ABSTRACT

Previous studies using unseparated normal human bone marrow cells have indicated that recombinant tumor necrosis factor α (rTNF-α) can inhibit the in vitro colony growth by normal granulocyte/macrophage (CFU-GM) and erythroid (BFU-E) progenitor cells in a dose-dependent manner. In the present studies, by using very low numbers of highly enriched normal bone marrow progenitor cell populations as target cells, we have extended these previous findings to provide convincing evidence that erythroid and myeloid colony growth suppression by rTNF-α is manifested by a direct interaction between rTNF-α and CFU-GM and BFU-E progenitor cells.

In addition, the sensitivity of normal peripheral blood and chronic myeloid leukemia bone marrow CFU-GM and BFU-E colony growth to inhibition by rTNF-α was examined and found to be comparable with that of normal bone marrow CFU-GM and BFU-E.

Although the continuous presence of high doses of rTNF-α (5000 units/ml) was required in methylcellulose cultures for maximal CFU-GM (90%) and BFU-E (70%) colony suppression, short-term exposure (24 to 72 hr) of normal bone marrow-enriched progenitor cells to rTNF-α, in the absence of hematopoietic growth factors, was sufficient to irreversibly suppress up to 50 to 65% of CFU-GM colony growth. In contrast, the number of BFU-E colonies was increased under these conditions. If, however, hematopoietic growth factors (Mo-T-cell-conditioned medium and erythropoietin) were present during preincubation of the cells with rTNF-α, BFU-E were then slightly suppressed while the extent of CFU-GM inhibition remained essentially the same.

The suppressive effect of rTNF-α on erythroid and myeloid progenitor cell growth appears to be most pronounced on the more primitive stages of committed progenitor cell development, since inhibition of CFU-GM- and BFU-E-derived colony growth progressively decreased with the delayed addition of rTNF-α to methylcellulose cultures.

[3H]Thymidine incorporation was also inhibited by rTNF-α in normal bone marrow-enriched progenitor cell populations stimulated to proliferate in liquid culture by colony-stimulating factors. This effect was transient, however, since the activity of rTNF-α declined after the first 24 h of culture at 37°C, particularly at low doses of rTNF-α where the activity was completely lost after 48 h of culture. This loss of activity appeared to be due to a decreased sensitivity of progenitor cells to the antiproliferative effects of tumor necrosis factor (TNF) after an initial exposure rather than a lack of available TNF.

The use of purified hematopoietic progenitor cells should facilitate further studies in elucidating the interactions occurring between TNF and these cells at the cellular level as well as molecular level.

INTRODUCTION

TNF, initially characterized by Carswell et al. (1) as a cytotoxin in the serum of *Bacillus Calmette-Guérin*-sensitized mice given injections of endotoxin, has recently been the subject of intense investigation due to its known ability to cause hemorrhagic necrosis and subsequent regression of experimental animal tumors (1-3).

Recently, purification and cloning of the gene for human TNF (3-6) have made possible further detailed studies of the biological effects of TNF. Such studies to date have demonstrated that TNF has a cytotoxic/cytostatic effect on certain human and murine tumor cell lines (2, 7); stimulates the growth of fibroblasts (7, 8); enhances monocyte and neutrophil cytotoxicity (9, 10); induces monocyte differentiation of human myeloid leukemic cell lines (11); stimulates the production of GM-CSF from normal human lung fibroblasts, vascular endothelial cells, and from cells of several malignant tissues (12); and inhibits lipoprotein lipase activity in fat cells which may lead to cachexia during parasitic and bacterial infections (13, 14).

Because of its demonstrated ability to induce tumor necrosis in vivo and cytotoxic/cytostatic specificity for some tumor cell lines in vitro, clinical trials evaluating the efficacy of TNF as an antineoplastic agent are currently under way. Of relevance to these trials are the recent studies of others (15-17) providing evidence indicating that TNF suppresses normal human myelopoiesis. Thus, it has been demonstrated that TNF profoundly inhibits colony formation in vitro by CFU-GM and erythroid progenitor cells (BFU-E and CFU-E) from normal human bone marrow. However, in these studies, since a relatively heterogeneous or partially purified population of bone marrow cells was used as target cells in their clonogenic assays, it could not be determined if the observed inhibitory effects on hematopoietic colony growth by TNF were the result of direct interaction with hematopoietic colony-forming cells or rather were mediated through contaminating accessory cells.

Recently, our laboratory (18, 19) as well as others (20-22) have demonstrated that a highly enriched population of hematopoietic progenitor cells devoid of accessory cells can be obtained from normal human bone marrow and peripheral blood by negative selection using a panel of monoclonal antibodies directed against myeloid, erythroid, and lymphoid maturation antigens followed by depletion of antibody-coated cells using well-established immunoadherence or immune-rosetting methods. The resulting enriched progenitor cell populations can be cultured at such low cell numbers that contamination by any other cell types that may influence the proliferative behavior of CFU-GM and BFU-E is greatly minimized, if not negligible. As such, they serve to facilitate a direct study of the stimulatory...
and inhibitory factors which have been implicated in regulating hematopoiesis at the progenitor cell level.

In the present studies, the in vitro effects of rTNF-α on CFU-GM and BFU-E colony growth were examined using highly enriched progenitor cell populations obtained from normal human bone marrow and peripheral blood, as well as bone marrow from patients with CML. Our results indicate that (a) rTNF-α exerts a direct dose-dependent suppressive effect on erythroid and myeloid colony growth in vitro, (b) this effect appears to decrease with cellular maturation in the cultures, (c) the continued presence of rTNF-α is required for maximal colony suppression and (d) the sensitivity to rTNF-α of CFU-GM and BFU-E progenitors from patients with CML and from normal donors is comparable.

MATERIALS AND METHODS

Bone Marrow Specimens. After appropriate humane protection committee validation and informed consent, posterior iliac crest bone marrow and peripheral blood specimens were obtained from normal volunteers and also four patients with Ph'-positive CML by aspiration containing-preneur cells, depleted of any known accessory cells, were obtained by negative selection, using immunoadherence and/or immune rosetting techniques as previously described in detail (19, 25, 26).

Enriched Normal Bone Marrow Progenitor Cells. Table 1 shows that colonystimulating Factor-dependent Myeloid Progenitor Cell Proliferation Assay in Liquid Culture. Proliferation of normal bone marrow-enriched myeloid progenitor cells was assayed as follows. Briefly, 10,000 normal bone marrow-enriched myeloid progenitor cells were cultured in 0.2 ml of IMDM containing 10% FCS and 10% MoCM for 24, 48, and 72 h in flat-bottomed microtiter 96-well plates (Costar) at 37°C and 5% CO₂ with or without various concentrations of rTNF-α. Four h before each cell harvest time point, 50 μl of IMDM containing 0.5 μCi of [³H]dThd (New England Nuclear, Boston, MA) were added. Cells were harvested onto glass fiber filters using an automatic cell harvester (Mini-mash II; Whitaker-M. A. Bioproducts, and [³H]dThd incorporation was measured in a Packard scintillation counter (Packard Instruments Co., Downer's Grove, IL). Assays were performed in triplicate, and the results were expressed as a mean ± SD.

Short-Term Cultures of Myeloid Progenitor Cells. In those studies in which normal bone marrow-enriched progenitor cells were placed in liquid culture for 24, 48, and 72 h prior to plating in methylcellulose, cells were plated in 12 x 75 mm polyplyethylene tubes at 10,000 to 30,000/ml in IMDM plus 10% FCS with or without 50,000 units/ml of rTNF-α. Cells were also preincubated in the presence of 10% MoCM and 1 unit of Ep/ml with or without 50,000 units of rTNF-α. At the time of plating, equal aliquots of cell suspension were removed, and the cells were washed twice and assayed for CFU-GM and BFU-E colony growth in the absence of rTNF-α.

Statistical Analysis. In some experiments, the probability of significant differences between samples was determined by the statistical method of analysis of variance.

RESULTS

Effect of rTNF-α on MoCM-induced Proliferation of Highly Enriched Normal Bone Marrow Progenitor Cells. Table 1 shows that, when highly enriched normal bone marrow progenitor cells are induced to proliferate in MoCM-containing liquid cultures, rTNF-α at concentrations of 5, 500, and 50,000 units/ml resulted in significant and comparable inhibition (approximately 50%) of [³H]dThd uptake at 24 h when compared to the control cultures (without rTNF-α). However, over the next 48 h of incubation, the extent of inhibition of [³H]dThd uptake gradually decreased at all concentrations of rTNF-α tested, with the least reduction in inhibition in cultures containing 50,000 units/ml (59% inhibition of [³H]dThd uptake at 24 h versus 34% and 45% inhibition at 48 and 72 h, respectively), and the most reduction in inhibition in cultures containing 5 units of rTNF-α (58% inhibition at 24 h versus 15% and 1% inhibition at 48 and 72 h, respectively).

In order to determine if the pronounced loss of inhibition of [³H]dThd uptake at 48 h and 72 h seen with 5 units of rTNF-α was simply due to a lack of sufficient TNF available after 24 h of culture, in one experiment an additional 5 units were added at both 24 h and 48 h of culture. As shown in Table 1, further addition of 5 units of rTNF-α at both these time points was not able to reverse the loss of inhibition of [³H]dThd uptake seen with 5 units of rTNF-α added only at the beginning of the culture period. These results suggest that bone marrow progenitor cells, induced to proliferate, become less sensitive toward the antiproliferative effects of rTNF-α after an initial exposure.

Effect of Continual Presence of rTNF-α on Hematopoietic Colony Formation in Cultures of Highly Enriched Progenitor Cell Populations Obtained from Normal and CML Bone Marrow and Normal Peripheral Blood. Since rTNF-α was able to suppress the proliferation of highly enriched normal bone marrow progenitor cells, we then examined its effects on hematopoietic
colony growth by enriched progenitor cell populations.

Fig. 1 shows that, when different concentrations of rTNF-α are incorporated into methylcellulose cultures containing enriched myeloid progenitor cell populations from either normal bone marrow, CML bone marrow, or normal peripheral blood, both Day 14 CFU-GM- and BFU-E-derived colonies from all three sources are comparably inhibited by rTNF-α in a dose-dependent manner. For CFU-GM, plateau levels of colony suppression (approximately 85 to 90%) were seen at high doses of rTNF-α (5000 units/ml) and up to 70% inhibition at 500 units. For BFU-E, a plateau level of colony suppression was also observed with 5000 units of rTNF-α (82 ± 15%, 65 ± 21%, and 73 ± 9% inhibition for CML bone marrow, normal bone marrow, and peripheral blood, respectively). It must be emphasized, however, that the suppressive effect of rTNF-α on BFU-E-derived colony growth appeared to be cytostatic, since we consistently observed that increasing concentrations of rTNF-α (beginning at 500 units/ml) resulted not only in an increased reduction in the number of colonies but also in an increased reduction in the average size of the erythroid colonies. This observed reduction in the size of erythroid colonies was not as readily apparent for GM colonies.

Effect of rTNF-α on Colony Growth by Normal Bone Marrow-enriched Progenitor Cells Stimulated with Varying Concentrations of MoCM. Since the growth of CFU-GM- and the optimal growth of BFU-E-derived colonies from highly enriched progenitor cell populations is absolutely dependent on an exogenous source of hematopoietic growth factors (19) such as those present in MoCM, and since rTNF-α can suppress colony formation, competition between the growth factors present in MoCM and rTNF-α was evaluated. Thus, in a set of experiments using highly enriched normal bone marrow progenitor cells as target cells, the amount of MoCM used to stimulate colony growth was 10%, 5%, and 2.5%. All concentrations produced the same plating efficiency of CFU-GM and BFU-E, although the average size of the erythroid and GM colonies was smaller in the presence of 2.5% MoCM. With all concentrations of MoCM tested, the inhibitory effects of r-TNF-α on GM colony growth remained the same at three different concentrations of r-TNF-α tested (Fig. 2, top). For BFU-E, r-TNF-α at 50 and 500 units/plate appeared to be more inhibitory at lower MoCM concentrations (Fig. 2, bottom). However, these differences, when compared to inhibition observed in the presence of 10% MoCM, were not statistically significant by analysis of variance.

Effect of Preincubation of Normal Bone Marrow-enriched Progenitor Cells with rTNF-α on Colony Growth. In order to determine whether the continued presence of rTNF-α was required during the culture period for colony suppression, normal bone marrow-enriched progenitor cells were incubated in short-term liquid culture at 37°C in the absence or presence of rTNF-α (50,000 units/ml). In addition, the effects of preincubation of these cells with rTNF-α were evaluated in the presence of MoCM and Ep. At 24-, 48-, and 72-h time points, equal aliquots of cell suspension were removed and washed twice prior to plating the cells in methylcellulose. The results of these experiments (Table 2) show that exposure of these enriched progenitor cells to rTNF-α for 24, 48, and 72 h resulted in a 46%, 55%, and 65% inhibition of CFU-GM colonies, respectively, when compared to the control cultures (without rTNF-α). A similar degree of irreversible inhibition of GM colony growth with exposure to rTNF-α over the 72-h liquid culture period was observed when MoCM and Ep were present during preincubation.

In contrast to the inhibitory effect seen on CFU-GM, prein-
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Fig. 2. Effect of TNF-α on hematopoietic colony growth by normal bone marrow-enriched myeloid progenitor cells in the presence of varying concentrations of MoCM. Highly enriched normal bone marrow progenitor cells were incubated in methylcellulose cultures with TNF-α at the concentrations indicated in the presence of 2.5% MoCM, 5.0% MoCM, and 10% MoCM. Columns, mean percentage of inhibition of CFU-GM (top) and BFU-E (bottom) colonies for three separate experiments; bars, SD. The mean number of colonies in the control plates (without TNF-α) in the presence of 10% MoCM was 57 ± 6 BFU-E and 58 ± 37 CFU-GM per 3 × 10⁴ cells. Essentially the same number of colonies was obtained in the control plates containing 5% and 2.5% MoCM, although the size of the colonies in the presence of 2.5% MoCM was noticeably smaller. CM, conditioned medium.

Table 2 Effect of various time exposures to TNF-α on highly enriched normal bone marrow CFU-GM and BFU-E maintained in liquid culture in the absence or presence of MoCM and erythropoietin

<table>
<thead>
<tr>
<th>Sample</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (cells)</td>
<td>45 ± 14ª</td>
<td>45 ± 13ª</td>
<td>27</td>
</tr>
<tr>
<td>1a (cells + TNF-α)</td>
<td>25 ± 11ª</td>
<td>-46ª (27–65ª)</td>
<td>-55 (44–61)</td>
</tr>
<tr>
<td>2 (cells + MoCM + Ep)</td>
<td>52 ± 22ª</td>
<td>64</td>
<td>85</td>
</tr>
<tr>
<td>2a (cells + MoCM + Ep + TNF-α)</td>
<td>28 ± 14ª</td>
<td>-44 (32–69)</td>
<td>36</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>BFU-E</td>
<td>24 h</td>
<td>11 ± 2ª (0–142)</td>
<td>25 ± 9ª (36–370)</td>
</tr>
</tbody>
</table>

* Mean ± SD of the colony number of 4 experiments.
ª Mean ± SD of the colony number of 3 experiments.
¢ Mean percentage of change from respective control cultures.
§ Numbers in parentheses, range.
* Mean ± SD of the colony number of 4 experiments.
of small erythroid colonies. No apparent inhibitory effects of rTNF-α on erythroid colony growth were observed when rTNF-α addition was delayed 7 days after the initiation of culture.

**DISCUSSION**

In the present studies, we have confirmed the previous observations of others (15–17) that rTNF-α suppresses erythroid and myeloid colony growth *in vitro* by normal human bone marrow cells in a dose-dependent manner. However, unlike these previous studies in which relatively high numbers (0.5 to 1 x 10⁵ cells/plate) of a heterogeneous or partially purified population of bone marrow cells were used as target cells in clonogenic assays to examine the effects of rTNF-α, in the present studies, very low numbers (800 to 2500 cells/plate) of highly enriched normal bone marrow progenitor cell populations were depleted of maturing myeloid, erythroid, and lymphoid cells, were used as target cells. By cloning low numbers of cells, the influence that any non-colony-forming cells may have in assessing the effects of rTNF-α on hematopoietic colony growth was greatly minimized. As such, our data extend the previous observations of others to strongly indicate that rTNF-α can suppress hematopoietic colony growth by direct interaction with CFU-GM and BFU-E progenitor cells. However, while all detectable accessory cells were removed and some of these enriched populations contained up to 17% of 14-day colony-forming units, definitive proof that rTNF-α acts directly on hematopoietic progenitor cells would require pure (100%) progenitor cell populations.

In addition, the effect of rTNF-α was examined on highly enriched normal peripheral blood CFU-GM and BFU-E, both of which are generally considered to be more primitive than the majority of bone marrow CFU-GM and BFU-E (31, 32). Our results (Fig. 1) show that the sensitivity of normal peripheral blood CFU-GM and BFU-E to inhibition by rTNF-α is comparable with that of normal bone marrow CFU-GM and BFU-E. This is, to our knowledge, the first report to describe the suppressive effects of rTNF-α on normal circulating hematopoietic colony-forming cells.

Previous studies comparing the regulation of CFU-GM colony growth by normal bone marrow and bone marrow from patients in the chronic phase of CML have demonstrated that, although there is a similar requirement for colony-stimulating factors in promoting CFU-GM colony growth (33), CFU-GM in patients with CML appear to be much less sensitive than normal CFU-GM to growth suppression by inhibitory molecules such as prostaglandin E (34, 35) and acidic isoferritin (36). Based on the latter findings, we compared the effects of rTNF-α on hematopoietic colony growth by highly enriched progenitor cell populations from normal bone marrow and bone marrow from CML patients. Although only four patients were tested, our results indicate that there are no apparent differences between the sensitivity of normal and CML BFU-E and CFU-GM to inhibition by rTNF-α.

As to the mechanism of action of rTNF-α, we have shown that the continuous presence of a high dose (5000 units) of rTNF-α can suppress up to 90% of GM colony growth (Fig. 1, top) by highly enriched normal bone marrow progenitor cells, and, moreover, that partial suppression (i.e., 46 to 65%) of CFU-GM growth is irreversible after 24-, 48-, and 72 h exposure to rTNF-α prior to plating the cells in methylcellulose in the absence of rTNF-α. The same extent of irreversible CFU-GM inhibition was observed when MoCM was present during preincubation of these enriched progenitor cells with rTNF-α.

In contrast to its effect in suppressing CFU-GM, in the absence of MoCM and Ep, we found that the number of BFU-E-derived colonies from highly enriched normal bone marrow progenitor cells was actually increased after exposure to a high dose of rTNF-α for 24, 48, and 72 h, respectively. This finding was quite unexpected in light of our observation that the continuous presence of high doses of rTNF-α in methylcellulose cultures resulted in suppression of BFU-E colony growth similar to that observed for CFU-GM (Fig. 1, bottom). However, if MoCM and Ep were present during short-term exposure of the cells to rTNF-α, there was no increase in the number of BFU-E-derived colonies when compared to control cultures (without rTNF-α) after 24 h; rather, some suppression of BFU-E growth was seen after 48 h and 72 h of preincubation, respectively. We have no clear-cut explanation as to why short-term exposure to rTNF-α has a suppressive effect on BFU-E in the presence of BFU-E growth-promoting and differentiation factors while having a stimulatory (or survival) effect on BFU-E in the absence of these factors. Further studies will be necessary to firmly establish the role of rTNF-α in affecting the growth or survival of BFU-E under certain conditions. Others have recently shown that rTNF-α can, in fact, act as a growth stimulator of human fibroblasts (7, 8).

With regard to those experiments in which the addition of rTNF-α was delayed at varying time intervals after the initiation of colony cultures, our results (Table 3) indicate that the susceptibility of normal bone marrow CFU-GM and BFU-E to growth inhibition by rTNF-α is decreased with progenitor cell maturation; however, this decreased sensitivity to rTNF-α is more pronounced for BFU-E than CFU-GM. Thus, if rTNF-α is added 4 days after the initiation of culture, inhibition of BFU-E growth is largely alleviated, whereas, if the addition of rTNF-α is delayed as late as 7 days after the initiation of culture, inhibition of CFU-GM, though diminished, is still marked.

In looking at the effects of rTNF-α on MoCM-induced
proliferation of highly enriched normal bone marrow progenitor cells in short-term liquid culture, our results (Table 1) indicate that rTNF-α has a transient suppressive effect rather than a cytotoxic effect on myeloid progenitor cells. Thus, we found that a low dose of rTNF-α (5 units) was just as effective as high doses (5,000 and 50,000 units) in significantly inhibiting [3H]dThd uptake by these cells during the first 24 h of culture. However, over the next 48 h of culture, the extent of inhibition of [3H]dThd uptake gradually declined at all concentrations of rTNF-α tested, this decline being most pronounced in cultures initially containing 5 units of rTNF-α in which no significant inhibition of [3H]dThd uptake was observed after 24 h of culture. This loss of inhibitory activity was not attributable to lack of availability of rTNF-α, suggesting a reduction in sensitivity of the target population after an initial exposure. Our observation is similar to the recent studies of Mannel (37) demonstrating that significantly higher concentrations of rTNF-α were required to induce a 50% reduction in cell viability when TNF highly sensitive L929 murine tumor cells had been exposed to rTNF-α previously. Mannel has suggested that TNF receptor, and it is clear from these studies (41-43) that high-affinity TNF receptors exist on the surface of a variety of cell types. The results of the studies reported herein strongly indicate that TNF receptors are present on myeloid progenitor cells as well. Although the intracellular events responsible for the antiproliferative effects of rTNF-α are not known, the use of purified recombinant TNF and purified myeloid progenitor cells will allow more in-depth studies at defining surface membrane and intracellular molecular events involved in the interaction of TNF and hematopoietic progenitor cells.

ACKNOWLEDGMENTS

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REFERENCES

EFFECT OF TUMOR NECROSIS FACTOR ON HUMAN PROGENITOR CELLS


Effects of Recombinant Human Tumor Necrosis Factor on Highly Enriched Hematopoietic Progenitor Cell Populations from Normal Human Bone Marrow and Peripheral Blood and Bone Marrow from Patients with Chronic Myeloid Leukemia

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