**In Vivo** Cisplatin-exposed Macrophages Increase Immunostimulant-induced Nitric Oxide Synthesis for Tumor Cell Killing

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Abstract

Mice pre-exposed to cisplatin increased their production of nitric oxide (NO) when treated with lipopolysaccharide (LPS). Peritoneal macrophages, isolated from mice 11 days after cisplatin treatment and cultured with LPS plus IFN-γ, increased NO production, whereas the macrophages isolated 2 days after cisplatin treatment decreased it. In both cases, NO was not produced without immunostimulant(s). Northern and Western Blot analysis showed that macrophages exposed to cisplatin for 11 days increased production of mRNA and protein expression of inducible nitric oxide synthase (iNOS). This result indicated that macrophages became more sensitive to LPS and IFN-γ when they were exposed to cisplatin in vivo. Peritoneal macrophages, when activated with LPS plus IFN-γ and then cocultured with several tumor cells, exhibited cytotoxic activity against both cisplatin-sensitive and cisplatin-resistant tumor cells. There was no difference in cytotoxicity between the paired cells. Under the same experimental condition, macrophages that were exposed to cisplatin for 11 days had significantly increased their cytotoxicity to the tumor cells. This cytotoxic activity was inhibited by the NOS inhibitor Nω-nitro-l-arginine, indicating that NO is a major effector for macrophage-mediated tumor cell killing. Treatment of tumor cells with S-nitroso-N-acetylpenicillamine, a NO-generating compound, showed the similar tumoricidal effect. These data demonstrated that injection of cisplatin into the mice can enhance the sensitivity of macrophages to the subsequent treatment of immunostimulant(s) for effective tumor cell killing through enhanced NO production.

Introduction

* cis-Dichlorodiamine platinum II (cisplatin), a widely used anticancer drug, activates the murine macrophages and natural killer cells to a tumoricidal state and, thus, functions as a potent biological response modifier (1, 2). Macrophages, one of the major effector cells with tumoricidal activity, produce a large amount of nitric oxide (NO) by cytosolic, NADPH-dependent inducible NO synthase (iNOS) from the terminal guanido nitrogen atom(s) of l-arginine after activation by cytokines and/or LPS (3). NO produced by activated macrophages is responsible for cytostatic or cytolytic activity for tumor cells in vivo and in vitro (4, 5). Many recent works have shown that NO, a small, free radical molecule, causes several metabolic alterations because of its high reactivity with iron- or thiol-containing biological molecules and, thus, inhibits TCA cycle, mitochondrial respiration, DNA synthesis, and antioxidative, as well as DNA repair enzymes (6, 7).

Immunostimulants such as interleukins, TNF, and IFN have potent antitumor activities, alone or in combination with cisplatin. The combination of cisplatin-based chemotherapy with an immunostimulant, referred to as chemoimmunotherapy, has shown a synergistic enhancement in the antitumor activity of tumor-bearing mice (8, 9) and in the in vitro cytotoxic activity of immune cells (10). Although the synergistic antitumor activity of cisplatin-based chemoimmunotherapy was thought to be associated with the activation of tumor-infiltrating macrophages (11), a therapeutic mechanism has not been elucidated. We have tested whether cisplatin can enhance the sensitivity of macrophages to immunostimulant(s) for tumor cell killing through enhanced NO synthesis. This hypothesis is tested by (a) measuring NO concentration in cisplatin-exposed mice and their peritoneal macrophages; and (b) coculturing cisplatin-sensitive and cisplatin-resistant tumor cells with peritoneal macrophages isolated from cisplatin-exposed mice and activated with LPS plus IFN-γ.

Materials and Methods

**Materials.** Cisplatin, LPS (Escherichia coli 0111:B4), Lowry protein assay kit (P5656), phenylmethylsulfonyl fluoride, and N-acetylpenicillamine were purchased from Sigma Chemical Co. Mouse recombinant IFN-γ was obtained from Amgen. Thiglycollate was purchased from DIFCO, Inc. (Detroit, MI). DMEM and other materials for cell culture were from GIBCO-BRL. SNAP was synthesized and checked routinely for stoichiometric S-nitrosothiol as described previously (7).

**Animals.** Six- to 8-week-old female BALB/c mice (Charles River Breeding Laboratory) were used in all experiments and given food and water ad libitum throughout the study.

**Tumor Cells.** Murine fibroblast cell line L929 was purchased from ATCC. Human ovarian carcinoma cell lines 2008 (cisplatin-resistant variant 2008/CP) and A2780 (cisplatin-resistant variant A2780/CP) were obtained from Drs. P. A. Andrews (Georgetown University, Washington, DC) and K. J. Scanlon (City of Hope National Medical Center, Duarte, CA), respectively. Human breast carcinoma cell line MCF-7 and its cisplatin-resistant variant MCF-7/CP were obtained from B. Teicher (Dana-Farber Cancer Institute, Harvard University, Boston, MA).

**Determination of NO Synthesis.** For in vivo studies, mice were given injections i.p. with cisplatin (10 mg/kg) or saline as a control. After various periods, LPS (15 mg/kg) or saline as a control was injected i.p., and 12 h later, blood was collected into a vacutainer containing EDTA by heart puncture, followed by centrifugation for 5 min at maximum speed in microcentrifuge to obtain plasma. Protein was precipitated with a double volume of 0.5 N NaOH and 10% ZnSO4. Total NO2− plus NO3− in the samples was determined by converting the NO3− to NO2− by using an automated high-performance liquid chromatography as described previously (7). For in vitro studies, NO2− accumulation, as an indicator of NO production, in the supernatant of cultured macrophages was determined by the Greiss reaction (4). One hundred μl of each sample were measured at A550 in triplicate by using a microplate reader.

**Culturing Macrophages.** Mice were injected with 2 ml of 4% thioglycolate broth. After 4 days, peritoneal exudate macrophages were isolated from peritoneal lavage of mice. Macrophage cells were cultured with DMEM supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 2 mM L-glutamine, penicillin (100 unit/ml), and streptomycin (100 μg/ml) in 5% CO2-95% air at 37°C for 4 h in 96-well plates (1 X 105 cells/well) for nitrite measurement, or 60-mm plastic dishes (1 X 106 cells/dish) for Northern and...
Western Blots. Cells were washed with DMEM to remove nonadherent cells and cultured with LPS (10 μg/ml) and IFN-γ (100 units/ml).

RNA Isolation and Northern Blot Analysis. RNA was isolated from macrophages cultured with LPS plus IFN-γ for 10 h using guanidium isothiocyanate (7). Total RNA (20 μg) was electrophoresed on 1% agarose gel containing 1% formaldehyde and then transferred to GeneScreen (NEN Research Products, Boston, MA). The membrane was hybridized with a probe isolated from murine macrophage iNOS cDNA and 18 S RNA as described previously (7). The membrane was exposed for 4 to 5 h to a Phospholmage screen (Molecular Dynamics), and radioactivity was analyzed by a Phospholmage.

Western Blot Analysis. Macrophages were harvested, washed with PBS containing 0.1 mM phenylmethylsulfonyl fluoride, and lysed by three cycles of freezing and thawing in liquid nitrogen. Cytosolic fraction was obtained from the supernatant of 12,000 rpm in a microcentrifuge at 4°C for 10 min. Protein was analyzed by the Lowry method. Samples (50 μg) were separated on 8% SDS-PAGE and transferred to nitrocellulose membrane (Schleicher & Schuell, Keene, NH). The membrane was blocked with PBS-Tween containing 5% nonfat milk for 1 h, washed quickly twice with PBS-Tween, and incubated with monoclonal mouse-antimacrophage iNOS IgG (Transduction Laboratories, KY) in 1% PBS-Tween for 1 h. After washing three times with PBS-Tween, the membrane was incubated with antimouse IgG conjugated peroxidase (DuPont NEN, Boston, MA) for 1 h. The membrane was washed seven times with PBS-Tween, incubated in ECL reagents for 1 mm, and exposed to Kodak X-ray film.

In Vitro Assay of NO-mediated Cytotoxicity. The NO concentration that reduced the number of viable cells to 50% of the control (IC50) was determined as follows. Tumor cells were cultured with complete medium (DMEM for L929 and MCF-7 cells and RPMI 1640 for A2780 cells) in 12-well plates to 70–80% confluency. Cells were exposed to different concentrations (0–3 mM) of SNAP in medium for 24 h, followed by staining with crystal violet. Plates were dried overnight, lysed with 1% SDS solution, and then A550 was measured. IC50 for tumor cells to SNAP was calculated from percentage viability against nontreated cells. For measurement of tumor cell cytotoxicity by NO generated from macrophages, macrophages isolated from mice at day 11 after i.p. injection of cisplatin were plated in 96-well plates (1 × 105 cells/well) and activated with 150 μl of LPS and IFN-γ for 8 h. Tumor cells harvested from culture plates were labeled with 0.2 ml of 51Cr (1 mCi/ml), followed by incubation with occasional shaking at 37°C for 1 h. Cells were washed three times with medium and resuspended in medium to a final concentration of 4 × 104 cells/100 μl. The activated macrophages were cocultured with 50 μl of 51Cr-labeled tumor cells (a final concentration of 1 × 105 tumor cells/well) for 16 h. Cultured cells were spun at 650 × g for 10 min. Supernatant (100 μl) was transferred into a tube and radioactivity was counted in gamma counter. Percentage of NO-mediated cytotoxicity was calculated by using the following formula:

\[
% \text{ of cytotoxicity} = \frac{\text{experimental counts} - \text{spontaneous counts}}{\text{total counts} - \text{spontaneous counts}} \times 100
\]

Results

Fig. 1 shows the time course of in vivo NO formation by LPS in serum after preinjection of cisplatin. NO production increased with time after initial suppression. The enhancement was significant at day 7 and reached the maximum at day 11 after cisplatin injection, only when an exogenous LPS was present. There was no NO production without LPS treatment. Interestingly, NO production at days 1 and 2 after cisplatin pretreatment was approximately one-half of the control, which remained constant with time at about 500 μM. The control represents saline (no cisplatin) pretreatment and LPS posttreatment into mice.

To find how cisplatin treatment increases the formation of NO, peritoneal macrophages were taken from mice at days 2 and 11 after cisplatin injection, cultured in the presence or absence of LPS plus IFN-γ, and then analyzed for the nitrite content of culture medium. As shown in Fig. 2, macrophages pre-exposed to cisplatin for 2 and 11 days did not produce NO without LPS plus IFN-γ. However, when cisplatin-treated macrophages for 11 days were cultured with LPS plus IFN-γ, in vitro NO formation increased to the level comparable with in vivo studies (see Fig. 1). On the other hand, the level of NO formation by macrophages taken at day 2 reduced about 50% with respect to the control. The control represents pretreatment of mice with saline followed by treatment of isolated macrophages with LPS plus IFN-γ in vitro. The NO level of the control also remained unchanged for 11 days. We investigated further the increase in NO formation at the molecular level by examining macrophage iNOS mRNA (Fig. 3A) and enzyme expression (Fig. 3B). iNOS mRNA level from macrophages increased approximately 2-fold when macrophages were exposed to cisplatin in vivo for 11 days and cultured with LPS plus IFN-γ, with respect to the control. mRNA level of the control was also constant with time. As expected, mRNA level, exposed to cisplatin for 2 days and then cultured with LPS plus IFN-γ, was less than one-half of the control. It was also clear that expression of iNOS protein from macrophages, pretreated with cisplatin in vivo and cultured with LPS plus IFN-γ, increased approximately 2-fold with respect to the control. There was no detectable iNOS expression when macrophages were cultured in the absence of the immunostimulants.
cisplatin, macrophages were in vivo exposed to cisplatin for 11 days, with several tumor cells for 16 h. As seen in Table 2, macrophages activated in vitro with LPS plus IFN-γ for 8 h, and then incubated with SNAP, was ineffective. To further determine whether peritoneal macrophages can effectively be induced for tumor cell killing by NO, produced by the activated macrophages, is an important mediator for tumoricidal activity in vivo (5). Peritoneal macrophages, activated by LPS, IFN, a combination of cytokines or tumor cell, induced NOS (3, 12, 13). NO is highly reactive with a variety of biological molecules involving in energy biosynthesis, such as aconitate and mitochondrial electron-transfer proteins (complexes one and two) containing iron (6). The FeS cluster in their active sites reacts with NO and forms iron-nitrosyl complexes, resulting in loss of their biological activities. Ribonucleotide reductase, a key enzyme of DNA synthesis, requires an iron and tyrosyl radical for catalytic activity, is also inhibited by NO (14). Poly(ADP-ribose)-synthesize activated by NO depletes cellular NAD level (15), which blocks ATP and DNA synthesis. Furthermore, NO can cause apoptosis of tumor cells (16). These biological actions of NO may be responsible for cytotoxicity for tumor cells.

Cisplatin also activated macrophages and increased production of several secretory as well as membrane-associated forms of IL-1 and IL-6. These cytokines were responsible for cytotoxicity for tumor cells.

Table 1 IC₅₀ for several carcinoma cell lines to NO

<table>
<thead>
<tr>
<th>Cells</th>
<th>IC₅₀ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7</td>
<td>1.28 ± 0.11</td>
</tr>
<tr>
<td>MCF-7/CP</td>
<td>2.15 ± 0.36</td>
</tr>
<tr>
<td>A2780</td>
<td>1.78 ± 0.48</td>
</tr>
<tr>
<td>A2780/CP</td>
<td>2.02 ± 0.23</td>
</tr>
<tr>
<td>MCF-7</td>
<td>2.37 ± 0.49</td>
</tr>
<tr>
<td>MCF-7/CP</td>
<td>2.37 ± 0.49</td>
</tr>
</tbody>
</table>

Table 2 Effect of cisplatin on macrophage-mediated cytotoxicity for several carcinoma cells

Peritoneal macrophages (1 x 10⁶ cells/well) in 96-well plates were activated with 150 μl of LPS plus IFN-γ for 8 h. Activated macrophages were cocultured with ²⁵³Cr-labeled tumor cells (a final concentration of 1 x 10⁶ cells/well) in the presence (+) or absence (−) of N⁵-monomethyl-L-arginine (NMA) for 16 h. Cytotoxicity was measured from radioactivity released into medium. See “Materials and Methods” for details.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Saline</th>
<th>NMA (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7</td>
<td></td>
<td>−NMA</td>
</tr>
<tr>
<td></td>
<td>2008</td>
<td>37.5 ± 4.2</td>
</tr>
<tr>
<td></td>
<td>2008/CP</td>
<td>32.7 ± 5.3</td>
</tr>
<tr>
<td>A2780</td>
<td></td>
<td>47.8 ± 5.3</td>
</tr>
<tr>
<td>A2780/CP</td>
<td>47.8 ± 5.3</td>
<td>7.30 ± 3.6</td>
</tr>
<tr>
<td>MCF-7</td>
<td>33.2 ± 2.5</td>
<td>5.30 ± 3.2</td>
</tr>
<tr>
<td>MCF-7/CP</td>
<td>28.4 ± 2.8</td>
<td>2.10 ± 1.8</td>
</tr>
</tbody>
</table>

To examine NO-mediated cytotoxicity for tumor cells, we treated the tumor cells with SNAP, a NO-generating compound, and measured their viability to calculate IC₅₀. As shown in Table 1, IC₅₀ ranged from 1.3 to 2.4 mM and were not significantly different between paired cisplatin-sensitive and cisplatin-resistant cells. Treatment of the cells with N-acetylpenicillamine, a parent compound of SNAP, was ineffective. To further determine whether peritoneal macrophages can effectively be induced for tumor cell killing by cisplatin, macrophages were in vivo exposed to cisplatin for 11 days, activated in vitro with LPS plus IFN-γ for 8 h, and then incubated with several tumor cells for 16 h. As seen in Table 2, macrophages isolated from the saline-injected (control) mice killed tumor cells about 30–50%, whereas macrophages from cisplatin-injected animals increased cytotoxicity about 40–70%. N⁵-monomethyl-L-arginine, competitive inhibitor of NO synthase, significantly reduced the cytotoxicity activity of macrophages, isolated from both cisplatin-injected and control animals, for tumor cells. Interestingly, there was no significant difference in the cytotoxicity between paired cisplatin-sensitive and cisplatin-resistant cells except the cytotoxicity of A2780 and A2780/CP to the cisplatin-exposed macrophage only (P < 0.01). Without any immunostimulants, however, cisplatin-exposed macrophages did not show any cytotoxicity to the cells tested.

Discussion

NO, produced by the activated macrophages, is an important mediator for tumoricidal activity in vivo (5). Peritoneal macrophages, activated by LPS, IFN, a combination of cytokines or tumor cell, induced NOS (3, 12, 13). NO is highly reactive with a variety of biological molecules involving in energy biosynthesis, such as aconitate and mitochondrial electron-transfer proteins (complexes one and two) containing iron (6). The FeS cluster in their active sites reacts with NO and forms iron-nitrosyl complexes, resulting in loss of their biological activities. Ribonucleotide reductase, a key enzyme of DNA synthesis, requires an iron and tyrosyl radical for catalytic activity, is also inhibited by NO (14). Poly(ADP-ribose)-synthesize activated by NO depletes cellular NAD level (15), which blocks ATP and DNA synthesis. Furthermore, NO can cause apoptosis of tumor cells (16). These biological actions of NO may be responsible for cytotoxicity for tumor cells.

Cisplatin also activated macrophages and increased production of several secretory as well as membrane-associated forms of IL-1 and IL-6. These cytokines were responsible for cytotoxicity for tumor cells.
TNF (17) and increased generation of cytotoxic active compounds, including reactive oxygen species such as superoxide and \( \mathrm{H}_2\mathrm{O}_2 \) (18, 19), and a number of lysozymes (20). Cisplatin is a potent antitumor agent against a wide variety of tumors in humans (21, 22) and animals (23). Our study showed that cisplatin activated peritoneal macrophages only when exogenous LPS or LPS plus IFN-\( \gamma \) was present. This finding is not consistent with the previous one in which bone marrow-derived macrophages, when treated with cisplatin in vivo, produced NO (24). Although the exact mechanism of cisplatin-induced macrophage activation is not clear, we demonstrated that the in vivo cisplatin-exposed macrophages enhanced expression of iNOS mRNA and iNOS protein only in the presence of exogenous immunostimulants. The enhancement of iNOS induction can be explained by three possibilities: (a) reactive oxygen species produced by cisplatin-treated macrophages may synergistically activate a transcription factor NF-\( \kappa \)B in the presence LPS (or cytokines; Refs. 18, 19, 25). Duval et al. (26) also showed a synergistic induction of iNOS expression by reactive oxygen generated by xanthine oxidase and cytokine; (b) cisplatin may increase an intracellular level of glutathione for responsible iNOS expression (26) by increasing glutathione S-transferase activity (27); and (c) cisplatin may sensitize macrophages to LPS and/or INF-\( \gamma \) through increased cytokine receptor for enhanced iNOS transcription. On the other hand, initial decrease in NO production for 2 days after cisplatin injection may be attributed to depletion of glutathione (28), a cytotoxic effect induced by cisplatin. Interestingly, when murine macrophage cell line RAW264.7 was continuously exposed to cisplatin in vitro, NO production did not increase even in the presence of LPS plus IFN-\( \gamma \) (data not shown). This result suggests that unknown biological factor(s), which may be induced by cisplatin, involve activation of macrophages by immunostimulants.

It is most likely that cytotoxicity of NO, produced by activated macrophages, depends on scavenging activities of cellular components such as MT (29). In a biological system, a RBC is very resistant to NO because it is a well-known biological NO scavenger. MTs, a family of low molecular weight metal-binding proteins, were overexpressed in cisplatin-resistant cells (30). Recently, MT-transfected endothelial cells showed protection from NO-mediated cytotoxicity (29). Nonetheless, our data have shown that both MT level, e.g., 208/CP and A2780/CP have higher contents of MT than do the parent cells, and cisplatin resistance were not related to NO-mediated cytotoxicity for the tumor cells tested. These results indicate that cisplatin-resistant tumor cells respond to NO in vitro, NO production did not increase even in the presence of LPS plus IFN-\( \gamma \) (data not shown). This result suggests that unknown biological factor(s), which may be induced by cisplatin, involve activation of macrophages by immunostimulants.

Several cytokines, such as interleukins, IFN and TNF, have been used in immunotherapy for cancer patients and tumor-bearing animals. Recent studies have shown that the combination of cisplatin-based chemotherapy with cytokines, chemoinmunotherapy, exhibited a synergistic enhancement of antitumor activity against both cisplatin-sensitive and cisplatin-resistant ovarian cell lines in animal models (8) and in vitro (10). Furthermore, ovarian tumor cells pre-exposed to cisplatin were more susceptible to liposome-mediated gene transfer (31). Although the mechanism involved in the phenomenon is not known, cisplatin chemotherapy, followed by cisplatin-induced cyto- kinase gene therapy can achieve not only therapeutic synergism but induction of long-lasting immunity against tumor growth. Therefore, a new therapeutic strategy, based on our findings, will provide an important insight for treating terminal cancer patients.

References

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