Direct Demonstration of Negative Regulation of Tumor Growth and Metastasis by Host-inducible Nitric Oxide Synthase

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Abstract

Inducible nitric oxide synthase (NOS) II expression can be induced in the tumor bed, predominantly in host cells that infiltrate and surround a tumor. However, the impact of this physiological NOS II expression in host cells on tumor growth and metastasis remains unclear because of a lack of appropriate experimental approaches. In the present study, three NOS II-null (NOS II−/−) tumor cell lines, M5076, B16-BL6, and MCA, were established and verified using Southern, Northern, and Western blot analysis, and nitric oxide production assays. Cells from these lines were then s.c. and i.v. injected into NOS II−/− and NOS II−/− C57BL/6 mice. NOS II protein expression and enzyme activity were clearly detected in the tumors that formed in NOS II−/− mice but not in those that formed in NOS II−/− mice. Consistent with the absence of NOS II expression in the tumor stroma, M5076, B16-BL6, and MCA cells produced much faster and produced many more experimental lung metastases in NOS II−/− mice than in NOS II−/− mice. Therefore, physiological expression of NOS II in host cells directly inhibits tumor growth and metastasis.

Introduction

Recent studies have indicated that NOS3 plays an important role in the regulation of tumorigenicity and metastasis (1–5). However, previous studies in this area were inconclusive and even controversial (1). There are many potential reasons for this. Generally, the various studies in this area were inconclusive and even controversial (1). Recent studies in this area were inconclusive and even controversial (1). Recent studies in this area were inconclusive and even controversial (1). Recent studies in this area were inconclusive and even controversial (1). The results of these studies were inconclusive and even controversial (1).

Materials and Methods

Reagents. Eagle’s MEM, HBSS, and FBS were purchased from M. A. Bioproducts (Walkersville, MD). Mouse recombinant IFN-γ (specific activity, 1 × 107 units/mg protein) was purchased from Genzyme (Cambridge, MA). Phenol-extracted Salmonella LPS, MCA, and olive oil were purchased from Sigma Chemical Co. (St. Louis, MO). Anti-NOS II antibody was purchased from Transduction Laboratories (Lexington, KY). [32P]dCTP (sp. act., 6000

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3 The abbreviations used are: NOS, nitric oxide synthase; NO, nitric oxide; FBS, fetal bovine serum; LPS, lipopolysaccharide; MCA, methylcholanthrene.

Arguably, these studies are not conclusive, because neither NOS II activators nor inhibitors are specific. For example, IFN-γ, a potent NOS II inducer, can produce both NO-dependent and -independent antitumor activity (12, 13). Nω-monomethyl-l-arginine, a NOS inhibitor, may influence tumor biology via alteration of l-arginine metabolism in addition to NO synthesis. Moreover, the use of different NOS II inhibitors and activators, and their treatment regimens often leads to different outcomes by mechanisms irrelevant to NO pathways (1, 6).

This discrepancy may also result from the use of different tumor types, as the genetic and epigenetic makeup of tumor cells may not only dictate the level of NOS II expression within them and surrounding host cells but also influence the sensitivity of tumor cells to NO-mediated cytotoxicity (1, 2, 5). For example, cells containing wild-type p53 were found to be more sensitive to NO-mediated apoptosis (2).

Moreover, most of the evidence comes from in vitro and ex vivo experiments, and may not reflect what occurs in vivo (1). Recent studies have shown that genetic disruption of host NOS II apparently enhances the growth and metastasis of NO-sensitive M5076 tumor cells but suppresses the metastasis of NO-resistant B16-BL6 tumor cells, suggesting that host-derived NO may differentially modulate tumor progression (14). However, tumor-associated NO is derived from both tumor and host cells. Specifically, M5076 and B16-BL6 cells both may express NOS II and produce NO, which may influence tumor growth and metastasis (14). In fact, M5076 cells produce NO at a much higher level than B16-BL6 cells do. The different levels of NO production may contribute to the different metastatic behaviors of the respective cell lines in NOS II−/− and NOS II−/− mice, because a high level of endogenous NO production may suppress metastasis (15).

Collectively, all of the evidence suggests that there is a close relationship between NO production and tumor progression. However, the causal effect of NOS II expression in host cells on cancer metastasis remains unclear. A crucial approach to addressing the role of NOS II expression in both tumor and host cells is the development of unequivocal in vitro and in vivo model systems. In the present study, we established several tumor cell lines with disruption of the NOS II gene, compared their tumorigenicity and metastasis in syngeneic NOS II−/− and NOS II−/− mice, and explored in great length the role of NO in cancer metastasis. We clearly demonstrated that NOS II expression was induced in tumor-infiltration macrophages, and inhibited tumor growth and metastasis.
Animals. Female NOS II-/- and knockout NOS II (NOS II+/+) C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The mice were housed in laminar flow cabinets under specific pathogen-free conditions and used when they were 8 weeks old. They 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 United States Department of Agriculture, United States Department of Health and Human Services, and NIH.

Tumor Cell Line Establishment. The MCA tumor-induction method used in this study was described previously. Briefly, NOS II-/- C57BL/6J mice were injected s.c. with 10 μg/g body weight of MCA dissolved in olive oil. When the tumor in each mouse had grown to 0.5–1.0 cm in diameter, it was excised, and parts of it were fixed in a formalin solution for histological examination or used for serial transplantation in the hosts of the origin strain. Tumor tissue was also obtained immediately after surgery and processed as follows. The tissue was first rinsed several times in cold (4°C) culture medium and then cut into fine fragments using a sterile scalpel. The fragments were then subjected to sequential enzymatic digestion for 30 min at 37°C in medium containing collagenase type I and DNase (Sigma Chemical Co.). After enzymatic dissociation, the cells were maintained at 4°C. Finally, the cell suspension was filtered through four-layer sterile gauze, washed three times in serum-free medium, and cultured in RPMI 1640 supplemented with 10% FBS. About 8–10 in vitro passages later, a tumor cell line was established and designated as KX-dw; using a similar method, we established the cell lines KX-dw4 and KX-dw7. These are all fibrosarcoma cell lines.

Cell Lines and Culture Conditions. The RAW 264.4 cell line was purchased from the American Type Culture Collection (Manassas, VA). All of the cell lines, including KX-dw1, KX-dw4, and KX-dw7, were cultured in tissue culture in RPMI 1640 supplemented with 10% FBS, sodium pyruvate, nonessential amino acids, L-glutamine, and vitamins (Flow Laboratories, Rockville, MD). The cell cultures were maintained in plastic flasks and incubated in 5% CO2–95% air at 37°C. The cultures were free of Mycoplasma infection.

Tumor Growth and Metastasis. Tumorigenic and metastatic ability of KX-dw1, KX-dw4, and KX-dw7 was determined as described previously (14).

Southern Blot Analysis. Genomic DNA isolated from cell cultures or mouse tail biopsy samples was digested with BamHI, separated via electrophoresis through a 0.8% agarose gel, transferred to a GeneScreen nylon membrane (DuPont Co., Boston, MA), UV-cross-linked using a UV-Stratalinker 1800 (Stratagene, La Jolla, CA), and hybridized using Rapid-hyb Buffer (Amersham plc, Buckinghamshire, United Kingdom). NOS II and neomycin-resistant gene (Neo) cDNA probes were labeled using a random primer labeling kit (Boehringer Mannheim Biochemicals, Indianapolis, IN) and used in hybridizations. Mutant and wild-type alleles were identified according to predicted restriction-fragment-size differences.

NOS II Expression. NOS II mRNA expression was measured by Northern blot analysis, NOS II protein expression by Western blot analysis, and NO production in vitro and in vivo was determined by measuring total nitrate/nitrite concentrations in serum or culture supernatants using sodium nitrite as a standard (13,14). Immunolocalization of NOS II expression and macrophage infiltration in growing tumors was performed as described previously (13).

NOS II Enzyme Activity. The NOS II enzyme activity was assayed according to the conversion of [14C]arginine to [14C]citrulline using a commercial NOS assay kit (Sigma Chemical Co.). Briefly, cells were detached using a cell scraper and suspended in 1× homogenization buffer containing 25 mM Tris-HCl, 1 mM EDTA, and 1 mM EGTA. Afterward, the cells were homogenized using a tissue grinder; the cell homogenate was pipetted into microcentrifuge tubes, and the tubes were spun in a microcentrifuge at full speed for 5 min at 4°C. The supernatant was then collected in a tube and kept on ice until they were used. For the NOS II enzyme activity assay, 25 μl of cell homogenate corresponding to ~25 μg of protein was incubated at 37°C for 1 h in a reaction mixture containing 50 mM HEPES (pH 7.4 at 37°C), 1 mM Mg2+, 0.5 mM NADPH, 5 μM flavin adenine dinucleotide, 5 μM flavin mononucleotide, 10 μg/ml calmodulin, and 50 μM [14C]-arginine (Amersham Pharmacia Biotech Inc., Piscataway, NJ). The calcium chelators EDTA and EGTA were used to measure the NOS enzymatic activity of calcium-independent NOS II. The reaction was stopped by adding 2 ml of ice-cold 20 mM HEPES (pH 5.5) containing 5 mM EDTA. The sample was applied onto a Dowex AG 50W-X8 column that had been pre-equilibrated with 20 mM HEPES (pH 5.5). The radioactivity of the eluate, which contained [14C]-citrulline, was quantified using a liquid scintillation counter and expressed as counts per min per milligram of protein per min.

Statistics. The significance of the in vitro data were determined using Student’s t test (two-tailed), whereas that of the in vivo data were determined using the two-tailed Mann–Whitney U test. A P of <0.05 was deemed significant.

Results

KK-dw Cell Line Characterization. To confirm the disruption of the NOS II gene and lack of functional NOS II protein in KK-dw cell lines, we performed a series of experiments to determine from gene structure to enzyme products. For Southern blot analysis, genomic DNA was digested by BamHI and probed with Neo-resistance gene cDNA. A specific 5-kb fragment indicated a mutant allele in KK-dw cell lines and tissues from NOS II-/- mice but not in tissues from NOS II+/+ mice (Fig. 1A). For Northern blot analysis, KK-dw1, KK-dw4, and KK-dw7 cells were incubated for 24 h in medium alone or containing 10 units/ml IFN-γ and 1 μg/ml LPS, and total RNA was extracted. A RAW 264.7 macrophage line was used as a positive control. A 4.4-kb NOS II transcript was detected in NOS II-/- RAW 264.7 cells (Fig. 1B1). Because of the disruption of the NOS II gene, two abnormal NOS II transcripts were detected in KK-dw cells using a mouse NOS II cDNA probe: one was constitutively expressed and slightly smaller (~4 kb), whereas the other was inducible and significantly larger (~5.1 kb; Fig. 1B1). Both abnormal transcripts also hybridized to a neomycin probe, suggesting that they contained a Neo gene coding sequence (data not shown). Therefore, the 4-kb transcript was assigned as NOS II mRNA.
NOS II Expression in Tumors from NOS II−/− and NOS II+/+ Mice. To provide direct evidence that NOS II was induced in host cells by tumor cells, KX-dw1, KX-dw4, and KX-dw7 cells were injected s.c. into syngeneic NOS II−/− (II−/−) C57BL/6 mice. NOS II expression was determined using cytosolic protein (A, Western blot; B, NOS activity) prepared from tumor tissue samples and additionally confirmed by measuring the serum nitrite/nitrate levels as determined using a Greiss reagent (C). RAW 264.7 cell culture (Raw) and Panc02-H7 tumors growing in NOS II−/− (II−/−) or NOS II+/+ (II+/+) C57BL/6 mice were used as controls for in vitro and in vivo experiments, respectively. This was a representative experiment of three with similar results and the bars represented the detection limits of the assays. D, Immunolocalization of NOS II protein. s.c. KX-dw1 tumors growing in syngeneic NOS II−/− (D1 and D3) and NOS II+/+ (D2 and D4) C57BL/6 mice were collected and processed. Snap-frozen tissue sections were fixed and stained for infiltration macrophages using an F4/80 antibody (D3 and D4) and for NOS II protein expression using a specific polyclonal rabbit anti-NOS II antibody (D3 and D4); bars, ±SD.

To additionally confirm the absence of NOS II protein, immunoblot analysis was performed using the lysate from LPS/IFN-γ-treated KX-dw cells and a polyclonal antimouse NOS II antibody. Lysates from KX-dw cells contained no detectable NOS II (Fig. 1C). Consistent with the absence of NOS II protein, no NOS II enzyme activity was detected in the cytosolic protein from KX-dw cells (Fig. 1D), and no NO production was detected in the culture medium of LPS/IFN-γ-treated KX-dw cells as determined using a Greiss reagent assay (Fig. 1E).

NOS II Expression in Tumors from NOS II−/− and NOS II+/+ Mice. To provide direct evidence that NOS II was induced in host cells by tumor cells, KX-dw1, KX-dw4, and KX-dw7 cells were injected s.c. into syngeneic NOS II−/− and NOS II+/+ C57BL/6 mice. Normal and tumor tissue samples were collected from NOS II−/− and NOS II+/+ mice. The level of NOS II protein expression was determined using Western blot analysis (Fig. 2A). NOS II protein was clearly detected in the tumor tissue samples from NOS II+/+ mice, whereas no significant NOS II protein expression was detected in tumor tissue samples from NOS II−/− mice. We additionally determined the level of NOS activity in tumor tissue samples by measuring the conversion of [14C]-l-arginine to [14C]-citrulline (citrulline conversion assay). A significant level of NOS II enzyme activity was detected in the tumor lysates from NOS II−/− mice but not in those from NOS II+/+ mice (Fig. 2B), which was consistent with serum nitrite/nitrate levels as determined using a Greiss reagent (Fig. 2C). To provide evidence that host NOS II expression differentially affects the fate of injected tumor cells, immunostaining was performed using tumor sections from NOS II−/− and NOS II+/+ mice. Macrophage infiltration was apparent in the sections from both types of mice (Fig. 2D). These data indicated that NOS II was induced in host cells on the interaction between tumor and host cells.

Tumor Growth and Metastasis in NOS II−/− and NOS II+/+ Mice. To investigate the influence of host NOS II activity on tumor growth and metastasis in vivo, NOS II+/+ and NOS II−/− mice...
recovered a s.c. injection of $2 \times 10^5$ KX-dw cells. Tumor growth in these mice was assessed by measuring the tumor size every 3–7 days (Fig. 3, A–C). In addition, to evaluate metastasis, $5 \times 10^4$ KX-dw cells were injected into the lateral tail vein of both NOS II and KX-dw mice; their lungs were collected 21 days after the injection, and the metastatic nodules in them were counted. The metastatic nodules in NOS II mice were many more than that in NOS II mice (Table 1).

**Discussion**

In this study, three NOS II−/− tumor cell lines, KX-dw1, KX-dw4, and KX-dw7, were established and verified. Cells from these lines were s.c. and i.v. injected into syngeneic NOS II−/− and NOS II−/− C57BL/6 mice. NOS II protein expression and enzyme activity were clearly detected in the tumors that formed in NOS II−/− mice but not in those that formed in NOS II−/− mice. Consistent with the absence of NOS II expression in the tumor stroma, KX-dw1, KX-dw4, and KX-dw7 cells grew much faster and produced many more experimental lung metastases in NOS II−/− mice than in NOS II−/− mice. Therefore, the physiological expression of NOS II in host cells directly inhibits tumor growth and metastasis.

Accumulating evidence suggests that there is a close relationship between NO production and tumor progression. However, the causal effect of NOS II expression on cancer metastasis remains inconclusive and even controversial (1–6). The effect is explained in part by the fact that NO is a pleiotropic molecule and that the apparently opposing roles of NO may be attributed to many other factors, including NOS isoforms and expression levels (1, 6). Two isoforms of NOS are involved in tumor-associated NO production: NOS II and NOS III. Although the potential influence of NO derived from these two isoforms may be investigated with activators and/or inhibitors, those presently available lack the degree of isoform selectivity that would allow unequivocal interpretation of in vivo data (1, 6). The alternate approach is to use animals lacking a functional NOS II gene, thus avoiding the contentious issues arising from the use of activators and/or inhibitors in vivo, such as the mode, duration, and selectivity of treatment and dose administered. NOS II−/− mice display a phenotype consistent with loss of the cytotoxic actions of NO, as they are susceptible to infection and show impaired macrophage cytotoxicity in tumor cells, indicating a potential role of NO in natural defense against tumorigenicity (15, 16).

This conclusion was additionally supported by our previous study showing that NOS II expression was induced in the tumor bed because of the tumor-host interaction and that the tumor microenvironment is the critical determinant for induction of NOS II expression. For example, in previous studies, elevated NOS II expression and NO production were clearly observed in the tumors formed by IFN-β-secreting tumor cells. NOS II expression was required for the antitumor activity of localized production of IFN-β, a potent NOS II inducer, because disruption of the NOS II gene impaired this antitumor activity (17). Moreover, NOS II expression was significantly decreased with accelerated tumor growth in mice having disruption of the IFN-γ gene (13), which is essential for synergistic NOS II induction (7). Therefore, elevation of the availability of NOS II-inducing cytokines can effectively increase NOS II expression and NO-mediated tumor suppression. However, the source of NOS II and potential interaction of it with tumor and host cells remains unclear. A recent study found that B16-F1 tumor cells in NOS II−/− mice did not express NOS II, although those in NOS II−/− mice did, suggesting that the presence of NOS II in the host allows tumor cells to express NOS II (18, 19). In contrast, Panc02-H7 cells did not express NOS II in vitro but clearly expressed it in NOS II−/− mice (20). Therefore,

![Fig. 3. Comparison of tumor growth in NOS II−/− and NOS II+/+ mice. The KX-dw1, KX-dw4, and KX-dw7 cell suspensions were injected s.c. into groups of 5 syngeneic NOS II−/− and NOS II+/+ C57BL/6 mice, and the tumor growth rate was determined by measuring the tumor sizes at 3–7-day intervals. Data were presented as mean; bars, ±SD. This was a representative experiment of two with similar results, and the * indicated statistic significance ($P < 0.05$).](image)

### Table 1 Metastatic ability of NOS II−/− KX-dw1, KX-dw4, and KX-dw7 cells in syngeneic NOS II−/− and NOS II+/+ C57BL/6 mice

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Incidence</th>
<th>Number (median)</th>
<th>$P$</th>
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<tr>
<td>KX-dw1</td>
<td>5/5</td>
<td>35, 55, 56, 78, 89 (56)</td>
<td>0.009</td>
</tr>
<tr>
<td>KX-dw4</td>
<td>5/5</td>
<td>3, 6, 22, 26, 34 (22)</td>
<td>0.016</td>
</tr>
<tr>
<td>KX-dw7</td>
<td>5/5</td>
<td>27, 33, 40, 45, 73 (40)</td>
<td>0.043</td>
</tr>
<tr>
<td>KX-dw1</td>
<td>4/5</td>
<td>7, 12, 19, 25, 29 (19)</td>
<td>0.026</td>
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<td>13, 16, 16, 22, 44 (16)</td>
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<tr>
<td>KX-dw5</td>
<td>5/5</td>
<td>3, 5, 6, 8, 16 (6)</td>
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*Number of mice with metastases/total number of mice.
the inducibility of NOS II in tumor cells in vivo may not be predicted simply by its inducibility in tissue culture.

Presumably, tumor-associated NOS II activity is a result of NOS II expression in both tumor and host cells. NOS II expression in tumor cells may also contribute to cancer progression. In fact, NOS II expression inversely correlates with decreased cell proliferation and increased caspases in NOS II and without an intact NOS II gene. We found that the KX-dw cells and metastasis of these cells were compared in syngeneic mice with calcification analysis indicated significant NOS II expression only in tumor growth and metastasis. The NOS II contribution of NO from NOS II expression in tumor cells, several mechanisms of NOS II expression and NO production negatively regulate tumor growth and metastasis. To provide direct evidence of the net impact of host-derived NOS II expression on tumor growth and metastasis, a clear-cut model system must be considered. In the present study, to eliminate the contribution of NO from NOS II expression in tumor cells, several NOS II−/− tumor cell lines (KX-dw) were established. The growth and metastasis of these cells were compared in syngeneic mice with and without an intact NOS II gene. We found that the KX-dw cells grew much faster and produced many more experimental lung metastases in NOS II−/− C57BL/6 mice than in NOS II+/+ C57BL/6 mice. The increased tumor growth and metastasis were correlated with a lack of NOS II protein expression and enzyme activity in tumor tissue samples obtained from NOS II−/− mice. These data clearly demonstrated that host-derived NOS II expression and NO production negatively regulate tumor growth and metastasis.

Because macrophages are the main source of host-derived NO production, we sought to determine whether macrophages are actively involved in the process of host antitumor activity. In a previous report, NO II immunoreactivity was differentially distributed in tumor tissue samples from mice and occurred more frequently at the invasive edge (1). This pattern of NOS II distribution was consistent with the distribution patterns of tumor-infiltration macrophages. The present study demonstrated a similar pattern of macrophage infiltration in tumors from both NOS II−/− and NOS II+/+ C57BL/6 mice. Localization analysis indicated significant NOS II expression only in tumors from NOS II+/+ mice. In contrast, increased NOS II expression was correlated with decreased cell proliferation and increased apoptosis (data not shown). Our data additionally support the hypothesis that NOS II plays an important role in host immunosurveillance against tumor transformation (7).

In summary, using NOS II−/− tumor cell lines and NOS II+/− mice, we are the first to provide direct evidence showing that the physiological expression of NOS II in host cells negatively regulates tumor growth and metastasis. The NOS II−/− tumor cell lines may be useful in additional investigation of the as-yet undescribed mechanisms of NOS II expression and NO production in tumor-infiltration host cells, and to clearly understand the causal antitumor activity of NOS II to help design novel preventive and therapeutic approaches to controlling tumor growth and metastasis.

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References

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