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

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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

NO3 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, NO 3 is a potent biological molecule that mediates a diverse array of

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 × 105 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). [125I]IdUrd (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 and 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% trypan blue exclusion, and only single-cell suspensions of >95% viability were used. To determine tumorigenic ability, tumor cells (1 × 105 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 × 105 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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2 The abbreviations used are: NO, nitric oxide; NOS II, NO synthase II; IL-1α, interleukin 1α; NMA, Nω-monomethyl-l-arginine; dThd, thymidine; IdUrd, 5-[(125)I]Iodo-2′-deoxyuridine.

3 The abbreviations used are: NO, nitric oxide; NOS II, NO synthase II; IL-1α, interleukin 1α; NMA, Nω-monomethyl-l-arginine; dThd, thymidine; IdUrd, 5-[(125)I]Iodo-2′-deoxyuridine.
Distribution and Fate of \[^{[2]}\text{H}\text{dUrd}\]labeled Cells after i.v. Injection. In vitro labeling of tumor cells with \[^{[2]}\text{H}\text{dUrd}\]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 anesthetized mice at a dose of \(1 \times 10^5\) cells/mouse. Distribution and survival of labeled tumor cells in vivo were determined according to a published procedure (16).

In Vitro Cytotoxicity 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). NO production was determined by measuring nitrite concentration using Griess reagents, and cytosolic protein was extracted for determination of NOS activity. Incubation of tumor cells with the combination of IFN-\(\gamma\) and IL-1\(\alpha\) 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 correlated 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 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 cysteine. 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 \[^{[3]}\text{H}\text{dThd}\]labeled tumor cells were incubated with 100 units/ml IFN-\(\gamma\) and 10 ng/ml IL-1\(\alpha\) for 48 h, and cytotoxicity was determined by measuring \[^{[3]}\text{H}\text{dThd}\]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 p53-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>NO expression ((\mu)M)</th>
<th>Experimental lung metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV-2237 P(^b)</td>
<td>3 ± 0.5</td>
<td>7 ± 0.5</td>
</tr>
<tr>
<td>UV-2237 M(^a)</td>
<td>9 ± 2.0(^c)</td>
<td>18 ± 1.2(^d)</td>
</tr>
<tr>
<td>UV-2237 G-15(^a)</td>
<td>3 ± 0.8</td>
<td>9 ± 1.0</td>
</tr>
<tr>
<td>UV-2237 C-25(^a)</td>
<td>12 ± 3.1(^e)</td>
<td>23 ± 2.3</td>
</tr>
<tr>
<td>UV-2237 C-38(^a)</td>
<td>3 ± 1.1</td>
<td>6 ± 0.8</td>
</tr>
<tr>
<td>UV-2237 C-39(^a)</td>
<td>15 ± 3.2(^e)</td>
<td>26 ± 3.5(^e)</td>
</tr>
</tbody>
</table>

\(^a\) Low metastatic clones.
\(^b\) Highly metastatic clones.
\(^c\) \(p < 0.05\).
Table 2  NO-mediated cytotoxicity in vitro and survival in vivo of the highly metastatic and poorly metastatic UV-2237 fibrosarcoma cells

<table>
<thead>
<tr>
<th>Clone</th>
<th>Cytokines</th>
<th>Cytokines/NMA</th>
<th>Survival in vivo (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV-2237 P</td>
<td>11 ± 2</td>
<td>&lt;5</td>
<td>89 ± 4 &lt;0.1</td>
</tr>
<tr>
<td>UV-2237 M</td>
<td>22 ± 3*</td>
<td>&lt;5</td>
<td>88 ± 7 5 ± 1*</td>
</tr>
<tr>
<td>UV-2237 C-15</td>
<td>9 ± 1</td>
<td>&lt;5</td>
<td>92 ± 3 &lt;0.1</td>
</tr>
<tr>
<td>UV-2237 C-25</td>
<td>16 ± 4*</td>
<td>&lt;5</td>
<td>90 ± 4 3 ± 2*</td>
</tr>
<tr>
<td>UV-2237 C-38</td>
<td>12 ± 5</td>
<td>&lt;5</td>
<td>83 ± 7 &lt;0.1</td>
</tr>
<tr>
<td>UV-2237 C-39</td>
<td>33 ± 4*</td>
<td>&lt;5</td>
<td>94 ± 3 7 ± 2*</td>
</tr>
</tbody>
</table>

* P < 0.05.

Therefore, determined the organ distribution and fate of [125I]IdUrd-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 nonmetastatic 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 vitro. 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.

Fig. 1. Tumorigenicity and metastasis of NOS II-infected C-39 cells. A, NOS II expression. C-39 P, C-39-LX, C-39-NOS.1, and C-39-NOS.2 tumor cells were cultured in medium with (●) or without (□) 2 mM NMA. After 48 h, the amount of nitrite in the culture supernatants was determined. Cellular mRNA was isolated. Samples (2 μg) were separated on 1% agarose gel, transferred to a GeneScreen Plus membrane, and probed with 32P-labeled NOS II or GAPDH cDNA (inset). B, NO-mediated cytotoxicity. [3H]IdUrd-labeled C-39 P, C-39-LX, C-39-NOS.1, and C-39-NOS.2 cells in 96-well plates were incubated with medium in the absence (□) or presence (●) of 2 mM NMA. Cytotoxicity was determined 48 h later. C, tumorigenicity. D, metastasis. C-39 P, C-39-LX, C-39-NOS.1, and C-39-NOS.2 cells were injected s.c. or i.v. into four groups of nude mice (five mice per group). Tumor weight and number of lung metastases were determined 35 days after tumor injection. Columns, means from one representative experiment of two; bars, SD. *, P < 0.05; **, P < 0.01.
(8, present study), we believe that the manipulation of NO production can be used to control cancer metastasis.

Acknowledgments

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References

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