Direct Correlation between Nitric Oxide Synthase II Inducibility and Metastatic Ability of UV-2237 Murine Fibrosarcoma Cells Carrying Mutant p53

Qian Shi, Suyun Huang, Weidong Jiang, Laura S. Kutach, Honnavara N. Ananthaswamy, and Keping Xie

Abstract

The relationship between nitric oxide synthase II (NOS II) inducibility and the metastatic ability of UV-2237 murine fibrosarcoma cells was determined. Highly metastatic cells survived to produce numerous lung metastases after i.v. injection in syngeneic C3H/HeN mice, whereas poorly metastatic cells did not. Highly metastatic clones exhibited higher levels of NOS II than did poorly metastatic clones in response to interleukin 1α and IFN-γ stimulation. Furthermore, both poorly and highly metastatic clones contained an identical p53 mutation. Overexpression of NOS II in a highly metastatic clone by transfection with NOS II gene retarded tumor growth and completely suppressed metastasis. Our data indicate that a low to moderate level of NOS II expression directly correlates with metastatic ability of UV-2237 fibrosarcoma cells carrying mutant p53 and that a high level of nitric oxide production suppresses tumor growth and metastasis.

Introduction

Nitric oxide (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 multitude of effects on many aspects of tumor biology. NO causes DNA damage and is a potential endogenous carcinogen (2, 3). Increased NO production may increase angiogenesis and contribute to tumor progression (4). However, the effects of NO on tumor cells are apparently output dependent and cell type specific (5). Overproduction of NO is cytotoxic, induces apoptosis (5, 6), and suppresses tumor growth, whereas a low output of NO may protect cells from apoptosis and promote tumor growth (5). Expression of NOS II has also been implicated in the process of cancer metastasis. The expression of the NOS II gene inversely correlates with metastatic ability in human colon cancer (7) and K-1735 murine melanoma cells (8). However, NOS II activity correlates with more advanced human tumors of the colon cancer (7) and K-1735 murine melanoma (8). However, a low output of NO may protect cells from apoptosis and promote tumor growth (5). Expression of NOS II has also been implicated in the process of cancer metastasis. The expression of the NOS II gene inversely correlates with metastatic ability in human colon cancer (7) and K-1735 murine melanoma cells (8). However, NOS II activity correlates with more advanced human tumors of the breast (9) and central nervous system (10). Transfection experiments have shown that overexpression of the NOS II gene inhibits metastasis of human renal cell carcinoma and mouse melanoma in part by accelerating cell death (5), whereas NOS II transfection promotes the growth of human colon cancer cells, although it is not clear whether metastatic ability is affected (11). The apparent discrepancy may be due to the use of different cell lines and different levels of NO production. Recent data suggest that p53 functional status can influence the fate of tumor cells exposed to both endogenous and exoge nous NO (3, 12). Consistent with this hypothesis, wild-type p53 has been detected in K-1735 murine melanoma, and NO causes accumulation of wild-type p53 protein and, hence, induces apoptosis (5, 12). However, it remains to be determined whether there is any relationship between NO production and the metastatic ability of tumor cells carrying mutant p53 (13). Here, we found that UV-2237 mouse fibrosarcoma cells contained a mutation in the p53 gene, and in response to IFN-γ and IL-1α stimulation, highly metastatic clones expressed higher levels of NOS II activity than did poorly metastatic clones. However, transfection of metastatic cells with the NOS II gene suppressed tumor growth and completely inhibited metastasis. Therefore, NOS II expression may contribute to the progression of tumor cells containing p53 mutations, whereas sustained NOS II overexpression still suppresses tumor growth and metastasis.

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 × 10⁷ units/mg protein) and recombinant mouse IL-1α were purchased from Genzyme (Cambridge, MA). NMA was purchased from Sigma Chemical Co. (St. Louis, MO). [3H]dThd (specific activity, 2 Ci/mmol) was purchased from ICN Biomedicals, Inc. (Costa Mesa, CA). [32P]dATP (specific activity, 2 Ci/mmol) was purchased from New England Nuclear Corp. (Boston, MA). All reagents used in tissue culture were free of endotoxin, as determined by the Limulus amebocyte lysate assay (sensitivity limit, 0.125 ng/ml), purchased from Associates of Cape Cod (Woods Hole, MA).

UV-2237 Fibrosarcoma Clones in Vitro Culture Conditions. The original UV-2237 fibrosarcoma cells induced in a C3H/HeN mouse by exposure to UV light were provided by Dr. Margaret L. Kripke (The University of Texas M. D. Anderson Cancer Center, Houston, TX). The parental tumor was cloned in vitro by a double-dilution method (14). Of the large number of clones thus isolated, clones 15 and 38 (designated C-15 and C-38) were classified as nonmetastatic or poorly metastatic (14). Clones 25 and 39 (C-25 and C-39) were highly metastatic and produced metastases in the lungs of syngeneic C3H/HeN mice. UV-2237 M was derived from a solitary experimental lung metastasis produced by the UV-2237 parental line (UV-2237 P) after i.v. injection (14) and was shown to be highly metastatic in syngeneic recipients (15). All tumor cell lines were cultured in tissue culture in MEM supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, L-glutamine, and 2-fold vitamin solution (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 (15, 16).

Growth and Metastasis. To prepare tumor cells for inoculation, we harvested cells in exponential growth phase by brief exposure to 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 (1 × 10⁶ cells/mouse) were injected s.c. into syngeneic C3H/HeN mice. Tumor weight was measured on day 35. To determine metastatic ability, 0.2 ml of tumor cell suspensions (1 × 10⁶ cells/mouse) was injected into the lateral tail veins of unanesthetized mice. The mice were killed 35 days after injection, and the lung nodules were counted with the aid of a dissecting microscope.

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1 Supported in part by Cancer Center Support Core Grant CA 16672 from the National Cancer Institute, NIH, and the University Startup Fund (to K. X.) and by NIH Grant CA-46523 (to H. N. A.). Q. S. is supported by the Smith Education Fund of The University of Texas M. D. Anderson Cancer Center, Houston, TX. 105 cells/mouse) were injected into the lateral tail veins of unanesthetized mice. The mice were killed 35 days after injection, and the lung nodules were counted with the aid of a dissecting microscope.

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Distribution and Fate of $[^{25}]$IdUrd-labeled Cells after i.v. Injection. In vitro labeling of tumor cells with $[^{25}]$IdUrd was carried out as described (16). Labeled cells from poorly metastatic or nonmetastatic P, C-15, and C-38 lines and highly metastatic M, C-25, and C-39 lines were injected i.v. into unanesthetized mice at a dose of $1 \times 10^7$ cells/mouse. Distribution and survival of labeled tumor cells in vivo were determined according to a published procedure (16).

Determination of NOS Activity and Nitrite Concentration. The NOS activity in vitro was measured and expressed in nmol per mg of protein as described previously (8). NO production was determined by measuring nitrite accumulation in culture supernatants by a microplate assay using Griess reagents as described previously (8).

In Vitro Cytotoxicity. Cytokine-mediated tumor cell lysis was measured by the release of radioactivity from $[^{3}]$HIdUrd-labeled UV-2237 cells. The labeled target cells were plated into 96-well plates at $4 \times 10^5$ cells per well and cultured in medium containing cytokines for 96 h. The cytotoxic activity of the cytokines was calculated as follows: cytotoxicity (%) = (A - B)/A $\times$ 100, where A is the cpm in target cells incubated with medium and B is the cpm in target cells incubated with cytokines.

NOS II Retrovirus and Infection. The full-length murine NOS II gene from pNOS-L8 (17) was subcloned into plXSN vector to generate plXSN-NOS II. Retrovirus was generated as reported previously (18). The metastatic UV-2237 C-39 cells were infected with plXSN-NOS II, and cells infected with plXSN were used as controls as described previously (18). Tumor cells were cultured in medium containing 3 mm NMA to prevent NO-mediated cytotoxicity. After selection with neomycin (G418), individual clones were collected and expression of NOS II was verified.

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

Results and Discussion

NO is a pleiotropic molecule, and so it is not surprising that tumor-derived NO may have multiple effects on metastasis (5). The apparently opposing roles of NO suggest that NO effects are very complex. The inconsistencies in reported tumor-associated NO activities may be partially due to the use of inconsistent methods to assess NOS expression and to the use of heterogeneous tumor tissues or cell lines (5). To understand the relationship between NOS expression and metastatic ability, it is crucial to use a clonal tumor population, given that metastatic cells are of clonal origin (19). In our study, we used the UV-2237 fibrosarcoma, which was originally induced in a C3H/HeN mouse by exposure to UV light. The parental tumor was cloned in vitro by a double-dilution method (14). Of the large number of clones thus isolated, C-15 and C-38 were classified as nonmetastatic or poorly metastatic, whereas C-25 and C-39 were classified as highly metastatic. UV-2237 M cell line was derived from a solitary experimental lung metastasis produced by UV-2237 P cells after i.v. injection into a mouse (15). The data regarding metastatic ability of these lines are summarized in Table 1. In close agreement with previous reports (14, 15), P, C-15, and C-38 cells produced no or few lung metastases, whereas M, C-25, and C-39 cells produced many lung metastases.

To determine whether the NOS II is differentially expressed in UV-2237 fibrosarcoma clones, the poorly and highly metastatic tumor clones were incubated for 48 h with 100 units/ml IFN-γ and 10 ng/ml IL-1α, the combination of which provides optimal conditions for NOS II induction (5, 8). Supernatant was collected for nitrite measurement using Griess reagents, and cytosolic protein was extracted for determination of NOS activity. Incubation of tumor cells with the combination of IFN-γ and IL-1α induced higher levels of NOS II activity and NO production in the highly metastatic M, C-25, and C-39 cells than in the poorly metastatic P, C-15, and C-38 cells (Table 1), suggesting that NOS II inducibility directly correlates with increased metastatic ability of UV-2237 murine fibrosarcoma cells.

The above data seemingly disagree with a previous report showing that NOS II activity inversely correlates with metastatic ability in the K-1735 murine melanoma cell lines (8). The apparent discrepancy may be due to the use of different cell lines and/or to different levels of NO induction. Recent data suggest that p53 mutation may also influence the fate of tumor cells exposed to endogenous NO (3). NO has been shown to cause DNA damage and induce wild-type p53 accumulation, which can lead to growth arrest and apoptosis (3, 5). To determine the involvement of p53 in the regulation of the metastatic ability by NO in UV-2237 tumor cells, p53 mutation status was determined as described before (20). Identical mutations in p53 were detected in all UV-2237 clones (data not shown). The p53 mutation was located at codon 270 of exon 8 with nucleotide change from CGT to TGT and, hence, predicted an amino acid change from arginine to cystidine. These data suggested that tumor cells containing p53 mutations may be resistant to NO-mediated cytotoxicity and may even usurp the increased NO production for growth and metastasis (13), although NO-mediated and p53-dependent apoptosis has been reported in many cell lines (3, 5).

To test the hypothesis that p53 mutation leads to NO resistance, endogenous NO-mediated cytotoxicity in the UV-2237 tumor cells was determined. The $[^{3}]$HIdUrd-labeled tumor cells were incubated with 100 units/ml IFN-γ and 10 ng/ml IL-1α for 48 h, and cytotoxicity was determined by measuring $[^{3}]$HIdUrd release. The treatment induced significant cytotoxicity in all clones. The cytotoxicity was totally inhibited by addition of the specific NOS II inhibitor NMA (Table 2). NO-mediated cytotoxicity correlated with NOS II activity and NO production (Table 1), suggesting that NO induced cytotoxicity in p53-mutant UV-2237 cells. Therefore, this finding was consistent with the hypothesis that NO mediates both p53-dependent and -independent cell death (6).

A previous study also showed that increased NO production inversely correlated with tumor cell survival in the circulation (8). We,

<table>
<thead>
<tr>
<th>Clone</th>
<th>NOS II expression</th>
<th>Experimental lung metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV-2237 P</td>
<td>3 ± 0.5</td>
<td>2/10</td>
</tr>
<tr>
<td>UV-2237 M</td>
<td>9 ± 1.0</td>
<td>10/10</td>
</tr>
<tr>
<td>UV-2237 G-15</td>
<td>12 ± 3.1</td>
<td>9/10</td>
</tr>
<tr>
<td>UV-2237 C-25</td>
<td>15 ± 3.2</td>
<td>0/10</td>
</tr>
<tr>
<td>UV-2237 C-38</td>
<td>3 ± 1.1</td>
<td>10/10</td>
</tr>
<tr>
<td>UV-2237 C-39</td>
<td>15 ± 1.0</td>
<td>10/10</td>
</tr>
</tbody>
</table>

* Low metastatic clones.

** Highly metastatic clones.

p < 0.05.

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Table 1 Direct correlation between expression of NOS II activity and production of experimental lung metastasis by the UV-2237 fibrosarcoma

Cells ($1 \times 10^7$) were seeded into 100-mm dishes in triplicate and treated with 100 units/ml IFN-γ and 10 units/ml IL-1α for 48 h. NOS II activity (nmol/mg protein) of cytosolic proteins was determined. The $[^{3}]$HIdUrd-labeled tumor cells were incubated with 100 units/ml IFN-γ and 10 ng/ml IL-1α for 48 h, and cytotoxicity was determined by measuring $[^{3}]$HIdUrd release. The treatment induced significant cytotoxicity in all clones. The cytotoxicity was totally inhibited by addition of the specific NOS inhibitor NMA (Table 2). NO-mediated cytotoxicity correlated with NOS II activity and NO production (Table 1), suggesting that NO induced cytotoxicity in p53-mutant UV-2237 cells. Therefore, this finding was consistent with the hypothesis that NO mediates both p53-dependent and -independent cell death (6).
therefore, determined the organ distribution and fate of $^{125}$IIdUrd-labeled poorly metastatic P, C-15, and C-38 and highly metastatic M, C-25, and C-39 cells at 10 min and 3 days after i.v. injection. There were no discernible differences in initial arrest for the poorly metastatic and highly metastatic clones in the lungs 10 min after injection (Table 2). The percentage of viable metastatic cells in the lung was <0.1% by day 3, whereas >3% of metastatic cells still survived in the lung 3 days after injection, suggesting that highly metastatic cells survive better than low metastatic cells in the circulation, regardless of NO inducibility and NO-mediated cytotoxicity. This raises the possibility that low NOS II expression may have been insufficient to affect cell survival in vivo. To test this hypothesis, highly metastatic C-39 (C-39 P) cells were infected with NOS II retrovirus (pLXSN-NOS II). Without stimulation, NOS II-infected cells produced NO and marginal cell death in vitro (Fig. 1, A and B). However, cell death of two NOS II-infected C-39 cell clones (C-39-NOS.1 and C-39-NOS.2) in vivo was clearly higher than that in C-39 P and control pLXSN-infected C-39 cells (C-39-LX; data not shown). Consistently, NOS II-infected cells grew somewhat slowly (Fig. 1C) and did not produce metastases in syngeneic mice (Fig. 1D). These data showed that NOS II gene transfer suppresses tumor growth and metastasis of tumor cells harboring the p53 mutation.

Obviously, some discrepancy exists between the effects of endogenous NOS II expression and recombinant NOS II transfer on tumor cells in vitro (cytotoxicity) and in vivo (metastasis). Metastatic cells engineered to release NO produced marginal cytotoxicity in vitro (Fig. 1, A and B), and those cells were nonmetastatic in syngeneic mice (Fig. 1D). In contrast, although cytokines increased cytotoxicity by a similar level of NO production, those cells were highly metastatic in syngeneic mice (Table 1). In fact, more cytotoxicity was induced in highly metastatic cells than in poorly metastatic cells (Table 2). These data suggested that the in vitro condition (cytokine treatment) for increasing NO production and cytotoxicity in highly metastatic cells might not exist in vivo, whereas the in vivo condition (constitutive NO production after gene transfer) for increasing cell destruction and suppressing metastasis might not lead to significant NO-mediated cytotoxicity in vitro. Therefore, it is possible that the level of NO induced, presumably by cytokines in vivo, may be very low and nontoxic and, more likely, may facilitate survival and metastasis of tumor cells. However, constitutive NO production after gene transfer may not be high enough to produce significant cytotoxicity in vitro but may be high enough to cause cell destruction in the circulation and suppression of metastasis in vivo, suggesting that NO reduces metastatic ability in other ways besides NO-mediated cytotoxicity. For instance, excessive NO production may render circulating tumor cells more fragile as they pass through the narrow lumen of the capillaries because increased NO production eliminates cell deformability [5].

In summary, we found a direct correlation between NO inducibility in vitro and the ability of circulating tumor cells to survive and metastasize in vivo, suggesting that NO synthesis may contribute to the high metastatic ability of tumor cells expressing mutant p53. Possible mechanisms may include increased tumor angiogenesis [21, 22]. It remains to be proved whether NO induction leads to increased survival and metastasis of tumor cells. However, NOS II gene transfer can increase cell death and metastasis suppression in vivo without inducing significant NO-mediated cytotoxicity in vitro. Because excessive NO production suppresses metastasis regardless of p53 status.
(8. present study), we believe that the manipulation of NO production can be used to control cancer metastasis.

Acknowledgments

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