Mechanism of Diethyldithiocarbamate Modulation of Murine Bone Marrow Toxicity

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ABSTRACT

Sodium diethyldithiocarbamate (DDTC) has been shown to modulate the myelosuppression that commonly occurs following treatment with anticancer drugs in mice. In order to investigate the mechanism of action of this myeloprotector, murine long-term bone marrow cultures were treated with DDTC alone or were preceded by the anticancer drug cis-diamminedichloroplatinum(II) (CBDCA), and the granulocyte/macrophage colony-stimulating activity of the supernatants was measured. The supernatants harvested from DDTC-treated cultures enhanced proliferation of granulocyte/macrophage progenitor cells almost 4-fold compared to cultures treated with no drug or with CBDCA alone. Pretreatment of cultures with CBDCA neither enhanced nor inhibited DDTC-induced colony-stimulating activity. Similar results were obtained by using marrow stromal cell cultures free of hematopoietic cells. Thus, DDTC may hasten bone marrow recovery by augmenting stromal cell production of a factor(s) with hematopoietic colony-stimulating activity.

INTRODUCTION

Myelosuppression is a common dose-limiting complication of chemotherapy and can result in significant patient morbidity and mortality. DDTC is a small organic anion that has been shown to modulate the myelosuppression associated with administration of structurally diverse cancer chemotherapeutic drugs and to induce increased proliferation of murine hematopoietic stem cells (spleen colony-forming units) and granulocyte/macrophage progenitor cells (GM-CFC) (1-6). However, DDTC does not alter the number of hematopoietic stem or progenitor cells proliferating in vivo in the absence of a myelotoxic insult (2, 5, 6), suggesting that damage to the marrow may be a prerequisite for the myeloprotective effect of DDTC. This effect appears to be selective for bone marrow in that DDTC does not stimulate tumor cell proliferation in vitro or in vivo and does not reduce the cytotoxic effect when given in combination with a number of anticancer drugs (4-6).

The mechanism of this myeloprotective effect is unclear. DDTC is effective in mice at doses as low as 300 μg/kg. In vivo it stimulates DNA synthesis in bone marrow cells of mice that have been treated with a myelotoxic drug but does not alter DNA synthesis in L1210 leukemia cells (6). This selective stimulation of hematopoietic cell proliferation is similar to that reported for several cytokines (7, 8). The following experiments were designed to assess whether DDTC might be acting via stimulation of growth factor production.

MATERIALS AND METHODS

Materials. CBDCA was the generous gift of Johnson-Matthey, Inc. (Malvern, PA). DDTC was obtained from Sigma Chemical Co. (St. Louis, MO). Fischer's medium, α-minimal essential medium, 1-glutamine, pokeweed mitogen, sodium bicarbonate (7.5% solution), gentamicin, and penicillin/streptomycin solution were purchased from GIBCO (Grand Island, NY). Horse serum and fetal bovine serum were purchased from HyClone Laboratories (Logan, UT). Salmonella typhosa lipopolysaccharide B was purchased from Difco Laboratories (Detroit, MI). Methylcellulose (4A premium grade) was kindly provided by Dow Chemical Co. (Midland, MI). Falcon Petri plates and microscope slides were purchased from Fisher Scientific Co. (Springfield, NJ). All other plastic culture supplies and test tubes were obtained from VWR (Rochester, NY).

Experimental Animals. Male 6- to 8-week-old C57BL/6J × DBA/2J F1 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were housed 10/cage in plastic cages and allowed food and water ad libitum. All mice were acclimatized for at least 7 days; the animals were then killed by cervical dislocation, and both femurs and tibias were harvested for these experiments.

Establishment of Murine LTBMC. Long-term bone marrow cultures were established according to a previously published method (9). One ml of growth medium [Fischer's medium (pH 7.0) supplemented with 25% horse serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin] was used to aseptically flush marrow cells from one murine tibia and femur into a 25-cm2 flask containing 9 ml of growth medium. The cultures were maintained in a fully humidified incubator, 5% CO2 atmosphere, at 33°C. Weekly feeding was performed by replacement of the spent medium and nonadherent cells with 10 ml of fresh medium. Where specified, the medium also contained 10^-5 M hydrocortisone sodium hemisuccinate, which is known to facilitate development and maintenance of the adherent cells (10).

Cultures of the stromal bone marrow cells were established in the same fashion. However, the horse serum (25%) was replaced with 20% fetal bovine serum as a supplement to Fischer's medium (pH 7.0) and the antibiotic solution as described above. These culture conditions do not allow survival of colony-forming units-granulocyte, erythrocyte, macrophage, myelocyte or GM-CFC (confirmed by removing the adherent cells from three cultures and testing for the presence of colony-forming units-granulocyte, erythrocyte, macrophage, myelocyte or GM-CFC). In all other respects the cultures were initiated and maintained as described above.

GM-CFC Assay. This assay was carried out by using the method previously described (2), but with the following modifications. Bone marrow cells were harvested from untreated mice, and in the LTBMC experiments the PWM-SCCM was replaced with 500 μl of supernatant harvested from the drug-treated (or control) LTBMC. Granulocyte/macrophage colonies > 50 cells were counted on day 7 with the aid of a dissecting microscope. The morphology of the cells in the colony was verified by removing the colonies from the media with a finely drawn pipet, resuspending the colony in 0.4 ml of media (α-minimal essential medium or Fischer's) supplemented with 1-5% serum (horse or fetal bovine serum), spinning the colony onto a slide with a Cytospin apparatus, and staining with Wright-Giemsa stain. Positive (maximally stimulating amounts of PWM-SCCM included in the culture medium) and negative (no mitogen added) controls were included with each assay. The formation of colonies under these conditions was indicative of colony-stimulating activity in the LTBMC supernatants.

Determination of CSA in Media of Drug-treated LTBMC. The cultures were allowed to grow for 5 to 6 weeks prior to experimentation. Twelve cultures were randomly divided into four groups (control, DDTC, CBDCA, and CBDCA followed by DDTC), 3 cultures/group.
Drug solutions were prepared immediately prior to use with unsupplemented medium and were filter sterilized. CBDCA (300 µM in 10 ml medium) was applied to CBDCA- and CBDCA/DDTC-treated groups while the control and DDTC-treated groups received medium only. Cultures were replaced in the incubator for 1 h. The medium/drug solution was then removed and DDTC (300 µM in 10 ml medium) was added to DDTC and CBDCA/DDTC groups, while the control and CBDCA cultures received medium only. After 1 h these solutions were removed, and 10 ml of supplemented Fischer’s medium were added to each culture. At the specified time this medium along with any non-adherent cell group was removed, and the cells were pelleted by centrifugation (800 × g for 5 min). The supernatants were evaluated for colony-stimulating activity in the GM-CFC assay as described above.

A similar procedure was used to determine the response of drug-treated cultures exposed to mitogen stimulation. In these experiments Salmonella typhosa lipopolysaccharide B (5 µg/ml) was added to the supplemented Fischer’s medium following drug treatment. At the specified times the medium was removed and tested for colony-stimulating activity as described above.

The CSA production stimulated by various doses of DDTC was determined by treating triplicate cultures with the specified concentration of DDTC or medium alone for 1 h. This solution was then replaced with supplemented Fischer’s medium. Forty-eight h later the medium was removed, nonadherent cells were pelleted by centrifugation, and the supernatant was tested for colony-stimulating activity.

RESULTS

Murine LTBMC were established by a modification of the standard procedure (9). After 5 weeks, cultures were treated with DDTC with or without prior treatment with CBDCA. At various times the supernatants were removed and the CSA of each supernatant was assessed by using it to replace the pokeweed mitogen-stimulated spleen cell-conditioned medium in the GM-CFC assay. Basal levels of granulocyte/macrophage CSA in control supernatants varied with each experiment; differences in serum constituents are known to affect the ability of LTBMC to support hematopoiesis (9). The results of a typical experiment are shown in Fig. 1, and enhancement of CSA in three separate experiments is summarized in Table 1. CSA was augmented almost 4-fold in supernatants from DDTC-treated cultures, and this level represented about 50% of the maximal stimulation observed with conditioned medium (PWM-SCCM). CBDCA had no significant effect on CSA either alone or when added just prior to DDTC treatment. DDTC enhanced CSA at concentrations from 100 to 1000 µM (Fig. 2); these concentrations are readily achieved in the plasma of patients treated with DDTC (11).

DDTC may be enhancing production of a factor(s) that stimulates progenitor cells. Alternatively, DDTC could be neutralizing the effect of an inhibitory factor(s). To address this question, two different agents known to have CSA (12) were evaluated in combination with DDTC. Addition of hydrocortisone hemisuccinate to the DDTC-treated cultures neither enhanced nor diminished DDTC-induced CSA compared to treatment with DDTC alone (data not shown). Supernatants from cultures treated with a maximally stimulating concentration of LPS (5 µg/ml) induced formation of 195 colonies/10⁶ cells; neither DDTC, CBDCA, nor the combination of CBDCA and DDTC significantly changed the CSA of these supernatants (190–210 colonies/10⁶ cells). These results suggest that DDTC is inducing production of a colony-stimulating factor(s) that is not additive with stimulation by either hydrocortisone or LPS.

The hematopoietic microenvironment is thought to play a pivotal role in the regulation of blood cell production and differentiation (13, 14). Stromal cells are most likely responsible for elaborating the colony-stimulating factors that regulate the LTBMC system. Thus LTBMC containing stromal cells (including monocytes/macrophages) were established (15). The cells growing in these cultures were plated in standard clonogenic assays, and no progenitor or stem cell growth was observed, thereby confirming the absence of hematopoietic progenitor cells. Untreated supernatants from these cultures had greater CSA compared to those obtained from the complete LTBMC, and DDTC treatment enhances CSA approximately 2-fold compared to untreated cultures (Fig. 3). Again, CBDCA treatment had no significant effect on untreated or DDTC-treated cultures. The data in Figs. 1 and 3 imply that DDTC
stimulation of CSA is most pronounced during the first 24 h after treatment. This assumption was confirmed by comparing the CSA of supernatants collected over varying time intervals. CSA was significantly enhanced by DDTC in supernatants collected between 0 and 8 h and 8 and 24 h after DDTC treatment but was not significantly different from untreated supernatants obtained during later time intervals (data not shown).

DISCUSSION

The above findings suggest that DDTC modulates hematopoietic toxicity by inducing stromal cell production of a factor or factors that stimulate hematopoiesis. Although DDTC stimulates proliferation of both stem cells and GM progenitors only after pretreatment with a myelotoxic drug in vivo (2, 6), CSA was increased by DDTC alone in vitro. Treatment of LTBM with a cytotoxic concentration of CBDCA had no effect on CSA in this system, and it neither enhanced nor inhibited the DDTC response in vitro. These results are consistent with a mechanism in which DDTC augments rather than initiates a proliferative response; the response is presumably initiated by cytotoxic drug in vivo and by the addition of fresh medium in vitro (9). The involvement of stromal cells in the DDTC response may also account for the variable results observed with different cytotoxic agents (1–6), because direct toxicity to stromal cells (16) would be expected to reduce the DDTC response.

Although the identity of the factor(s) responsible for the CSA induced by DDTC is not known, several cytokines may be potential candidates. IL-1 has been shown to trigger the release of factors stimulating proliferation of pluripotent and GM colonies (7, 8), and the effect is modulated through the hematopoietic microenvironmental cells (17). Furthermore, LPS stimulates production of IL-1, and the levels of IL-1 induced are dependent upon culture conditions (18). The absence of additivity between DDTC and LPS is consistent with a common cytokine (21), it is not apparent that this factor directly stimulates stem cell proliferation, so it is difficult to explain the marked effect of DDTC on CFU-S in vivo based exclusively on GM-CSF production. LPS has been reported to stimulate GM-CSF production by stromal cells in vitro, but this effect may be secondary to LPS-induced release of IL-1 (22). Thus GM-CSF may play a role in the DDTC response, but its effects may be secondary to release of another cytokine (23). These results represent the first example of bone marrow proliferation resulting from induction of colony-stimulating factor(s) by a small molecule. Although the proliferative effects in vitro are independent of cytotoxic drug treatment, proliferation of both stem cells and GM progenitors is induced by DDTC in vivo only after a toxic insult to the marrow, suggesting that production of or response to the factor(s) is dependent upon the preexisting proliferative and/or regulatory state of the bone marrow. Thus DDTC may offer a unique clinical alternative to the administration of recombinant colony-stimulating factors in the treatment of myelosuppression resulting from high-dose chemotherapy.

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