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, KX-dw1, KX-dw4, and KX-dw7, 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−/− 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, 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 impacts of altered NO production may reflect the complexity of NO production and its pleiotropic nature of action, as well as the complexity of cancer metastasis (1, 6). Firstly, there are three distinct isoforms of NOS, all of which catalyze the conversion of L-arginine to the free radical NO (7, 8). The endothelial and neuronal NOS proteins are expressed constitutively and produce a trace amount of NO (low pSi for seconds to minutes) that mediates physiological functions such as neuronal transmission and vascular tone regulation (9, 10). In sharp contrast, the NOS II protein is primarily expressed in activated macrophages (7). Once stimulated, NOS II generates large amounts of NO throughout the life of the active enzyme (μM for hours to days). Presumably, differential expression of these isoforms may play very different roles in tumor growth and metastasis (1, 6).

Many experimental studies have indicated a direct or indirect influence on cancer metastasis by tumor-associated NOS II expression, and NO production using NOS II inhibitors and activators (11).

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).

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of the United States Department of Agriculture, United States Department of Health and Human Services, and NIH.

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 in plastic flasks and incubated in 5% CO2 at 37°C. The culture was free of endotoxins as determined by the limulus amebocyte lysate assay (sensitivity limit, 0.125 ng/ml), which was purchased from Associates of Cape Cod (Woods Hole, MA).

Cell Lines and Culture Conditions. The RAW 276.4 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, 1-glutamine, and vitamins (Flow Laboratories, Rockville, MD). The cell cultures were maintained in plastic flasks and incubated in 5% CO2 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 a mouse NOS II cDNA probe: one was constitutively expressed and two abnormal NOS II transcripts were detected in KX-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 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 KX-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...
represented the one fused between the Neo and partial NOS II transcripts (exons 14–19) under the control of the SV40 promoter, whereas the 5.1-kb transcript represented the one fused between the Neo and partial NOS II transcripts (exons 1–11 and 14–19) under the control of the NOS II promoter (16). We additionally confirmed this finding via Northern blot analysis using mRNA (Fig. 1B).

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 antiserum 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 were injected s.c. into syngeneic NOS II+/− (II+/−) and NOS II+/− (II+/−) C57BL/6 mice. We additionally confirmed this finding 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]L-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...
received 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, $5 \times 10^6$ KX-dw cells were injected into the lateral tail vein of both 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 ($P < 0.05$).

**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 isomor selective 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,
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 the ability of tumor cells to survive and produce metastases. This was demonstrated using the well-characterized K-1735 melanoma system (1), which clearly indicated that loss of NOS II expression in tumor cells correlates with a gain in metastatic potential. Apparently, the ultimate effect of tumor-associated NOS II activity on tumor growth and metastasis may be dictated by multiple sources and levels of NOS II expression. In general, macrophages have much higher levels of NOS II expression than tumor or other host cells do (7). Thus, host-derived NOS II expression may be the dominant source of tumor-associated NO production (11, 13, 14). However, whether this source has any positive or negative effects on tumor growth and metastasis was not established, which is very important when designing novel preventive and therapeutic approaches to controlling 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, NOS 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. Colocalization 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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