Nitric Oxide Boosts Chemoimmunotherapy via Inhibition of Acid Sphingomyelinase in a Mouse Model of Melanoma

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Abstract

Cisplatin is one of the most effective anticancer drugs, but its severe toxic effects, including depletion of immune-competent cells, limit its efficacy. We combined the systemic treatment with cisplatin with intratumor delivery of dendritic cells (DC) previously treated ex vivo with a pulse of nitric oxide (NO) released by the NO donors (z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl)amino]-diazene-1-ium-1,2-diolate or isosorbide dinitrate. We found that this chemoimmunotherapy, tested in the B16 mouse model of melanoma, was significantly more efficacious than cisplatin alone, leading to tumor regression and animal survival at low doses of cisplatin that alone had no effect. Tumor cure was not observed when combining cisplatin with DCs not exposed to NO donors, indicating the key role of the pretreatment with NO. We investigated the mechanisms responsible for the synergic effect of NO-treated DCs and cisplatin and found that NO-treated DCs were protected both in vitro and in vivo from cisplatin-induced cytotoxicity. Cisplatin triggered DC apoptosis through increased expression and activation of acid sphingomyelinase; pretreatment of DCs with NO donors prevented such activation and inhibited activation of the downstream proapoptotic events, including generation of ceramide, activation of caspases 3 and 9, and mitochondrial depolarization. The effects of NO were mediated through generation of its physiologic messenger, cyclic GMP. We conclude that NO and NO generating drugs represent promising tools to increase the efficacy of chemoimmunotherapies in vivo, promoting the survival and increasing the function of injected cells by targeting a key pathway in cisplatin-induced cytotoxicity. [Cancer Res 2007;67(16):7559–64]

Introduction

Delivery of cytotoxic chemotherapeutic agents in the treatment of human cancer is often accompanied by acute and cumulative toxicities to normal tissues that limit the dose and duration of treatment. One promising strategy to reduce the toxicity of chemotherapy maintaining its efficacy is the combination of chemotherapy with immunotherapy. Such strategy could exploit the debulking effects of chemotherapy to treat cancers, because chemotherapy and immunotherapy have different mechanisms of action (1–3).

Among immunotherapies, approaches based on dendritic cell (DC) vaccines are particularly promising. These professional antigen-presenting cells can exploit chemotherapy-induced apoptosis of tumor cells to elicit improved antitumor immunity through the acquisition of tumor-associated antigens from apoptotic tumor cells; in addition, they have direct cytotoxic effects on cancer cells (1, 2).

A major limitation for combination chemoimmunotherapy is that the chemotherapeutic drugs induce apoptotic death of immune competent cells used for immunotherapies (1, 4). This reduces significantly the efficacy of this kind of treatments. A strategy that protects selectively DCs, but not tumor cells, from the cytotoxic action of chemotherapeutics would therefore significantly enhance the efficacy of combined chemoimmunotherapy, allowing reduced dosage of the chemotherapeutics to limit their toxic effects against normal tissues. No strategy to reach this goal, however, has been envisaged thus far.

To address the issue, we took advantage of the properties of nitric oxide (NO). NO enhances resistance of DCs to the toxic effect of the tumor environment in vivo and increases DC cytotoxic, endocytic, and antigen-presenting functions (5–8). In addition, NO inhibits activation of the sphingomyelin-hydrolyzing enzyme acid sphingomyelinase (A-SMase), whose activation triggers apoptosis in DCs (9). A-SMase is activated by most chemotherapeutics (10, 11).

DCs were exposed to a single pulse of NO ex vivo, using either isosorbide dinitrate or (z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl)-amino]-diazene-1-ium-1,2-diolate (DETA-NO), a NO donor that releases NO constantly at defined concentrations (12). The NO donor was removed thoroughly before DC injection at the tumor site to obtain a selective effect on DCs and avoid the in vivo effects of NO that in the case of tumors are complex and often conflicting (13, 14). The treatment was tested in vivo on the highly tumorigenic and poorly immunogenic B16 mouse melanoma in a combined chemoimmunotherapy with cisplatin (cis-diammine-dichloro-platinum), one of the most effective, but also toxic, anticancer drugs (15). NO-treated DCs significantly enhanced the therapeutic efficacy of cisplatin so that tumor regression and animal survival were obtained at doses of cisplatin that were otherwise ineffective. NO inhibited cisplatin-triggered activation of A-SMase and generation of the proapoptotic sphingolipid ceramide in DCs and protected these cells from apoptosis. Furthermore, NO-treated DCs resisted the toxic effect of the tumor environment. NO seems, therefore, as a simple strategy to enhance efficacy of chemoimmunotherapy, by increasing survival and function of DCs.
Materials and Methods

Animals. Female C57BL/6 (H-2b) and C57BL/6 A-SMase−/− mice (6–8 weeks old; ref. 16) were treated in accordance with the European Community guidelines and with the approval of the Institutional Ethical Committee.

Cell culture and characterization. B16-F1 C57BL/6-derived melanoma cells and DCs from mouse bone marrow precursors were obtained, cultured, and characterized as described (5, 8, 9). The purity of DCs was in all experiments no less than 80%. Treatments of DCs with isosorbide dinitrate, DETA-NO, 8-Br-cyclic guanosine 3′,5′-monophosphate (cGMP), or 1H-[1,2,4] oxadiazolo-[4,3-a]quinazolin-1-one (ODQ) were for 6 h, and the compounds were then fully removed by washing. After the washout, the medium did not contain residual bioactive NO because it was unable to stimulate generation of cGMP in PC12 cells, an extremely sensitive cGMP-reporter cell system that can detect generation of NO in the low nanomolar range (ref. 17; Supplementary Materials and Methods). cGMP concentrations in DCs were assessed as described (6).

Intratumoral DC injection. C57BL/6 mice (five animals per group) received 1 × 10⁵ B16-F1 cells (i.e., 10-fold the minimal tumorigenic dose, determined in previous experiments to be 1 × 10⁴ cells) s.c. in the lower-right flank on day 0 (8). Tumors reached the appropriate size (−10 mm²) after 6 days. On days 6 and 9, mice received cisplatin or vehicle (PBS) i.p. and DCs (1 × 10⁷) or their vehicle (PBS) were injected i.t. within 1 h from cisplatin treatment; tumor growth was monitored twice a week by a caliper. Mice were sacrificed when their tumors reached 15 mm on either diameter. To reveal injected DCs at tumor site, DCs were previously labeled with 2 µmol/L fluorescent dye 5-chlormethylfluorescein (CMFDA) for 30 min at 37°C (8). Tumors were collected 24 and 72 h after DC injection. Single-cell suspensions were prepared as described (8), decorated with a phycoerythrin-conjugated anti-CD11c monoclonal antibody and analyzed by flow cytometry.

Measurement of A-SMase activity and expression and ceramide generation. A-SMase activity was determined by measuring conversion of sphingomyelin to phosphorylcholine in homogenates of DCs (2 × 10⁶ cells/mL) treated with cisplatin (10 µg/mL) as described (9). Western blot analyses were carried out on 50 µg of DC lysates (8). Relevant bands were immunodecorated using rabbit polyclonal primary antibodies specific for A-SMase (Eurogentech), followed by incubation with horse radish peroxidase–conjugated goat polyclonal anti-rabbit IgGs. Immunoreactive bands were visualized by the enhanced chemiluminescence procedure (Amersham) and quantified by microdensitometry using a Molecular Dynamics Imagequant apparatus. Ceramide generation was measured by the diacyl-glycerol kinase assay and revealed by TLC (9).

To detect intracellular A-SMase by flow cytometry, cells were fixed with paraformaldehyde (4% in PBS for 10 min at room temperature) and then permeabilized for 20 min at room temperature in PBS supplemented with 1% bovine serum albumin and 0.1% saponin. The primary antibody was detected using a FITC-labeled goat anti-rabbit secondary antibody. An isotype-matched antibody was used as control (9).

Measurement of variables of apoptosis. Phosphatidylserine exposure in propidium iodide (PI)–excluding cells was monitored by staining for 15 min at room temperature with FITC-labeled Annexin V (2 µg/mL). The mitochondrial membrane potential was evaluated by flow cytometry in DCs (2×10⁶ cells/sample) stained with 500 nmol/L of potential-sensitive fluorescent dye, tetramethylrhodamine ethyl ester (TMRE; ref. 8). Caspase-9 and caspase-3 activities were assessed measuring the cleavage of the fluorogenic caspase-9 and caspase-3 substrates Ac-LEHD-7-amc and Ac-DEVD-7-amc with a Perkin-Elmer LS50 fluorometer as described (8).

Statistical analysis. The results are expressed as means ± SE; n represents the number of individual experiments. Statistical analysis was done using the Student’s t test and the log-rank statistic. All data were considered statistically significant for P < 0.05. Double and triple asterisks or crosses in the figures refer to P values of <0.01 and <0.001, respectively.

Results

Combined chemoimmunotherapy with cisplatin and NO-treated DC yields tumor regression and animal survival. A and B, naive C57BL/6 mice (5 animals per group) were injected in the right flank with 1 × 10⁶ B16-F1 cells. After 6, 7, and 8 d, cisplatin (CDDP; 1 mg/kg when not otherwise indicated) or vehicle was injected i.p., whereas vehicle or DCs were treated for 6 h in the absence (UT) or presence of DETA-NO (10 µmol/L) and then washed free of the drug were injected i.t. A, points, tumor size (n = 6); bars, SE. B, percentage survival of animals (in one experiment of six reproducible ones). C, same treatments as in A and B but using CMFDA-loaded DCs. DCs were injected immediately after the removal of DETA-NO or 2 and 4 h later. Tumor mass was recovered 24 and 72 h after DC injection; cells were dispersed, stained with the DC marker CD11c, and analyzed by flow cytometry. Columns, number of tumor infiltrating, double-positive DCs (n = 5); bars, SE. Asterisks and crosses in A and C indicate statistical significance, measured as indicated in Materials and Methods, versus vehicle and UT DCs + CDDP, respectively.

C57BL/6 mice were treated in the presence or absence of 10 µmol/L DETA-NO that yielded a steady-state concentration of 11 ± 0.1 nmol/L NO (n = 4) as measured by an NO electrode (6). All treatments were for 6 h, and agents were removed thereafter. C57BL/6 mice were injected s.c. with 10-fold the minimal tumorigenic dose of B16-F1 cells (1 × 10⁶). On days 6 and 9 after tumor implantation, cisplatin (1 mg/kg) or vehicle was injected i.p. and vehicle, untreated DCs, or DETA-NO–treated DCs were
injected i.t. in the s.c. growing B16 melanomas. A parallel group of animals received 4-fold the dose of cisplatin (4 mg/kg), but not DCs. In the groups receiving injection of DETA-NO–treated DCs, tumor growth was significantly reduced (Fig. 1A) with respect to that observed in all other groups, including the one receiving high-dose cisplatin alone. All the animals in the other groups eventually died because of the tumor, whereas, in the group receiving DETA-NO–treated DCs, animal survival was significant (87 ± 4%; P < 0.001 versus cisplatin-treated animals receiving untreated DCs, n = 6; Fig. 1B). In all surviving animals, tumor regression was observed. The effect of DETA-NO was due neither to an action of the corresponding amine, DETA, nor to the stable decomposition product of NO, nitrite, because if the compound was allowed to decompose fully, it had no effects (not shown; ref. 12).

The effect of NO on DC intratumor survival in cisplatin-treated animals was also evaluated. To this end, DCs were stained with the dye CMFDA (8) before their injection into tumors. Tumors were recovered 24 and 72 h after injection, cells were dispersed and analyzed by flow cytometry after staining for the DC marker CD11c. Treatment ex vivo of DCs with DETA-NO resulted in the recovery of significantly higher numbers of CMFDA+/CD11c– cells at the tumor site (Fig. 1C). The effect of DETA-NO pretreatment was persistent, because the yield of DETA-NO–treated DCs retrieved from tumor site did not change even when DC injection was delayed for 2 and 4 h after the end of the treatment with DETA-NO and the removal of the drug (Fig. 1C). CMFDA+/CD11c– cells accounted for <0.1% of the recovered cells and did not change significantly with the various treatments (not shown). Results similar to those of DETA-NO

**Figure 2.** Cisplatin induces apoptosis of DCs by increasing activity and expression of A-SMase. wt and A-SMase–/– DCs were treated with cisplatin (10 μg/mL when not otherwise indicated) for 5 min, 12 and 24 h (A, C), or 12 h (B and C). A, A-SMase activity and expression. A-SMase activity was measured 5 min after cisplatin administration both as enzymatic activity, expressed as phosphorylcholine formation (filled circles), and ceramide generation (empty circles), quantified by TLC. Points and columns, values (n = 4); bars, SE. The filled and empty arrows indicate the position of the standard ceramide processed and loaded in parallel and of the origin, respectively, on one representative thin layer chromatogram. A-SMase expression was revealed after 12 and 24 h by both Western blotting and flow cytometry; the latter expressed as the percentage (±SE) of cisplatin-treated DCs over respective untreated, control DCs (n = 4). B, evaluation by flow cytometry of Annexin V (AnxV) expression in PI-excluding cells or of the mitochondrial membrane potential, measured using the potential-sensitive mitochondrial dye TMRE. Results are from one experiment representative of eight reproducible ones. Numbers reported in the various diagrams, percentage fluorescence values measured for the eight experiments in the region; bars, SE. C, caspase-3 (Casp-3) and caspase-9 (Casp-9) activity values are reported as percentage (±SE) of those observed in untreated wt or A-SMase–/– DCs. Values were 11 ± 0.2 and 9.8 ± 0.3 pmol/min mg−1 for caspase-9 and 6.3 ± 0.1 and 5.1 ± 0.1 pmol/min mg−1 for caspase-3 in untreated wt and A-SMase–/– DCs, respectively (n = 5). Asterisks, statistical significance measured as indicated in Materials and Methods versus untreated wt and A-SMase–/– DCs.
were observed when the pretreatment was carried out using isosorbide dinitrate (50 μmol/L) as the NO donor (Supplementary Fig. S1).

Thus, a combined therapy with cisplatin and NO-treated DCs increases numbers of DCs at tumor site and leads to tumor regression and animal survival at doses of cisplatin that otherwise have no therapeutic effects.

**NO pretreatment prevents activation of A-SMase by cisplatin and protects DCs from cisplatin-induced apoptosis.**

Cisplatin triggers apoptosis of tumor cells through activation of A-SMase (11). We investigated whether cisplatin triggered this apoptogenic pathway in DCs and assessed the effect of NO on it. Cisplatin (10 μg/mL) induced concentration-dependent increases in A-SMase activity and ceramide generation and a time-dependent increase in A-SMase expression by DCs from wt animals (wtDCs; Fig. 2A); wtDCs cultured for 8 h in the presence of cisplatin died via apoptosis, as shown by the exposure on the outer leaflet of the plasma membrane of phosphatidylserine assessed by Annexin V staining in PI-excluding cells, by the decrease in the mitochondrial membrane potential assessed by staining with TMRE, and by the increased activity of caspase-3 and caspase-9 (Fig. 2B and C; ref. 8). By contrast, cisplatin modified none of the above variables nor induced apoptosis of DCs obtained from A-SMase null (A-SMase−/−) mice (Fig. 2B and C).

To evaluate the role of NO, wtDCs were treated as described above in the presence or absence of DETA-NO, isosorbide dinitrate, the cell permeable analogue of cGMP, 8-Br-cGMP (3 mmol/L), or DETA-NO combined with ODQ (1 μmol/L), a selective guanylate cyclase inhibitor that prevents NO-dependent cGMP generation (6, 8). The NO donors inhibited the increases in expression and activation of A-SMase and ceramide generation induced by cisplatin in wtDCs and protected wtDCs from apoptosis as shown by reduced Annexin V staining, maintenance of mitochondrial membrane potential, and activation of caspase-3 and caspase-9 (Fig. 3A–C; Supplementary Fig. S1). Sensitivity to apoptosis, however, was restored by administration of exogenously added C2 ceramide (10 μmol/L) or A-SMase (2 units/mL), indicating that inhibition of A-SMase accounted for the protective effect of NO. All the effects of NO depended on generation of cGMP because they were mimicked by 8-Br-cGMP and prevented by ODQ. This was confirmed by the observation that DCs, although devoid of endogenous NOS isoforms (ref. 9; Supplementary Fig. S2), are endowed with guanylate cyclase activity (6, 9) and respond to DETA-NO with generation of cGMP; values in DETA-NO–treated wtDCs, control, and DETA-NO plus ODQ–treated wtDCs were 4.39 ± 0.11, 0.55 ± 0.07, and 0.69 ± 0.08 pmol/mg min−1, respectively (P < 0.001 in DETA-NO–treated cells versus control; n = 4). cGMP generation ceased after DETA-NO removal, whereas its effects on signaling were long lasting, as shown by the persistent increase in Bel-XL (Supplementary Fig. S3), consistent with the persistency of the effect of DETA-NO (Fig. 1C). DETA-NO, 8-Br-cGMP and ODQ had effects neither on basal A-SMase activity/expression nor on the various variables of apoptosis measured (not shown).

**NO pretreatment prevents activation of A-SMase by cisplatin and protects DCs from cisplatin-induced apoptosis in vivo.**

We evaluated the role of A-SMase activation and its inhibition by NO on DCs in vivo. To this end, we used untreated A-SMase−/− DCs and wtDCs treated with or without DETA-NO ex vivo. In the groups receiving cisplatin and injection of
A-SMase−/− DCs, tumor growth was significantly reduced (Fig. 4A) and animal survival and tumor regression were significant (69 ± 3% and 76.2 ± 4.23%, respectively, at 30 days; P < 0.001 versus cisplatin-treated animals receiving wtDCs; n = 6; Fig. 4B) in the range of that observed using DETA-NO–treated wt and A-SMase−/− DCs. Moreover, intratumor survival of CMFDA+/CD11c−/A-SMase−/− DCs was not significantly different from that of DETA-NO–treated wtDCs (Fig. 4C). Of importance, no additional protective effects were observed in DETA-NO–treated versus untreated A-SMase−/− DCs.

These results indicate that NO protects DCs from the apoptotic effect of cisplatin in vivo through inhibition of A-SMase.

Discussion

In this study we show that intratumor delivery of DCs briefly exposed ex vivo to NO enhances significantly the therapeutic efficacy of cisplatin. The protocol we used was designed to expose DCs to NO selectively and only ex vivo to minimize the systemic effects of NO in vivo. These effects of NO in the case of tumors are complex, resulting from a combination of cytotoxic and cytostatic actions on tumor cells, and protumorigenic functions, including stimulation of angiogenesis and down-regulation of immune responses (13, 14); in addition, systemic NO may change sensitivity to anticancer drugs in vivo (18–23).

The combined chemoimmunotherapy with NO-treated DCs and cisplatin not only specifically and significantly reduced the growth of the highly tumorigenic and poorly immunogenic B16 melanoma but also yielded almost 90% animal survival and tumor regression at doses of the chemotherapeutic that do not have a therapeutic effect per se. The effect of the combined chemoimmunotherapy is significantly greater than that of NO-treated DCs alone (8) and opens the way to possible therapeutic strategies that minimize the toxic effects of the chemotherapeutic while enhancing their efficacy.

The effect of NO seems due to inhibition of the ability of cisplatin to kill DCs in vivo. The cytotoxic effect of cisplatin is mediated through A-SMase activation and ceramide generation, as showed by resistance of A-SMase−/− DCs to cisplatin-induced death. Our results clearly show that inhibition of A-SMase is the mechanism whereby NO increases the numbers of DCs at tumor site in vivo. A-SMase is activated by most chemotherapeutics (10, 11) and is important in determining tumor sensitivity/resistance to them (24). The observation that A-SMase is also the target of the protective action of NO suggests that the therapeutic efficacy of NO-treated DCs is not restricted to cisplatin but may conceivably increase the efficacy of most chemotherapeutics.

Whereas survival at tumor site was similar for NO-treated wtDCs and untreated A-SMase−/− DCs, tumor regression and animal survival were greater with NO-treated DCs than with untreated A-SMase−/− DCs; this suggests that other A-SMase–independent actions of NO on DCs contribute to its antitumor effect. Indeed NO increases DC cytotoxic action against tumor cells, enhances DCs antigen-presenting functions, and increases their ability to release antitumor cytokines, such as IL-12 (5, 6, 8, 25).

The treatment with NO shows two characteristics: (a) it is persistent, because a brief exposure of DCs yielded long-lasting antitumor action; (b) it depends on the generation of its physiologic messenger cGMP after activation of guanylate cyclase, an enzyme known to be activated by nanomolar concentrations of NO and mediate physiologic actions of the gas. The persistency and cGMP dependency of the effects of NO are consistent with NO/cGMP–induced changes in gene expression profile (26), as also indicated by the persistent increases in Bcl-XL levels and/or changes in lipid homeostasis (27) that still need to be investigated. Both persistency and cGMP dependency of the effects of NO are crucial from a therapeutic standpoint because antitumor action is obtained with a short-term drug exposure compatible with minimal manipulation of DCs ex vivo and because the concentrations of NO exerting pharmacologic effects are low. In addition, because of its simplicity, the treatment might be easily combined with other strategies of DC manipulation, including loading with tumor-specific antigens (1), to yield an enhanced therapeutic effect of combined chemoimmunotherapies.


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