Influence of Nitric Oxide Synthase II Gene Disruption on Tumor Growth and Metastasis

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Abstract

The relationship between nitric oxide (NO) synthase II (NOS II) expression and the metastatic ability of tumor cells is inconclusive. We determined the role of host NOS II expression in the growth and metastasis of the B16-BL6 murine melanoma and M5076 murine ovarian sarcoma cell lines. The cells were either s.c. or i.v. injected into syngeneic C57BL/6 mice. Both cell lines produced slightly larger s.c. tumors in NOS II+/− mice than in NOS II−/− mice. However, B16-BL6 cells produced more and larger experimental lung metastases in NOS II+/− mice than in NOS II−/− mice, whereas M5076 cells produced fewer and smaller experimental lung metastases in NOS II+/− mice than in NOS II−/− mice. After activation with IFN-γ and lipopolysaccharide, macrophages isolated from NOS II+/− C57BL/6 mice produced NO-dependent cytotoxicity in sarcoma cells, whereas macrophages from NOS II−/− C57BL/6 mice did not. In contrast, activated macrophages produced little to NO-mediated cytotoxicity in melanoma cells. Immunostaining analyses indicated that NOS II expression was apparent in the metastases growing in NOS II+/− mice and correlated with increased cell proliferation in B16-BL6 lung metastases but with decreased cell proliferation in M5076 liver metastases. Our data suggest that disruption of host NOS II expression enhanced the growth and metastasis of NO-sensitive tumor cells but suppressed the metastasis of NO-resistant tumor cells, proposing that host-derived NO may differentially modulate tumor progression.

Introduction

NO is a potent biological molecule that mediates a diverse array of activities, including vasodilatation, neurotransmission, iron metabolism, and immune defense (1). Increasing evidence suggests that NO has a pleiotropic effect on diverse aspects of tumor biology. For example, NO is a potential endogenous carcinogen because it causes DNA damage (2, 3). Also, increased tumor-associated NO production may alter tumor blood supply by changing vascular tone and/or formation, thereby influencing tumor progression (4, 5). In addition, tumor-associated NO can be contributed by tumor and/or host cells (e.g., macrophages) that infiltrate tumors. NOS II expression and NO production within tumor cells can directly or indirectly influence the fate of the tumor cells themselves (3, 5, 6). For example, overproduction of endogenous NO is autotoxic through the induction of apoptosis (6) and suppresses tumor growth and metastasis, whereas low production of NO may protect tumor cells from apoptosis and promote tumor growth (5). Expression of NOS II in tumor cells has also been implicated as an important factor in cancer metastasis. Expression of the NOS II gene inversely correlates with the metastatic ability of human colon cancer (7) and K-1735 murine melanoma cells (8). Conversely, tumor-related NOS II activity correlates with more advanced human tumors of the breast (9) and central nervous system (10). In fact, NOS II expression directly correlates with the metastatic potential of UV-2237 murine fibrosarcoma cells (11). Transfection experiments have shown that overexpression of the NOS II gene inhibits metastasis of human renal cell carcinoma, K-1735 murine melanoma, and UV-2237 murine fibrosarcoma, in part by accelerating cell death (6, 11), whereas low expression of NOS II promotes the growth of human colon cancer cells, although it is not clear whether the metastatic ability of tumor cells is affected (5, 12). In summary, the effects of endogenous NO on tumor cells are output dependent and cell type specific (6), often depending on the p53 functional status of the tumor cells exposed to NO (3, 11, 13).

Quantitatively, the major source of tumor-associated NO may be host cells (e.g., macrophages) that infiltrate the tumors (1, 6, 14). It is known that both activated macrophages and endothelial cells may produce cytotoxic levels of NO in vitro. However, how this source of NO may influence metastasis is unclear. Macrophage- and endothelium-derived NO may prevent tumor growth and metastasis, presumably by killing tumor cells passing through vascular lumens (6). Alternatively, macrophages may promote tumor growth and metastasis by releasing NO, which is known to induce immune suppression, vasodilatation, and angiogenesis (1, 15). In the present study, we found that B16-BL6 mouse melanoma cells were resistant to cytotoxicity mediated by macrophages derived from both NOS II+/+ and NOS II−/− mice, whereas M5076 sarcoma cells were highly sensitive to NO-dependent cytotoxicity mediated by macrophages derived from NOS II−/− mice. Consistent with their differential sensitivity to NO-mediated cytotoxicity in vitro, B16-BL6 cells produced more and larger metastases in syngeneic NOS II+/+ mice than in NOS II−/− mice, whereas M5076 cells produced fewer and smaller metastases in syngeneic NOS II+/+ mice than in NOS II−/− mice. Therefore, physiological expression of host NOS II appears to negatively regulate the growth and metastasis of NO-sensitive tumor cells, whereas NO-resistant tumor cells may escape from or even usurp this physiological expression of host NOS II.

Materials and Methods

Reagents. Eagle’s MEM, HBSS, and fetal bovine serum were purchased from M. A. Bioproducts (Walkersville, MD). Mouse recombinant IFN-γ (specific activity, 1 × 10^7 units/mg protein) was purchased from Genzyme (Cambridge, MA). Phenol-extracted Salmonella LPS and AG were purchased from Sigma Chemical Co. (St. Louis, MO). [3H]Thymidine (specific activity, 2 Ci/mmol) was purchased from ICN Biomedicals, Inc. (Costa Mesa, CA). All reagents used in tissue cultures were free of endotoxins as determined using the Limulus amebocyte lysate assay (sensitivity limit, 0.125 ng/ml), which was purchased from Associates of Cape Cod (Woods Hole, MA).
Tumor Cell Lines and in Vitro Culture Conditions. The B16-BL6 murine melanoma and M5076 murine ovarian sarcoma cell lines were provided by Dr. Isaiah J. Fidler (The University of Texas M. D. Anderson Cancer Center). The original M5076 cell line was established in the laboratory of Dr. W. F. Dunning (Papanicolaou Cancer Research Institute, Miami, FL) and found to produce organ-specific metastases after i.v. injection into syngeneic C57BL/6 mice (16). The B16-BL6 line was established using in vitro selection and was shown to be highly invasive and to produce lung metastases after i.v. injection into syngeneic C57BL/6 mice (17). Both cell lines are reported to be nonimmunogenic (18, 19). All tumor cell lines were cultured in tissue culture in MEM supplemented with 10% fetal bovine serum, sodium pyruvate, non-essential amino acids, L-glutamine, and vitamins (CMEM; Flow Laboratories, Rockville, MD). Cell cultures were maintained in plastic flasks and incubated in 5% CO2–95% air at 37°C. Cultures were free of Mycoplasma.

Growth and Metastasis. To prepare tumor cells for inoculation, cells in exponential growth phase were harvested by brief exposure to a 0.25% trypsin/0.02% EDTA solution (w/v). Cell viability was determined by trypan blue exclusion, and only single-cell suspensions of >95% viability were used. To determine tumorigenic ability, tumor cells were injected s.c. into syngeneic NOS II+/+ or NOS II−/− C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME). Latency of tumor formation and tumor diameters were measured. To determine metastatic ability, 0.2 ml of the tumor cell suspensions was injected into the lateral tail veins of unanesthetized mice. The mice were killed 21 days after injection, and their pulmonary and hepatic metastatic nodules were counted using a dissecting microscope.

Determination of Nitrite Concentration. NO production was determined by measuring nitrite accumulation in culture supernatants using a microplate assay with Griess reagent, as described previously (11). In brief, 50-μl culture samples were harvested from conditioned medium and allowed to react with an equal volume of Griess reagent (1.0% sulfanilamide, 0.1% naphthylethylene diamine dihydrochloride, and 2.5% H₃PO₄) at room temperature for 10 min. The absorbance at 540 nm was monitored using a microplate reader. The nitrite concentration was determined using sodium nitrite as a standard.

Macrophage-mediated Cytotoxicity. Mouse peritoneal exudate macrophages were collected by peritoneal lavage from mice given an i.p. injection of 1.5 ml of thioglycollate broth (Baltimore Biological Laboratories, Cockeysville, MD) 3 days before harvesting (20). Purified cultures of mouse macrophages were incubated at 37°C for 18 h with 0.2 ml of medium alone or containing 10 units/ml IFN-γ and 0.01 μg/ml LPS. After incubation, the macrophage cultures were thoroughly washed, and 1 × 10⁴ [3H]thymidine-labeled B16-BL6 and M5076 target cells were added to achieve a population density of 2500 macrophages and 250 tumor cells/mm². At this population density, untreated macrophages were not cytotoxic to tumor cells (20). After a 48-h incubation, the cultures were washed twice with PBS, and adherent viable cells were lysed with 0.1 ml of 0.1 N NaOH. The lysates were harvested using a Harvester 96 (Tomtec, Orange, CT) and counted using a liquid scintillation counter. Maximal in vitro macrophage-mediated cytotoxicity in this assay was obtained after 48 h of incubation with target cells, assessed by measuring the release of radioactivity from DNA of target cells as described previously (20), and calculated as follows: cytotoxicity (%) = [(A – B)/A] × 100, where A is the cpm in cultures of control macrophages and target cells and B is the cpm in cultures of test macrophages and target cells.

Cytotoxicity Mediated by Tumor Cells Producing NO. C4.8L cells that were stably transfected with a full-length NOS II gene and constitutively produced NO or C4.S2 cells that were stably transfected with a truncate NOS II gene and did not produce NO (6) were plated into 96-well plates (2.5 × 10³ well) and incubated for 18 h in medium alone or with 2 μM AG (a specific NOS II inhibitor). [3H]Thymidine-labeled B16-BL6 or M5076 target cells (1 × 10⁴) were added in the absence or presence of 2 μM AG. After a 48-h incubation, cytotoxicity against B16-BL6 and M5076 cells was determined as described above.

Immunohistochemistry. Tissue sections (5-μm thick) of formalin-fixed, paraffin-embedded lung and liver specimens were processed using a standard procedure. Sections were stained for infiltration macrophages (F4/80 antibody; Ref. 21), NOS II (21), and PCNA (22). The sections were also stained for apoptotic cells using a terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (23). A positive reaction was indicated by a reddish-brown precipitate in the cytoplasm (F4/80 and NOS II) or in the nucleus (PCNA and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling).

Statistical Analyses. The in vitro data were analyzed for significance using Student’s t test (two-tailed).

Results and Discussion

NO is a pleiotropic molecule; therefore, it is not surprising that tumor-associated NO may have diverse effects on tumor progression (1–6). The apparently opposing roles of NO have been attributed to many factors, including NOS isoforms, expression levels, measurement methods, and heterogeneous tumor tissues or cell lines (6). Moreover, tumor-associated NO represents a mixture of both tumor and host infiltration cell-derived NO activities (1, 6, 9, 13). The functional NOS II status of tumor-associated macrophages or other infiltration cells may differ from tumor to tumor, and different tumor cells apparently express different levels of NOS II as well (6). Therefore, 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 produce much higher levels of NOS II expression than do tumor cells or other host cells and thus become a dominant source of NO production (1, 6, 9, 11). To determine the role of host NOS II in tumor growth and metastasis, we used the well-characterized B16-BL6 melanoma and M5076 sarcoma cell lines, which are syngeneic to C57BL/6 mice (16, 17).

In the first set of experiments, we measured the growth and metastasis of both B16-BL6 melanoma and M5076 sarcoma cells in syngeneic NOS II+/+ and NOS II−/−C57BL/6 mice. B16-BL6 or M5076 cells (5 × 10³ cells/mouse) were injected s.c. into the mice. The average latency period for B16-BL6 and M5076 cells was 9 and 12 days, respectively, in NOS II+/+ C57BL/6 mice but 7 and 10 days, respectively, in NOS II−/− C57BL/6 mice. Consistent with the slightly earlier tumor onset in NOS II−/− C57BL/6 mice, the average...
tumor size was also slightly larger in NOS II-/- C57BL/6 mice than in NOS II+/- C57BL/6 mice (Table 1). However, the difference in both tumor latency and tumor size was not statistically significant.

To determine the extent of experimental lung metastasis, B16-BL6 cells (5.0 x 10^4 cells/mouse) and M5076 cells (5.0 x 10^3 cells/mouse) were injected i.v. into syngeneic NOS II+/- and NOS II-/- C57BL/6 mice. As shown in Table 1, B16-BL6 cells produced fewer and smaller experimental lung metastases in NOS II-/- C57BL/6 mice than in NOS II+/- C57BL/6 mice. In sharp contrast, M5076 cells produced more and larger experimental liver metastases in NOS II-/- C57BL/6 mice than in NOS II+/- C57BL/6 mice. Therefore, we found that disruption of the host NOS II gene differentially affected the growth and metastasis of B16-BL6 and M5076 cells.

Because host macrophages may be the major source of host cell-derived NOS II activity in the tumor (1, 6, 9), we sought to determine the role of macrophage NOS II disruption in altered tumor growth and metastasis. Early work suggested that NO derived from macrophages produced cytosostasis in target tumor cells (14). Recent studies have shown that overactivated macrophages can also lead to NO-dependent cytosis of B16-F10 murine melanoma cells (20). Additionally, in...
cell coculture systems, macrophage-derived NO has induced apoptosis in P815 cells (24) and Meth A tumor cells (25). Similarly, activated microvessel endothelial cells can also produce NO-dependent lysis of tumor cells (26). This has been further supported by results of several recent studies. For example, LPS/cytokine-activated endothelial cells were shown to express NOS II and produce NO, which lead to thymocyte apoptosis (27). Similarly, human erythroleukemic K562 cells were shown to undergo apoptosis after coculture with rodent vascular smooth muscle cells or endothelial cells expressing NOS II and producing NO in the presence of IFN-γ and tumor necrosis factor-α (28). Indeed, increased production of NO by murine, rat, and human NK cells has been shown to be responsible, at least in part, for destruction of target cells by NK cells and NK cell-mediated DNA fragmentation and cell lysis (6).

In the present study, the tumoricidal activity of macrophages obtained from the peritoneal cavity of NOS II+/+ and NOS II−/− C57BL/6 mice was determined. Purified macrophages obtained from NOS II+/+ and NOS II−/− mice were pretreated with 10 units/ml IFN-γ and 0.01 μg/ml LPS for 18 h, as reported previously (20). The [3H]thymidine-labeled melanoma and sarcoma cells were then added and incubated for another 48 h in the presence or absence of 2 mM AG. Cytotoxicity was determined by measuring [3H]thymidine release. The treatment induced NO production in NOS II+/+ macrophages, which was inhibited by AG, whereas it did not induce NO production in NOS II−/− macrophages (Fig. 1A). The preactivated NOS II+/+ macrophages induced significant cytotoxicity in M5076 sarcoma cells (Fig. 1B) but not in B16-BL6 melanoma cells (Fig. 1C), whereas macrophages without activation did not (Fig. 1A). The cytotoxicity of M5076 cells was associated with the induction of NO (Fig. 1B). Both NO production and cytotoxicity were totally inhibited by the addition of the specific NOS II inhibitor AG, suggesting that activated macrophages mediated NO-dependent cytotoxicity. In sharp contrast, the same treatment did not induce NO production and cytotoxicity by NOS II−/− macrophages (Fig. 1, B and C). Similar results were obtained using macrophages isolated from lung and liver tissues (data not shown). These data clearly indicate that disruption of NOS II impairs the antitumor ability of macrophages, which may in part be responsible for the enhanced growth and metastasis of M5076 sar-

Fig. 3. Immunohistochemistry. Both B16-BL6 and M5076 cells (5 × 10^4 cells/mouse) were injected i.v. into NOS II+/+ (A–C and G–I) or NOS II−/− (D–F and J–L) C57BL/6 mice. Lungs and livers were harvested and processed for staining for macrophages (A and D), NOS II (B, E, G, and J), PCNA (C, F, H, and K), and apoptotic cells (I and L). Arrowheads, tumor lesions.
comata cells and conversely for the suppressed metastasis of B16-BL6 melanoma cells. Whether NOS II disruption also affects the functions of other effector cells (e.g., vascular endothelial and NK cells) remains to be determined.

To further confirm the different sensitivity of tumor cell lines to NO-mediated cytotoxicity, both [3H]thymidine-labeled B16-BL6 and M5076 cells were coincubated for 48 h in the presence or absence of 2 mM AG with C4.1.L8 cells that were stably transfected with a full-length NOS II gene and constitutively produced NO or C4.52 cells that were stably transfected with a truncate NO II gene and did not produce NO (Fig. 2A; Ref. 6). Cytotoxicity was determined as described above. As shown in Fig. 2B, significant cytotoxicity was produced in M5076 cells and was completely inhibited by AG, whereas no cytotoxicity was produced in B16-BL6 cells. Furthermore, both B16-BL6 and M5076 cells were incubated for 24 h with different concentrations of a NO donor, SNAP. M5076 cells were highly sensitive to SNAP, whereas B16-BL6 were not (Fig. 2C). Similar results were also obtained using other NO donors (e.g., sodium nitroprusside, GEA5024, and NOC-12; data not shown). These data clearly show that these cell lines have a different intrinsic sensitivity to NO-mediated cytotoxicity. Whether the cellular functional status, such as that of the p53 tumor suppressor gene, plays a role is currently under investigation.

To provide evidence that host NOS II expression differentially affect the fate of injected tumor cells, immunostaining was performed on tumor sections. Macrophage infiltration was apparent in the B16-BL6 lung metastases in NOS II and and NOS II and NOS II mice (Fig. 3, A and B). NOS II expression was only detected in B16-BL6 lung metastases and M5076 liver metastases in NOS II mice (Fig. 3, B and G) but not in NOS II mice (Fig. 3, E and J), suggesting that NOS II was mainly expressed in the host infiltration cells. The decreased NOS II expression was correlated with decreased cell proliferation in B16-BL6 lung metastases (Fig. 3, F versus C). In contrast, decreased NOS II expression was correlated with increased cell proliferation (Fig. 3, K versus H) and decreased apoptosis (Fig. 3, I versus L) in M5076 liver metastases. These data suggested that host NOS II expression differentially affects the survival and/or proliferation of tumor cells. It is likely that NOS II disruption and then decreased physiological level of NO production lead to decreased blood supply to the growing metastases of B16-BL6 cells resistant to NO-mediated cytotoxicity (3, 5). To that end, we are currently investigating the effect of NOS II disruption on B16-BL6 melanoma angiogenesis and the role of organ-specific tumor-host interaction in NOS II expression and tumor cell survival.

In summary, we found that disruption of the host NOS II gene differentially affects the metastasis of tumor cells, depending on their sensitivity to NO-mediated cytotoxicity. NOS II disruption enhanced the metastasis of NO-sensitive M5076 mouse ovarian sarcoma cells, whereas it suppressed the metastasis of NO-resistant B16-BL6 murine melanoma cells. These data suggest that physiological expression of the host NOS II gene may differentially modulate tumor progression (3, 5, 6). However, it remains to be determined whether disruption of host NOS II gene expression influences tumor growth and metastasis by other mechanisms in addition to impaired production of cytotoxic effectors, such as angiogenesis (3–6). Collectively, we have demonstrated that host NOS II gene expression regulates tumor growth and metastasis, suggesting that the modulation of host NOS II gene expression can be manipulated to control tumor growth and metastasis.

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