Tumor Necrosis Factor-alpha Strongly Potentiates Interleukin-3 and Granulocyte-Macrophage Colony-Stimulating Factor-Induced Proliferation of Human CD34+ Hematopoietic Progenitor Cells

By Christophe Caux, Sem Saeland, Catherine Favre, Valérie Duvert, Patrice Mannoni, and Jacques Banchereau

Previous studies have shown that tumor necrosis factors (TNFαs) inhibit the proliferative effects of crude or purified colony-stimulating factors (CSFs) on low density human bone marrow cell fractions. In the present study we investigated the effects of TNFα on the growth of highly purified CD34+ human hematopoietic progenitor cells (HPC) in response to recombinant CSFs. In short-term liquid cultures (5 to 8 days), TNFα strongly potentiates interleukin-3 (IL-3) and granulocyte-macrophage colony-stimulating factor (GM-CSF)-induced growth of CD34+ HPC, while it has no proliferative effect on its own. Within 8 days, the number of viable cells obtained in TNFα-supplemented cultures is threefold higher than in cultures carried out with IL-3 or GM-CSF alone. Secondary liquid cultures showed that the potentiating effect of TNFα on IL-3-induced proliferation of CD34+ HPC does not result from an IL-3-dependent generation of TNFα responsive cells. Limiting dilution analysis indicates that TNFα increases both the frequency of IL-3 responding cells and the average size of the IL-3-dependent clones. The potentiating effect of TNFα on IL-3- and GM-CSF-dependent growth of CD34+ HPC is also observed in day 7 colony assays. Under these short-term culture conditions, TNFα does not appear to accelerate cell maturation as a precursor morphologic change is not recognized. Finally, TNFα inhibits the relatively weak growth-promoting effect of granulocyte-CSF (G-CSF), which acts on a more committed subpopulation of CD34+ HPC different from that recruited by IL-3 and GM-CSF. TNFα displays the same modulatory effects as GM-CSF. Thus, TNFs appear to enhance the early stages of myelopoiesis.

THE IN VITRO proliferation of human hematopoietic progenitor cells (HPC) depends upon the presence of exogenous growth factors such as interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF), G-CSF, and M-CSF. In contrast, soluble factors such as interferons (IFNs), transforming growth factor β (TGF-β), and tumor necrosis factors (TNFαs) have been shown to inhibit in vitro hematopoiesis, although their mode of action is not well characterized.

TNFα is a protein produced by various cell types and that was originally shown to induce necrosis of tumors in vivo. A cDNA clone coding for human TNFα has been isolated, and the availability of recombinant preparations has revealed that this cytokine displays a broad spectrum of activities (reviewed in reference 17). Among those activities, TNFα, alone or in synergy with IFN-γ, has been reported to inhibit in vitro hematopoietic colony formation by granulocytic and monocytic, erythroid, and multipotential progenitors when tested on total mononuclear bone marrow cells or fractions thereof depleted of mature cells.

Recently, we and others have shown that IL-3, GM-CSF, and G-CSF can induce the proliferation of purified HPC expressing the CD34 antigen. This population, which represents 1% to 2% of bone marrow low density mononuclear cells, contains most of the hematopoietic progenitors, including multipotential self-renewing progenitors, as CD34+ HPC can fully reconstitute lethally irradiated baboons. In this context, we have investigated the effects of other growth regulators on CD34+ progenitors. In the present study, using short-term proliferative assays of CD34+ HPC, we found unexpectedly that TNFα, which has no growth-promoting effect per se, strongly enhances both IL-3- and GM-CSF-dependent proliferation of CD34+ HPC. We further demonstrated that the potentiating effect of TNFα on IL-3-induced proliferation of CD34+ HPC is direct and the consequence of both increased frequency and growth-rate of IL-3 responsive cells.

MATERIALS AND METHODS

Hematopoietic factors. Recombinant human IL-326 (provided by Dr S. Tindall, Schering-Research, Bloomfield, NJ) and G-CSF (provided by Dr N. Arai, DNAX, Palo Alto, CA) were purified to homogeneity. Specific activities of IL-3 and G-CSF were 5 x 10^4 U/mg and 2 x 10^4 U/mg, respectively; one unit corresponding to half maximal proliferative activity as determined by (methy1-3H) thymidine (3H-TdR). Specific activity 25 Gi/mmol (CEA Saclay, France) uptake by human HPC in liquid cultures. IL-3 and G-CSF were used at a saturating concentration of 10 ng/mL (50 U/mL) and 25 ng/mL (50 U/mL), respectively. Human GM-CSF27 (provided by Drs P. Trotta and T.L. Nagabhushan, Schering-Research) was expressed in Escherichia coli and subsequently purified to homogeneity. Specific activity was 2 x 10^4 U/mg, as previously defined. GM-CSF was used at a saturating concentration of 100 ng/mL (200 U/mL). Supernatant of Cos 7 cells transfected with the cDNA of human TNFβ was used at a concentration of 0.5% (vol/vol), and mock supernatant derived from Cos 7 cells transfected with unrelated cDNA was used as negative control (provided by Dr N. Arai, DNAX, Palo Alto, CA). Recombinant purified TNFα was purchased from Genzyme (Boston, MA) (specific activity 2 x 10^4 U/mg). In most experiments, it was used at a saturating concentration of 25 ng/mL (500 U/mL).

Collection and fractionation of cord blood and bone marrow cells. Umbilical cord blood and bone marrow samples were collected according to institutional guidelines. Cord blood was collected during normal full-term deliveries in glass vessels containing preserv...
ative-free grade-1 sodium heparin (Sigma, St Louis, MO) at a final concentration of 20 IU/mL. Marrow was obtained by iliac crest aspiration from adult bone marrow transplant donors free of hematological disease. Light-density mononuclear cells were isolated by Ficoll-Hypaque gradient separation at a density of 1.077 g/mL. Mononuclear fractions were depleted of adherent cells by overnight incubation at 37°C in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 1% wt/vol tissue culture grade bovine serum albumin (BSA; Boehringer Mannheim, FRG). Cells bearing CD34 antibodies were isolated from nonadherent mononuclear fractions through positive selection by indirect immune "panning" using anti-My10 monoclonal antibody (MoAb) (1HPCA-1; Becton-Dickinson, Mountain View, CA), as reported elsewhere.

When the purity of the isolated cells, determined by FACScan flow cytometric analysis, was below 95%, a second purification step was performed using immunomagnetic beads. Briefly, after overnight incubation at 37°C, the CD34+ "panned" cells were resuspended at 107 cells/mL in a cocktail of MoAbs (Leu-M3 [CD14], Leu5b [CD2], Leu3 [CD4], Leu11a [CD16], and Leu16 [CD20] from Becton-Dickinson, at 2 μg/mL each) for 15 minutes, washed twice, and incubated for 30 minutes with immunomagnetic beads (107 beads/mL) coated with anti-mouse immunoglobulins (Dynabeads M450; Dynal, Oslo, Norway). The beads were removed using a magnet, and the CD34+ cells were recovered in suspension. Thus, in all experiments, the isolated cells were 95% to 99% CD34+, as judged by staining with the anti-My10 MoAb.

Cell cultures in liquid medium. Cell cultures were established in the presence of IL-3 (10 ng/mL) or IL-3 plus TNFa (25 ng/mL) in medium consisting of RPMI 1640 supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS; Flow Laboratories, Irvine, UK), 10 mmol/L HEPES, 2 mmol/L L-glutamine, 5 x 10-5 mol/L 2-mercaptoethanol, penicillin (100 U/mL), and streptomycin (100 μg/mL); hereafter designated complete medium. CD34+ cells were seeded in 24-well culture plates (Linbro; Flow Laboratories, McLean, VA) for expansion. Optimal conditions were maintained by splitting these cultures every 4 to 5 days. At different timepoints as indicated, cells were collected for counting and establishment of secondary cultures. For initiation of secondary cultures, cells were washed 4 times and further incubated for 3 hours in complete medium in order to eliminate growth factors. Subsequent proliferation assays were then carried out as described below.

For proliferative assays, cells were distributed in 96-well round-bottom microtest tissue culture plates (Nunc) for expansion. Optimal conditions were maintained by splitting these cultures every 4 to 5 days. At different timepoints as indicated, cells were collected for counting and establishment of secondary cultures. For initiation of secondary cultures, cells were washed 4 times and further incubated for 3 hours in complete medium in order to eliminate growth factors. Subsequent proliferation assays were then carried out as described below.

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RESULTS

TNFa potentiates IL-3-induced proliferation of CD34+ HPC. The effects of TNFa on CSF-induced proliferation of CD34+ HPC were examined in a liquid culture system. Results shown in Fig 1A indicate that TNFa (25 ng/mL) strongly enhances the IL-3-dependent proliferation of CD34+ HPC, whereas it has no proliferative effect per se. The enhancement of "H-TdR incorporation was observed at all times tested. Figure 1B shows that the enhanced "H-TdR incorporation (Fig 1A) reflects an increased cell proliferation, since more viable cells are recovered in IL-3 plus TNFa
IL-3-induced CD34+ HPC proliferation. CD34+ HPC cultured in TNFa (-+-I, TNFa 0.025 ng/mL) with IL-3 for various periods of time with IL-3, and proliferative responsiveness to TNFa and CSFs was subsequently assayed as 3H-TdR incorporation in secondary liquid culture. The TNFa-induced enhancement of the proliferative responses to GM-CSF and G-CSF, we wondered whether IL-3 would induce the proliferation of CD34+ HPC. Finally, TNFa alone or in dual combinations. Data shown in Table 1 indicate that TNFa is able to enhance the GM-CSF-dependent proliferation of CD34+ HPC, and, as observed with IL-3, TNFa approximately doubles the level of 3H-TdR incorporation. The recovery of viable cells is also enhanced (data not shown). In contrast, TNFa inhibits the G-CSF-dependent proliferation of CD34+ HPC. Finally, TNFa displays the same activities as TNFa on CSF-induced proliferation of CD34+ HPC.

IL-3 does not induce the generation of cells proliferating in response to TNFa. Since we previously showed that preculturing CD34+ HPC with IL-3 results in a subsequent enhancement of the proliferative responses to GM-CSF and G-CSF, we wondered whether IL-3 would induce the appearance of a cell population proliferating in response to TNFa alone. Thus, CD34+ HPC were precultured for various periods of time with IL-3, and proliferative responsiveness to TNFa and CSFs was subsequently assayed as measured by 3H-TdR incorporation in secondary liquid cultures. Results shown in Fig 3 indicate that TNFa alone has no direct proliferative effect on IL-3 precultured cells, while the cells remain responsive to IL-3, GM-CSF, and G-CSF. The same results were obtained when preculturing CD34+ HPC with IL-3 plus TNFa (data not shown).

TNFa increases the frequency and size of IL-3-responsive clones. We next addressed whether the potentiating effect of TNFa on IL-3-dependent cell growth was due to an increased frequency of IL-3 responding cells, to an enhanced growth rate of cells already responding to IL-3, or to both. To answer this question, clonogenic day 7 CFU assays in methylcellulose and limiting dilution studies in liquid medium were carried out with CD34+ HPC.

First, day 7 CFU, from cord blood or bone marrow CD34+ HPC (Table 2) show that TNFa enhances the total number of colonies induced by IL-3 and GM-CSF, but blocks the G-CSF-dependent generation of colonies. In addition, colonies generated in response to combinations of IL-3 and TNFa or GM-CSF and TNFa were larger than those generated in the presence of IL-3 or GM-CSF alone (not shown).

Second, limiting dilution studies were done where CD34+ HPC were seeded at various concentrations into wells of microtitration plates with IL-3 and with or without TNFa. The number of growing colonies was determined after 6 days of culture. As shown in Fig 4, TNFa increases the frequency of IL-3 responding cells (1 of 3.2 cells generates clones in the presence of IL-3 and TNFa, whereas 1 of 4.5 cells generates clones in the presence of IL-3 alone). The combination of IL-3 and TNFa generates 20% to 40% more clones than IL-3 alone (three separate experiments). The fact that the potentiating effect of TNFa is seen at very low cell concentration (0.5 cells per well) and the linear slope of the frequency analysis curves demonstrates that TNFa acts directly on the CD34+ HPC. We further determined the number of cells in positive wells (seeded with 1.5 cells) to estimate clone size (Fig 5). Cultures done in the presence of TNFa and IL-3 yielded a higher number of large clones (greater than 32 cells) and a lower number of small clones (less than 32 cells) than those done with IL-3 alone. This is illustrated by a shift of clone size distribution towards the larger size clones (Fig 5) and by a higher average clone size in the combination of IL-3 plus TNFa (59 cells per clone, experiment 1 and 53 cells per clone, experiment 2) than in IL-3 alone (34 cells per clone, experiment 1 and 32 cells per clone, experiment 2).

![Graph](image-url)
Fig 3. The potentiating effect of TNFα on IL-3-induced proliferation of CD34+ HPC is not a consequence of an IL-3-dependent induction of TNFα responding cells. Purified CD34+ HPC were precultured with IL-3 (10 ng/mL); and at various time points, cells were harvested, washed, processed as described in Materials and Methods, and subsequently assayed for secondary proliferative responsiveness to IL-3 (■), GM-CSF (□), G-CSF (■), TNFα (○), or medium alone (○). Responsiveness was measured by 3H-TdR uptake, 6 days after initiation of secondary cultures.

DISCUSSION

This study demonstrates that tumor necrosis factors strongly potentiate the proliferative effects of IL-3 and GM-CSF on CD34+ HPC isolated from cord blood and bone marrow. Within 8 days, the number of cells generated in TNFα-supplemented liquid cultures of CD34+ HPC is three times higher than in cultures carried out with IL-3 alone. This reflects a true potentiating effect as TNFα per se does not act as a growth factor for CD34+ HPC. In our hands, TNFα appears to be the most potent activator of IL-3-dependent proliferation of CD34+ HPC, since combinations of IL-3 with GM-CSF, G-CSF, IL-1 (data not shown), and IL-6 (data not shown) are always less potent than the combination of IL-3 and TNFα. As expected, TNFβ, which uses the same receptor as TNFα, also potentiates IL-3-induced proliferation of CD34+ HPC.

As shown by secondary liquid cultures, the observed potentiation is not the consequence of an IL-3-dependent generation of cells proliferating in response to TNFα alone. Rather, colony assays and limiting dilution studies indicate that the effect of TNFα results from both an increased frequency and an increased growth rate of IL-3 responding cells. Furthermore, limiting dilution analysis demonstrates a direct effect of TNFα on its target cells. Although TNFα strongly potentiates the proliferative effects of IL-3, no morphologic differences were observed in terms of cell maturation between day 7 colonies generated with IL-3 and those generated with IL-3 and TNFα (data not shown). In

Table 2. Effects of TNFα in Combination With CSFs on the Induction of Day 7 CFU From Cord Blood and Bone Marrow CD34+ HPC

<table>
<thead>
<tr>
<th>Factors</th>
<th>Medium</th>
<th>IL-3</th>
<th>GM-CSF</th>
<th>G-CSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td>Medium</td>
<td>0</td>
<td>241</td>
<td>169</td>
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<tr>
<td></td>
<td>TNFα</td>
<td>0</td>
<td>245</td>
<td>141</td>
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<td>0</td>
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<td>259</td>
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<td>0</td>
<td>343</td>
<td>251</td>
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<tr>
<td>Experiment 2</td>
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<td>240</td>
<td>154</td>
</tr>
<tr>
<td></td>
<td>TNFα</td>
<td>8</td>
<td>222</td>
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<td></td>
<td></td>
<td>2</td>
<td>356</td>
<td>276</td>
</tr>
</tbody>
</table>

Duplicate dishes plated with 5 × 10⁵ cells in the presence of Epo (1 U/mL) and CSFs with or without TNFα (25 ng/mL) or in medium alone were scored for number of colonies on day 7 (colonies ≥ 50 cells). For CSF concentrations, see Materials and Methods. Experiment 1, cord blood; Experiment 2, bone marrow.

Fig 4. TNFα increases the frequency of IL-3 responsive CD34+ HPC. Replicate wells (120) were seeded at 13.5 cells per well, (240) at 4.5 cells per well, and (360) at 1.5 and 0.5 cells per well. The presence of clones was assessed on day 6. Single-hit kinetics were obtained, and the slopes were drawn by linear regression analysis. The shaded region represents the 95% confidence limit. The clone frequencies obtained in this experiment were 1:4.5 = 0.22 (P = .65) for IL-3 (□) and 1:3.2 = 0.31 (P = .46) for IL-3 + TNFα (○). TNFα or medium alone did not induce the development of any clones. The experiment presented here is representative of three.
of CD34' committed progenitors. Thus, the potentiating and antiproliferative effects of TNFα may be indirect through the release of factors by accessory cells,17 factors that may act directly or in concert with TNFα to inhibit hematopoiesis.40,30 Thus, the use of heterogeneous bone marrow cell fractions or conditioned medium as a source of CSFs in previous studies could explain the apparent discrepancies with our current results, obtained with recombinant factors and CD34+ HPC, a population that is highly enriched for progenitor cells and devoid of accessory cells. In keeping with the generally described inhibitory effects of TNFα, we have found (in other experiments to be submitted) that TNFα can block cell growth in long-term cultures (14 to 35 days) of CD34+ HPC, when well-differentiated cells are present in the cultures.

Our data on the potentiating effect of TNFα in short-term cultures are supported by the recent observations that TNFα can enhance GM-CSF–induced in vitro growth of cells from some acute myeloid leukemia (AML) specimens.31,32 As previously shown for IL-3 and GM-CSF,20,33 such observations further indicate similarities in cytokine responsiveness of AML cells and normal hematopoietic cells.

Additional studies are required to determine the mechanism of the potentiation observed in our present investigation. As TNFα has been shown to upregulate the expression of the IL-2 receptor on activated T cells,24 it is tempting to speculate that TNFα might upregulate the expression of IL-3 and GM-CSF receptors on CD34+ HPC. However, TNFα may also mediate its effect by activating a specific second messenger, thus amplifying mitotic signals given by IL-3 or GM-CSF alone. Conversely, the inhibitory effect of TNFα on G-CSF–dependent proliferation of CD34+ HPC may result from downregulation of the G-CSF receptor or from a blocking of intracellular pathways activated by G-CSF.

TNFα, primarily identified as a cytotoxic factor was later demonstrated to display growth-stimulatory properties on different cell types.34,37 Such a capacity to display both proliferative and antiproliferative effects is indeed shared by many factors.18 In this study, we showed that TNFα, originally described as an inhibitor of hematopoiesis, strongly enhances the early stages of myelopoiesis. This finding should be taken in consideration when clinical use of cytokines is proposed in order to control the proliferation of leukemias or tumors.

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