Transforming growth factor-β-mediated down-regulation of antitumor cytotoxicity of spleen cells from MOPC-315 tumor-bearing mice engaged in tumor eradication following low-dose melphalan therapy

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Received: 1 September 1993 / Accepted: 28 October 1993

Abstract: We have previously shown that treatment of mice bearing a large MOPC-315 plasmacytoma with a low dose of the anticancer drug melphalan (L-phenylalanine mustard; L-PAM) results in the acquisition of a potent CD8+ T-cell-mediated anti-MOPC-315 cytotoxic T lymphocyte (CTL) activity by the hitherto immunosuppressed tumor bearers, and this immunity contributes to complete tumor eradication. In the studies presented here, we sought to determine how the acquisition of this antitumor immunity following low-dose chemotherapy is possible, in light of the report that MOPC-315 tumor cells produce transforming growth factor-β (TGF-β), an immunosuppressive cytokine that can down-regulate the generation of CTL responses. We found that the acquisition of CTL activity following low-dose L-PAM therapy is not due to a chemotherapy-induced decrease in the sensitivity of MOPC-315 tumor bearer spleen cells to TGF-β-mediated inhibition of CTL generation. Moreover, even spleen cells from MOPC-315 tumor-bearing mice, which had received L-PAM therapy 7 days earlier and had acquired CTL activity in vivo, were sensitive to the inhibitory activity of TGF-β upon culture for as little as 1 day, with or without stimulator tumor cells. However, the production of TGF-β by MOPC-315 tumors decreased drastically as a consequence of the low-dose chemotherapy. Thus, the curative effectiveness of low-dose L-PAM therapy for MOPC-315 tumor-bearing mice may be due, at least in part, to a reduction in TGF-β production that enables the development of tumor-eradicating immunity.

Key words: Immunomodulation - Low-dose chemotherapy - TGF-β

Introduction

The therapeutic effectiveness of anticancer drugs can be facilitated by antitumor immunity [2, 5, 6, 14–17, 19–23, 29, 32]. Consequently, situations should exist under which a low, non-immunosuppressive dose of drug can be as effective therapeutically as a high, immunosuppressive dose of drug, even though the lower dose exerts a substantially weaker direct antitumor effect. Indeed, several investigators have shown that a low dose of drug in cooperation with the host antitumor immunity, which emerges shortly after the chemotherapy, can be as effective, or even more effective, than a high dose of drug in the treatment of tumor bearers [2, 6, 14, 15, 17].

The importance of antitumor immunity for the therapeutic outcome of low-dose chemotherapy has been studied extensively in the MOPC-315 tumor system [2, 5, 17, 19, 20, 29, 32, 33]. These studies established that a low-dose of L-phenylalanine mustard (L-PAM) is as effective as a high dose of L-PAM for the cure of mice bearing a large s.c. tumor and extensive metastases, because of the participation of CD8+ T-cell-dependent antitumor immunity in tumor eradication. Moreover, as a consequence of low-dose L-PAM therapy, there is an 80-fold increase in the number of CD8+ T cells present in the primary tumor nodule, which is associated with a 2000-fold increase in anti-MOPC-315 cytotoxic T-lymphocyte (CTL) activity.

In light of the recent report that MOPC-315 tumor cells produce transforming growth factor-β (TGF-β) [3], which is an effective inhibitor of the generation of CTL responses [4, 9, 10, 13, 22, 26, 28, 30, 31], we sought to determine...
how such a potent CD8+ CTL response develops after low-dose chemotherapy of mice bearing a large MOPC-315 tumor and extensive metastases. Accordingly, we examined whether, as a consequence of low-dose L-PAM therapy, (a) the sensitivity of tumor bearer spleen cells to TGF-β-mediated inhibition of CTL generation is decreased, and/or (b) the production of TGF-β by the tumor is decreased.

Materials and methods

Tumors. We have employed the weakly immunogenic [27] MOPC-315 plasmacytoma, which was maintained in vivo, as previously described [5, 33], in female BALB/c mice 7–10 weeks old (Charles River Breeding Laboratories, Wilmington, Mass.). Routinely, mice were inoculated s.c. with 1 × 106 viable tumor cells, a dose that is at least 300-fold the minimal lethal tumor dose, and leads to the appearance of a palpable tumor in 4–5 days. Therefore, the tumors grow progressively, killing the mice in approximately 16 days.

Chemotherapy. A fresh stock solution of 10 mg/L-PAM/ml (Burroughs Wellcome Co., Triangle Park, N.C.) was prepared just prior to injection, as previously described [2], and was further diluted with phosphate-buffered saline (PBS), pH 7.2, to the desired concentration. A dose of 2.5 mg L-PAM/kg body weight (low-dose) was administered i.p. to BALB/c mice bearing a large (approx. 20-mm) tumor that resulted from the s.c. inoculation of 1 × 106 MOPC-315 tumor cells 10 days earlier. This dose of drug is the lowest dose that is curative for almost all (at least 90%) BALB/c mice bearing a large (approx. 20-mm) MOPC-315 tumor [2], and requires the participation of CD8+ T-cell-dependent antitumor immunity in tumor eradication [2, 19, 29]. MOPC-315 tumor-bearing mice treated with 2.5 mg/kg L-PAM are referred to as L-PAM TuB mice.

Lymphoid cell suspensions. Single-cell suspensions were prepared, as previously described [18], by mechanical disruption between glass slides. Splenies were obtained from normal BALB/c mice, tumor-bearing mice that had been injected with 1 × 106 MOPC-315 tumor cells 10 days earlier, or MOPC-315 tumor-bearing mice that had received low-dose L-PAM therapy 3 or 7 days earlier (day 3 or 7 L-PAM TuB mice) at a time when the mice bore large, day 10 tumors. CD8+ splenic T-cells from tumor-bearing mice acquire anti-MOPC-315 cytotoxicity in vivo as a consequence of low-dose chemotherapy [19, 33]. This antitumor cytotoxicity, which is first detected 5 days after chemotherapy and is highest 7 days after chemotherapy, is specific for MOPC-315-associated antigens; no natural killer activity is evident [19, 33].

Preparation of tumor cell supernatants. Tumor cell supernatants were prepared according to the method of Berg and Lynch [3], with slight modifications. Briefly, MOPC-315 tumor cells were obtained from the s.c. tumor nodule of mice that had been injected 10–13 days earlier with 1 × 106 tumor cells, or from tumor-bearing mice that had been injected with 1 × 106 tumor cells 13 days earlier but treated on day 10 with 2.5 mg/kg L-PAM. The cells were cultured at 2 × 106 cells/ml in RPMI-1640 medium [Grand Island Biological Co. (Gibco), Grand Island, N.Y.] supplemented with 50 units/ml penicillin and 50 μg/ml streptomycin (Gibco). Culture supernatants were collected after 48 h and 100 mM phenylmethylsulfonyl fluoride (Sigma, St. Louis, Mo.), dissolved in dimethyl sulfoxide (Sigma) was added to a final concentration of 1.0 mM. Although latent TGF-β can be converted in vivo and in vitro into a biologically active form, and in fact latent TGF-β added exogenously was shown to inhibit CTL generation to a similar extent as acid-activated TGF-β [31], TGF-β activity in tumor cell supernatants as measured by the MvILu assay was reported in some situations to be greater following acid activation [28]. Therefore, to maximize the detection in the MvILu assay of TGF-β with potential inhibitory activity for CTL generation, the MOPC-315 supernatants were dialyzed against 1.0 M acetic acid [12]. Subsequently, the supernatants were centrifugally concentrated using a Centriprep-10 (W.R. Grace and Co., Beverly, Mass.). Bovine serum albumin (Sigma) was added to a final concentration of 1.0 mg/ml, and the samples were dialyzed against 4.0 mM HCl, and then against Dulbecco’s PBS, pH 7.2 (Gibco). Final preparations were 1/10 of the starting volume. The preparations were then divided into aliquots and stored at −70°C until they were employed in experiments. The supernatant dilutions provided in the figures represent those of the concentrated preparations.

TGF-β. Platelet-derived human transforming growth factor-β1 was obtained from Collaborative Biomedical (Bedford, Mass.), and a stock solution was made by diluting to a concentration of 250 ng/ml in 36% acetonitrile (Sigma), 0.3% trifluoroacetic acid (Sigma). Aliquots of stock solution were stored at −70°C, thawed immediately prior to use, and diluted to the desired concentration in RPMI-1640 medium or PBS. As a control, in all experiments in which TGF-β was used, corresponding dilutions of the TGF-β solvent (acetonitrile/trifluoroacetic acid solution) were also employed.

TGF-β bioassay. As commonly done [3, 4, 28, 34, 35], we determined the presence of TGF-β in culture supernatants of MOPC-315 tumor cells (according to the method of Ikeda et al. [8]) by determining their inhibitory activity for MvILu proliferation. Briefly, MvILu cells (American Type Culture Collection, Rockville, Md.) were cultured in flat-bottomed 96-well strip plates (Costar, Cambridge, Mass.) at (1–3.5) × 103 cells/well in 200 μl final volume of a solution comprised of (a) 100 μl of minimal essential medium (Gibco) supplemented with 20% heat-inactivated fetal bovine serum (FBS; Sigma), 100 μ/ml penicillin, 100 μg/ml streptomycin, and 2% nonessential amino acids (Gibco), and (b) 100 μl of PBS (pH 7.4) containing concentrated tumor cell supernatants or platelet-derived TGF-β. Turkey anti-TGF-β antibody (Collaborative Biomedical) or a pan-specific rabbit anti-TGF-β (i.e., anti-TGF-β1, 1.2, 2, 3, and 5) IgG and normal rabbit IgG (R & D Systems, Minneapolis, Minn.) were incubated in some experiments with the concentrated tumor cell supernatants or MvILu tumor cells in TGF-β for at least 60 min prior to the addition of MvILu cells. Further culture at 37°C for 56 h, 1 μC [3H]thymidine (Amersham, Arlington Heights, Ill.) was added in 25 μl culture medium to each well; after an additional 7 h incubation, the samples were harvested and counted by beta scintillation. Each group consisted of at least seven but usually ten replicates; each experiment was performed at least three times.

In vitro stimulation. Spleen cells were stimulated in vitro with MOPC-315 tumor cells according to the method we have previously described for the in vitro generation of CTL activity by normal or L-PAM TuB spleen cells [18, 32]. Briefly, 40 × 106 spleen cells were cultured in vitro at 37°C for 5 days in the presence or absence of 1.33 × 106 mitomycin-C-treated (50 μg/ml for 30 min) MOPC-315 tumor cells in a 30-ml tissue culture flask (Corning Glass Works, Corning, N.Y.) in a final volume of 20 ml RPMI-1640 medium supplemented with 5% FBS, 1% nonessential amino acids, 50 units/ml penicillin, 50 μg/ml streptomycin and 50 μM 2-mercaptoethanol (Sigma). TGF-β (or solvent control) was always added at the time of initiation of the 5-day culture.

Antitumor cytotoxicity assay. The lytic activity of fresh spleen cells as well as of cultured spleen cells was determined as previously described [18], by the 3.5-h 51Cr release assay. Briefly, 5 × 104 51Cr-labeled MOPC-315 tumor cells were incubated with various numbers of spleen cells in 12 × 75-mm plastic tubes. At the end of the incubation period, the cells were pelleted and both supernatants (Sup) and pellets (P) were counted in an Auto-Gamma scintillation counter. The percentage of 51Cr release for each sample was calculated as follows:

\[ 51\text{Cr release}(\%) = 100 \times \frac{(\text{Sup} + \text{CPM in Sup})}{(\text{CPM in Sup} + \text{CPM in P})} \]

Subsequently, the percentage of specific 51Cr release was calculated by the following formula:

- Percentage of specific 51Cr release = \( 100 \times \frac{(T-C)}{(C-M)} \)

where T is the percentage release with test lymphocytes, C is the mean of three replicates of the percentage of spontaneous release (which