Immunostimulatory Effects of Low-Dose Cyclophosphamide Are Controlled by Inducible Nitric Oxide Synthase

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Abstract
Cyclophosphamide is a widely used chemotherapeutic drug that was recently applied as either an antiangiogenic/antivasculogenic or an immunostimulatory agent in combination with cancer immunotherapies. It has been previously shown that cyclophosphamide augments the efficacy of antitumor immune responses by depleting CD4+CD25+ T regulatory cells and increasing both T-lymphocyte proliferation and T memory cells. Furthermore, cyclophosphamide was shown to mediate killing of circulating endothelial progenitors. However, the molecular basis for these observations has not yet been elucidated. We show here that the cyclophosphamide-mediated inhibition of inducible nitric oxide synthase is directly linked to its immunostimulatory but not to its antivasculogenic effects. Moreover, combined application of cyclophosphamide with a novel, oral DNA vaccine targeting platelet-derived growth factor B (PDGF-B), overexpressed by proliferating endothelial cells in the tumor vasculature, not only completely inhibited the growth of different tumor types but also led to tumor rejections in mice. These findings provide a new rationale at the molecular level for the combination of chemotherapy and immunotherapy in cancer treatment. (Cancer Res 2005; 65(12): 5027-30)

Introduction
Cyclophosphamide is a chemotherapeutic agent used to treat various types of cancer. Although high doses of the drug lead to immunosuppression, low-dose protocols have been shown to lead to enhanced immune responses against various tumor antigens (1). This immunostimulatory effect has been linked to the ability of cyclophosphamide not only to decrease the number but also to the functionality of CD4+CD25+ T regulatory cells (2, 3). Moreover, cyclophosphamide has been shown to trigger lymphocyte proliferation and increase the number of T lymphocytes with a memory phenotype by up-regulation of CD44hiCD4+ and CD44hiCD8+ (4). Cyclophosphamide was also reported to have antivasculogenic effects (5), which at least in part can be attributed to the suppression of circulating endothelial progenitors (CEP; ref. 6). The molecular mechanisms of both the immunostimulatory and antivasculogenic effects of cyclophosphamide have yet to be defined.

Platelet-derived growth factor B (PDGF-B) is a 241–amino acid protein that is mainly expressed in proliferating endothelial cells and various tumor cells. Besides its effects on pericytes, it has been implicated in autocrine growth stimulation, formation of tumor stroma, and control of the interstitial tumor pressure therefore making it a promising target for tumor vaccinations (7).

We show here for the first time that the cyclophosphamide-mediated inhibition of inducible nitric oxide synthase (iNOS) is directly linked to its immunostimulatory but not to its antivasculogenic effects. Furthermore, we show that the newly described mechanism applies to tumor bearing mice and can be employed in different tumor models to greatly enhance the antitumor effect of an oral DNA vaccine targeting PDGF-B, delivered by attenuated Salmonella typhimurium to secondary lymphoid tissue.

Materials and Methods
Animals, bacterial strains, cell lines, and plasmid vectors. Female C57BL/6J iNOS−/− and iNOS+/+ mice were purchased at 6 weeks of age from The Jackson Laboratory (Bar Harbor, ME). Female BALB/c mice, 6 to 8 weeks of age, were obtained from The Scripps Research Institute’s Rodent Breeding Facility. All animal experiments were done according to the NIH Guide for the Care and Use of Laboratory Animals and approved by our Animal Care Committee. The attenuated S. typhimurium strain RE87 (AroA-) was provided by Remedyne Corp. (Santa Barbara, CA). Murine D2F2 breast carcinoma cells were a gift from Dr. Wei-Zen Wei (Department of Immunology, Karmanos Cancer Institute, Detroit, MI). MOPC-315 plasmacytoma cells and D121 Lewis lung carcinoma (LLC) cells were kindly provided by Dr. Lea Eisenbach (Weizmann Institute of Science, Rehovot, Israel). The cDNA encoding murine PDGF-B was purchased from Invitrogen (San Diego, CA) and the pcDNA3.1/Zeo control vector from Invitrogen (San Diego, CA).

Oral immunization, tumor cell challenge, and treatment with cyclophosphamide and antibodies. Mice were challenged either by s.c. injection of 3 × 10^4 MOPC-315 plasmacytoma cells in the left front flank, o.t. injection of 3 × 10^5 D2F2 breast cancer cells in the left, second lowest mammary fat pad, or by i.v. injection of 7.5 × 10^6 LLCs in the lateral tail vein. Tumor volumes were measured in 2 dimensions and calculated as length / 2 × width^2. Treatment with cyclophosphamide was done in experimental groups of 8 mice with 175 mg/kg cyclophosphamide (Sigma-Aldrich, St. Louis, MO) as described previously (1), given i.p. every 6 days, starting on days 3, 4, or 6 after tumor cell challenge with D2F2, MOPC-315, or LLC cells, respectively. One hour after cyclophosphamide treatment, antibodies (500 μg) directed against either CD4 (clone GK 1.5) or CD8 (clone 24.43), both from the National Cell Culture Center (Minneapolis, MN), were injected i.p. every 6 days to deplete the respective CD4+ and CD8+ T cells. Mice were immunized 24 hours thereafter by gavage with 100 μL PBS containing 10^7 S. typhimurium (AroA-) transformed with the plasmid vectors indicated and as described previously (8).

Determination of serum nitrite/nitrate concentration. After collection of the respective blood samples, serum was obtained according to standard procedures and incubated for 15 minutes at 80°C. Thereafter, the colorimetric assay was done according to the manufacturer’s protocol (Roche Applied Science, Indianapolis, IN).

Flow cytometry. For the determination of different T-cell subpopulations, spleenocytes were stained for CD4, CD8, CD25, and Ly-6C (BD PharMingen, San Diego, CA) by four-color flow cytometric analysis.
and $10^5$ cells were counted for each analysis. To enumerate CEPs, 200 µL heparinized peripheral blood were collected, BBC lysed with ACK lysis buffer (Walkersville, MD), and cells stained for CD3, CD34, and flk-1 (BD Pharmingen) in three-color flow cytometric analyses with $2.5 \times 10^5$ cells used for each analysis.

**Statistical analysis.** The statistical significance of differential findings between experimental groups and controls was determined by Student's t test. Findings were regarded significant, if $P < 0.05$.

**Results and Discussion**

Based on the findings of Vig et al. (9), who established a link between the lack of iNOS and higher frequencies of memory T cells in response to peptide immunizations, we hypothesized that nitric oxide could be a mediator of immunostimulatory and/or antivascularogenic effects of cyclophosphamide. To this end, iNOS$^{-/-}$ mice were used to compare their generation of NO with that of C57Bl/6j (iNOS$^{+/+}$) control mice after either mock or low-dose cyclophosphamide treatment. As shown (Fig. 1A), treatment with low-dose cyclophosphamide inhibited the generation of NO in iNOS$^{-/-}$ controls but not in iNOS$^{-/-}$ mice, showing a major role for iNOS in the cyclophosphamide-mediated suppression of NO production (10). Moreover, cyclophosphamide was able to increase the percentage of CD8$^+$ T cells with a memory phenotype by increasing the level of Ly-6C expression in iNOS$^{+/+}$ but not in iNOS$^{-/-}$ mice (Fig. 1B). We could also show that the cyclophosphamide-induced decrease of T cells with a regulatory phenotype (CD4$^+$CD25$^+$) is mainly mediated by iNOS as we observed a marked decrease of regulatory T cells in iNOS$^{-/-}$ mice treated with cyclophosphamide but not in iNOS$^{-/-}$ mice (Fig. 1C). The decrease observed in T cells with a regulatory phenotype can be explained by differences in the susceptibility of various T-cell subpopulations to NO (11). In contrast to these two immunostimulatory effects of cyclophosphamide, there was no detectable iNOS dependence of cyclophosphamide-mediated antivascularogenic effects as shown by the lack of any significant difference in the decrease of CEPs, indicated by CD3$^+$/CD34$^+$/flk-1$^+$ cells (12), between iNOS$^{-/-}$ and iNOS$^{-/-}$ mice after cyclophosphamide treatment. This finding can be explained by recent reports that treatment with low-dose cyclophosphamide can lead to up-regulation of the antiangiogenic factor thrombospondin-1 (Tsp-1; ref. 5), which is known for its ability to mediate apoptosis of endothelial cells in association with increased expression of proapoptotic Bax and decreased expression of antiapoptotic Bcl-2 molecules (13). Furthermore, Tsp-1 is capable of sequestering vascular endothelial growth factor (14), thereby preventing it from exerting such proangiogenic functions as the induction of an increased expression of survivin, an inhibitor of apoptosis protein in endothelial cells (15), and the expansion (16) and mobilization of CEPs (17).

To determine, whether the immunomodulatory effects of low-dose cyclophosphamide therapy also apply to tumor-bearing mice, cyclophosphamide therapy was combined with a DNA vaccine against PDGF-B, which is overexpressed by proliferating endothelial cells in the tumor microenvironment but not by any of the tumor cell lines used in our studies (data not shown). Three different preclinical mouse tumor models were applied. In the first experiment (Fig. 2A), we took advantage of the murine plasmacytoma model MOPC-315 and challenged syngeneic BALB/c mice s.c. with MOPC-315 cells. Although mice were challenged with 50 times the minimal lethal dose, a marked delay in the onset of tumor growth in such mice was shown when treated with either chemotherapy (cyclophosphamide) or immunotherapy. In our approach, the immunotherapeutic entity consisted of an orally given DNA vaccine encoding PDGF-B, carried by attenuated S. typhimurium (Aro$^+$). Following gavage, these avirulent bacteria proved to be efficient in delivering plasmid DNA to such secondary lymphoid tissues as Peyer's patches in the small intestine. As observed previously, this resulted in the subsequent induction of a robust CD8$^+$ T cell-mediated immune response against target cells overexpressing the respective antigen (8). Although this inhibition of tumor growth was quite significant, the mice had to be sacrificed due to the huge tumor burden that ultimately reached the level of that in untreated or control-treated mice. In contrast to single therapy with the DNA vaccine, the combination of cyclophosphamide with this same vaccine could completely inhibit further growth progression of these highly aggressive tumors for 50 days (Fig. 2A). In a second experiment, BALB/c mice were challenged o.t. with D2F2 cells in a syngeneic murine breast cancer model (Fig. 2B). In this case, the treatment with cyclophosphamide was initiated at a time point when the tumors were still small (~10 mm$^3$) compared with the previous experiment and thus more closely resembled clinical conditions. The group of mice treated with the combination therapy not only showed an inhibition of tumor growth for 80 days but more importantly revealed complete regression of tumors in three of eight mice. In view of the recently proposed three-stage model of cancer immunomodulating (18), consisting of tumor cell elimination via cancer immunosurveillance, equilibrium, and escape, we conclude that our combination treatment was sufficient to revert tumors from phase 3 which escaped immune recognition and destruction, to tumors in phase 1 as indicated by the complete remission in 37.5% of the mice challenged with D2F2 breast cancer cells, or to phase 2 of stable disease as indicated by the majority of tumors not regrowing even

**Figure 1.** Effects of cyclophosphamide on iNOS$^{+/+}$. A. NO serum concentrations from iNOS$^{+/+}$ or iNOS$^{-/-}$ mice treated with cyclophosphamide (CTX) or PBS as a control. The respective percentages of CD8 Ly-6C$^+$ T cells (B) and CD4 CD25$^+$ T cells (C) as well as the number of CD3 CD34$^+$flk-1$^+$ cells (D) were determined by flow cytometry. Columns, mean (n = 3); bars, ±SD.
In a third approach, C57BL/6J mice were challenged i.v. with a lethal dose of syngeneic D121 LCC cells to induce experimental pulmonary metastases and groups of such animals were depleted in vivo of either CD8+ or CD4+ T cells with the corresponding antibodies (Fig. 2C). This combination treatment led to a significant increase in life span compared with control- or single therapy-treated mice. More importantly, the in vivo depletion experiments indicated that the increase in life span observed in the group of mice subjected to the combination therapy depended mainly on CD8+ T cells, suggesting that the improvement of the antitumor effect achieved by adding cyclophosphamide depends mainly on its immunostimulatory properties and not on any added antivasculogenic effect. We therefore asked ourselves whether cyclophosphamide was capable of exerting its immunomodulatory and antivasculogenic effects also in mice bearing tumors in the tumor escape phase of immunoediting. To this end, the different control and treatment groups of mice were analyzed for their percentages of T cells exhibiting either a memory or a suppressor phenotype as well as for the number of CEPs. As indicated (Fig. 3), cyclophosphamide, either given alone or in combination with the DNA vaccine against PDGF-B, was capable of lowering the serum concentration of NO (Fig. 3A), thereby augmenting the percentage of memory T cells (Fig. 3B) and decreasing both the percentage of suppressor T cells (Fig. 3C) and the number of CEPs (Fig. 3D).

Taken together, we were able to show that the cyclophosphamide-mediated inhibition of iNOS is directly linked to its immunostimulatory effects but not to its antivasculogenic effects. Importantly, we showed that the mechanism described applies to tumor bearing mice in vivo by combining cyclophosphamide with after stopping the combination therapy. In fact, a decrease in the percentage of CD4+CD25+ T regulatory cells has been shown at least in two cases to be sufficient for a conversion from tumor escape to tumor rejection (19, 20).
a novel DNA vaccine against PDGF-B, which is expressed on proliferating endothelial cells. This combinational therapy was able to completely suppress tumor growth in two therapeutic tumor models and significantly enhance the life span in a therapeutic survival model. These findings provide a new rationale for combined treatments with agents, which, in addition to cyclophosphamide, inhibit iNOS or the production of NO and therefore lead to active and effective cancer immunotherapy.

References
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