methods

Reagents, Cell Lines, and Culture Conditions. MEM and FBS were purchased from Sigma. All reagents used in tissue culture were free of endotoxins as determined by using the Limulus amebocyte lysate assay (sensitivity limit, 0.125 ng/ml), which was purchased from Associates of Cape Cod. 253J-BV, A375SM, COLO357-L3.3, SKOV3.ip1, Km12SM, MDA-453, PC3M, and SN2PM6 human cancer cell lines were provided by Isaiah J. Fidler (M. D. Anderson Cancer Center, University of Texas). HT-1080 and DLD-1 were purchased from the American Type Culture Collection. All tumor

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Abbreviations: NOS, nitric oxide synthase; moi, multiplicity of infection; AG, aminoguanidine; l-NAME, N-nitro-l-arginine-methyl ester; PI, propidium iodide; MVD, microvessel density.

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cell lines were cultured in MEM supplemented with 10% FBS, sodium pyruvate, nonessential amino acids, l-glutamine, and a twofold vitamin solution (Flow Laboratories). The cell cultures were maintained in plastic flasks and incubated in 5% CO2/95% air at 37°C. Cultures were free of Mycoplasma.

Animals. Female athymic BALB/c nude mice were purchased from The Jackson Laboratory. The mice were housed in laminar flow cabinets under specific pathogen-free conditions and used when they were 8 weeks old. The animals were maintained in facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care in accordance with the current regulations and standards of the U.S. Department of Agriculture, Department of Health and Human Services, and National Institutes of Health.

Tumor Growth and Metastasis. To prepare tumor cells for inoculation, cells in the exponential growth phase were harvested by brief exposure to a 0.25% trypsin/0.02% EDTA solution (wt/vol). Cell viability was determined by trypsin blue exclusion, and only single-cell suspensions >95% viable were used. To evaluate tumor growth and metastasis, 0.05 ml of tumor cell suspensions (1 × 10⁶ cells per mouse) was ectopically injected into the subcutis or orthotopically injected into the organ in which the tumor originated of nude mice (The Jackson Laboratory) as described in refs. 18 and 19. The animals were killed at the indicated number of days after tumor implantation or when they had become moribund. Next, the primary tumor in the subcutis or other organ was harvested and weighed. Regional lymph nodes (at least five in each mouse) were collected and examined for tumor metastasis by histopathology as described by refs. 18 and 19. Metastasis was expressed as percentage incidence by using the following formula: metastasis incidence (%) = [mice with metastasis/total mice used] × 100, in which metastasis was regional and/or distant.

Generation of Ad.NOS II and Adenoviral Transduction of Tumor Cells. Full-length murine NOS II cDNA was obtained from C. Nathan (Weill Medical College, Cornell University, New York) (1, 20, 21). Point mutations of the NOS II gene were generated with a QuickChange mutagenesis kit (Stratagene). Ad.NOS II-Wt harbors the full-length murine NOS II gene; Ad.NOS II-M1, Ad.NOS II-M2, Ad.NOS II-M3, Ad.NOS II-M4, and Ad.NOS II-M5 harbor differentially mutated murine NOS II genes. Also, Ad.CMV carries the CMV promoter sequence, and Ad.EGFP carries the EGFP gene expression cassette. Replication-deficient recombinant adenoviruses were generated, and cells were transduced as described in ref. 22.

Determination of NO Production and Analysis of Gene Expression. NO production was determined by measuring nitrate/nitrite accumulation in the culture supernatants by using Griess reagent after conversion of nitrate into nitrite by nitrate reductase (20, 22). The NOS II enzyme activity was assayed according to the conversion of [¹⁴C]arginine to [¹⁴C]citrulline (18). Northern blot analysis was performed to determine the level of VEGF, IL-8, and NOS II mRNA expression, and equal loading of mRNA was monitored by rehybridizing each membrane filter with a β-actin cDNA probe. Western blot analysis was performed with appropriate first antibodies, and equal protein loading was monitored by rehybridizing the membrane filter with a β-actin antibody. Quantitative analyses were performed with the use of densitometry and standardized according to β-actin (19–22).

In Vitro Endothelial Cell Tube Formation Assay and in Vivo Assessment of Tumor Angiogenesis. The tube formation assay was performed by using human umbilical vein endothelial cells as described in ref. 23. The degree of tube formation was assessed as the percentage of cell surface area vs. total surface area. Control cell cultures were given arbitrary values of 1 (23). Tumor angiogenesis was assessed by standard microvessel density (MVD) counting after CD31 staining as described in ref. 23.

Analysis of Cell Growth and Apoptosis in Vitro. Tumor cells were seeded at 4 × 10⁶ cells per well in six-well culture plates. Twelve hours later, the cells were incubated for 2 h at 37°C in serum-free media or serum-free media with Ad.NOS II or Ad.EGFP at a multiplicity of infection (moi) of 20. After being washed with serum-free medium, the transduced cells were replenished with DMEM and incubated for 1–4 days. The cell numbers were counted daily via the trypan blue exclusion method with a hemocytometer. Apoptosis was quantitatively determined by measuring internucleosomal DNA fragmentation, and the apoptotic cells were further visualized by TUNEL according to our published procedures (22, 24).

Annexin V–Propidium Iodide (PI) Assay. Annexin V-fluorescein and PI staining were used to determine the percentage of cells undergoing apoptosis and necrosis, respectively, and were done according to the manufacturer’s instructions (Roche Applied Science, Indianapolis). Briefly, cells were trypsinized and washed twice with ice-cold PBS. Cells were resuspended and incubated for 15 min at room temperature in Hepes buffer containing Annexin V-fluorescein and PI labeling reagent. Cells were then analyzed with the aid of flow cytometry to determine the percentage of cells undergoing apoptosis and necrosis (25).

Statistics. All experiments were performed at least twice with similar results, and representative experiments are presented. The significance of the differences between the median values of the in vitro data were determined by using the two-tailed Mann–Whitney or Kruskal–Wallis test, whereas the significance of the differences between the mean values of the in vitro data were determined with the two-tailed Student t test. In all of the tests, P < 0.05 was defined as statistically significant.

Results

Up-Regulation of Multiple Angiogenic Molecules by NOS II Gene Transfer. Studies by our group and others have suggested that NO production leads to altered expression of genes important to tumor angiogenesis and metastasis (8, 16, 24). In the present study, we aimed to determine whether NOS II gene transfer affects the angiogenic phenotype of tumor cells through modulation of the expression of VEGF and IL-8, which are critical angiogenic molecules in human pancreatic cancer (24, 26, 27). The adenoviral vector was used to mediate efficient NOS II gene transfer into and expression in various human tumor cells (>95% transduction efficiency). We found that only Ad.NOS II transduction significantly increased VEGF and IL-8 expression (Fig. 1a), an effect that was completely reversed by blocking NO production with aminoguanidine (AG) and N-nitro-l-arginine-methyl ester (l-NAME), NOS II inhibitors (Fig. 1b, c, and e), suggesting that NO mediated time-dependent induction of VEGF and IL-8. By using a tube formation assay, we found that the supernatant from NOS II-transduced cells was more angiogenic than that from control cells and the effect was reversed by addition of AG (Fig. 1d) and l-NAME (Fig. 1f), suggesting that NOS II transfer and NO production did indeed increase the angiogenic potential of the tumor cells.

Suppression of Tumor Growth and Metastasis by NOS II Gene Transfer. To determine the effects of NO-mediated elevated angiogenic potential on tumorigenic and metastatic potential, L3.3 human tumor cells were transduced with Ad.NOS II as described above for Fig. 1 and NOS II expression was determined by Northern blot analysis (Fig. 2 a Inset–i Inset). Control cells or cells transduced with Ad.CMV or Ad.EGFP grew progressively in nude mice, whereas
metastasis to distant organs (data not shown). Our data clearly demonstrated that the NOS II gene can be transferred into and expressed in human tumor cells and that the production of NO strongly suppresses tumor growth and metastasis.

**Induction of Apoptosis by NOS II Gene Transfer.** L3.3 cells were transduced with Ad.NOS II or Ad.EGFP or Ad.CMV in the presence or absence of 1 mM AG or L-NAME. NO-mediated cytotoxicity in these cells was determined by measuring cell viability and internucleosomal DNA fragmentation. Transduction of Ad.NOS II significantly inhibited cell growth as determined by cell counting (Fig. 3a) and induced apoptotic cell death as determined by DNA fragmentation assay (Fig. 3b) and PI-staining/FACS analysis (Fig. 3c). The apoptotic cells were further visualized by TUNEL assay (Fig. 3d) and confirmed by Annexin V and PI staining (Fig. 3e). These data indicated that transfer of NOS II and production of NO suppressed tumor growth by inducing apoptosis.

**Generation of NOS II Gene Mutants with Different Enzymatic Activities.** To quantitatively define the effect of NO production on tumor biology, various mutant NOS II genes were constructed (Fig. 4a). The adenovirus harboring various NOS II genes was transduced into L3.3 cells. As compared with Ad.NOS II-Wt, the mutant Ad.NOS II-M1, Ad.NOS II-M2, Ad.NOS II-M3, Ad.NOS II-M4, and Ad.NOS II-M5 exhibited decreasing levels of NOS II activities as determined by measuring NO production (Fig. 4b and 4b Inset) and NOS II enzymatic activity (Fig. 4c), although the levels of NOS II protein expression were very similar as determined by Western blot analysis (Fig. 4c Inset).

**Impact of Defined Levels of NO Production on Tumor Growth in Vitro.** L3.3 cells were transduced in triplicate with an adenovirus harboring wild-type or mutant NOS II genes. The growth of the cells was...
determined by direct cell counting (Fig. 5a) and PI staining and FACS analysis 48 h after transduction (Fig. 5b). Clearly, cytotoxicity produced by adenoviral transduction depended directly on the NOS II enzymatic activity. The control Ad.EGFP did not produce significant cytotoxicity. In contrast, Ad.NOS II-Wt totally suppressed tumor growth and eventually killed all of the tumor cells, whereas Ad.NOS II-M1 had significant but reduced cytotoxic activity, which was proportional to its enzymatic activity level. Other mutants only showed marginal tumor-suppressive activities. Therefore, this was the first biological system to demonstrate concentration-dependent NO cytotoxicity.

Impact of Defined Levels of NO Production on Tumor Growth and Metastasis in Vivo. To determine the impact of defined levels of NO production on tumor growth and metastasis, we used two complementary systems. For tumor-growth kinetics, the L3.3 cells transduced with an adenovirus harboring wild-type or mutant NOS II were injected into the subcutis of nude mice (n = 10). Ad.NOS II-Wt totally suppressed tumor growth as expected, whereas the antitumor activities of the NOS II mutants were proportional to their NOS II activity levels (Fig. 5c). The NOS II mutants with a low level of activity only produced marginal antitumor activity; tumor promotion was not observed in the NOS II mutants with a low or high level of NOS II activity. To determine the effect of NOS II activity on tumor growth and metastasis, the L3.3 tumor cells were injected into the pancreas of nude mice (n = 10). Consistent with the s.c. nude mouse model, Ad.NOS II produced NO-level-dependent suppression of primary tumor growth and distant metastasis to the liver and/or regional metastasis to lymph nodes (Fig. 5d). Angiogenesis in xenograft tumors was further determined by CD31 staining and MVD. As shown in Fig. 5e and f, there was no significantly increased or decreased MVD in tumors formed by cells transduced with NOS II genes. Because of complete tumor suppression, no tumors were formed by NOS II-Wt, II-M1, and II-M2 transduced cells for MVD assessment. Our data indicated that NO is cytotoxic in vitro and in vivo and that the extent of the cytotoxicity depends on its level of production, and NO production did not significantly alter tumor angiogenesis.

Discussion

Induction of NOS II expression in tumor and/or stromal cells and the subsequent biological impact of NO production on tumor biology have been inconclusive (5–7, 28). Before logically designing an effective preventive and therapeutic strategy through targeting tumor-associated NOS II/NO, the as yet unclear mechanisms of NOS II expression and NO production and their actions in the tumor bed must be clearly understood. In the present study, we showed that production of NO significantly up-regulated multiple angiogenic molecules but failed to enhance tumor growth and metastasis in ectopic and orthotopic xenograft nude mouse models. The dramatic loss of malignancy was due to NO-mediated apoptosis. More importantly, we generated a series of adenoviral vectors harboring mutant NOS II genes that expressed mutant NOS II proteins with defined levels of enzymatic activity. Transduction with these genes produced different levels of NO and antitumor activity in vitro and in vivo, and no tumor promotion effects were observed.

Whether NOS II expression is elevated or induction of NOS II is actually impaired in human tumors remains open for debate (5, 15). Many studies have indicated an elevated level of NOS II expression in human tumor specimens. However, the constitutive and inducible levels of NOS II expression are very low in most human tumor cell cultures (8, 9, 29). To recapitulate NOS II expression and its impact on human cancer biology, many studies have been designed and performed to determine the effects of different concentrations of NO donors on the survival and proliferation of a variety of cell types. It is apparent that the cell type and NO concentration are important determinants of the biological outcome (4, 5, 7, 29). However, it is difficult to interpret their biological relevance. As an alternative, gene transfer has been used to restore NOS II expression in and assess its impact on human cancers provided that the cancers in question had decreased or lost NOS II induction (5, 20). Unfortunately, the NOS II transgene in the receiving cells is very unstable. Our previous studies showed that cell lines with relatively high levels of NOS II expression only survive in culture medium with a continuing presence of NOS II inhibitors (1, 20). In contrast, because of the cytotoxicity of NO,
was further confirmed by Western blot analysis. Supernatant (b) and NO activity (c). (Inset) A photograph of microtiter plate chambers showing Griess reaction. (c) (Inset) NO II protein was further confirmed by Western blot analysis.

stable NO II transfection of various human colon cancer cell lines without using an inhibitor only yields rare clones with low levels of NO II expression (16, 17). Clearly, the potential impacts of different methods of restoration of NO II expression on human cancer cells are very different, and their biological relevance remains to be validated. In the present study, we effectively transduced a variety of human tumor cell lines with the NO II gene. Although the levels of NO II protein expression and NO production differed from cell line to cell line, all of the cells producing NO failed to produce tumors and metastases in animal models. Our data were consistent with those in our previous reports but differed from those in other reports (16, 17). This lingering discrepancy may have been due to several plausible factors, such as the cellular p53 status, NO output, and, most importantly, different methods of restoration of NO II expression.

Ambs et al. (9) originally proposed the possibility that the cellular p53 status influences the fate of tumor cells exposed to exogenous or endogenous NO. Along with other evidence, we have shown that endogenous and exogenous NO activates the wild-type p53 gene, which is closely associated with apoptosis (5, 30). These findings have been confirmed in numerous studies (5, 14, 29). The resulting growth inhibition may provide selection pressures for clonal expansion of cells with mutant p53 (9). However, several studies have demonstrated that NO produces p53-dependent and -independent apoptosis in tumor cells (5, 31). In the present study, the cell lines used exhibited diverse p53 functions, from wild-type (e.g., A375SM) and mutant (e.g., Km12SM) forms of the gene to loss of it (e.g., COLO357-L3.3). Yet, they were all susceptible to NO II-mediated tumor suppression. Therefore, mutation of p53 may not necessarily render tumor cells more resistant to NO and growth advantage for those with mutated p53 gene.

NO output is considered another important determinant of the biological outcome. Numerous studies have shown that high concentrations of NO induce apoptosis in susceptible cells, whereas low concentrations of it can be antiapoptotic. However, it remains unclear what might be the spatial and temporal concentration and production rate of NO in tumor bed (32). In our current study, various human tumor cells were transduced to produce NO. Some cell lines produced NO at higher levels than HT-29-NOS II cells did, whereas others produced NO at lower or similar levels. Although the reported level of NO production in HT-29-NOS II cells had a tumor-promoting effect (16), we did not see NO at particular levels promoted tumor growth. To exclude the possibility that different cell lines exhibited different sensitivity to NO, we generated multiple NO II mutants with defined levels of NO II enzymatic activities, which produce NO at a range that could possibly occur under both physiological and pathological circumstances. By using this unique system, we found no evidence that either low or high levels of NO production promoted tumor growth in vitro and in vivo. The tumors were weighed, and regional and distant metastasis were determined. The tumor tissues were further processed for quantitative evaluation of MVD status (e), and representative photos of MVD in tumor sections were taken at magnification of 100 (f).
The most likely reason for our observed dose-dependent antitumor activity might be the NOS II gene transfer procedure. With stable transfection without the use of a NOS II inhibitor, adaptation to NO-induced cytotoxicity may occur during NOS II transfection and culture maintenance, and the resulting cell lines may be a subpopulation that gains more tumorigenic potential through the development of NO resistance and induction of expression of genes important to tumor survival and growth, such as IL-8 and VEGF (26–28). Therefore, cell lines forced to produce NO may already have had significant phenotypic modifications during in vitro culture (5, 16, 33, 34). This notion was supported by our current study. We showed that transduction of tumor cells with the NOS II gene significantly increased the expression of VEGF and IL-8, two major protumor factors for human pancreatic cancer (26–28). However, these cells were clearly nontumorigenic and nonmetastatic in animal models, suggesting that up-regulation of protumor molecules was insufficient to increase the malignant phenotype in vivo. One simple explanation may be that the cytotoxic effect outweighed the protumor activity of NO; thus, the net effect was tumor suppression. Nevertheless, this result has very significant implications, because in a heterogeneous tumor, sustained, high NO production may eliminate tumor cells producing NO but spare and support the growth of tumor cells not producing NO by inducing overproduction of protumor molecules (secondary and/or bystander effects). The lack of tumor growth in the present study may have been due to 100% gene transfer efficiency, i.e., there were no tumor cells not producing NO to take advantage of this putative secondary and/or bystander effects. Conversely, tumor cell cultures contain various cell subpopulations. If clonal selection occurs during the lengthy process of NOS II gene transfer and selection, the resulting cell line may be a subpopulation that intrinsically resists NO and survives. This particular subpopulation may be highly tumorigenic even before NOS II transfer and intrinsically express antiapoptotic genes, such as Bcl-2 and Bcl-x, and/or angiogenic molecules, such as VEGF and IL-8 at a high level. Indeed, a previous study has shown that exposure cells to a small dose of NO for 8–12 h induces a phenotypic change in the cells whereby they become markedly resistant to a normally lethal exposure to a higher NO (or oxidant) exposure (35). We are currently testing whether more malignant tumor cells undergo clonal selection, dominance, and expansion in vitro and in vivo and/or undergo epigenetic adaptation under the selection pressure imposed by NO.

In summary, we have applied variable levels of endogenous NOS II activity in tumor cells without long-term use of NOS inhibitors and transfection/selection and have tracked simultaneously antitumor and protumor activities in vitro and in vivo. The production of NO significantly up-regulated multiple protumor factors but suppressed tumor growth and metastases, regardless of NO production level and tumor type. This demonstration using a relevant biological system showed that NO produced dose-dependent antitumor activity in vitro and in vivo. How NO suppresses tumor growth in vivo and whether NOS II can be used for cancer therapy warrant further investigation.

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