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 (TNFs) 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 TNFa 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), TNFa 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 per se. Within 8 days, the number of viable cells obtained in TNFa-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 TNFa on IL-3–induced proliferation of CD34\(^+\) HPC does not result from an IL-3–dependent generation of TNFa responsive cells. Limiting dilution analysis indicates that TNFa increases both the frequency of IL-3–responding cells and the average size of the IL-3–dependent clones. The potentiating effect of TNFa 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, TNFa does not appear to accelerate cell maturation as a precursor morpholgy is retained. Finally, TNFa 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. TNFa displays the same modulatory effects as TNFβ. Thus, TNFs appear to enhance the early stages of myelopoiesis.

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MATERIALS AND METHODS

Hematopoietic factors. Recombinant human IL-3\(^26\) (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\(^6\) U/mg and 2 x 10\(^4\) U/mg, respectively; one unit corresponding to half maximal proliferative activity as determined by (methyl-\(^3\)H) thymidine (\(^3\)H-TdR; specific activity 25 Ci/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-CSF\(^27\) (provided by Drs P. Trotta and T.L. Nagabushan, Schering-Research) was expressed in Escherichia coli and subsequently purified to homogeneity. Specific activity was 2 x 10\(^5\) 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 TNFa 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 TNFa was purchased from Genzyme (Boston, MA) (specific activity 2 x 10\(^5\) 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-I 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 antigens 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.\(^{(9,20)}\)

When the purity of the isolated cells, determined by FACSscan 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 10^7 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 (10^7 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 TNFα (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 mM HEPES, 2 mM L-glutamine, 5 x 10^-5 mol/L 2-mercaptoethanol, penicillin (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) in Iscove’s modified Dulbecco’s medium (IMDM) containing 30% FBS, 10^-5 mol/L 2-mercaptoethanol, 0.5% wt/vol BSA neutralized with NaHCO₃, and L-glutamine and antibiotics as indicated above. For each cell concentration used, ranging from 0.5 to 40.5 cells per well, and for each factor tested, aliquots of 50 μL were seeded in 120 to 360 wells. After 5 to 7 days of culture (37°C, 5% CO₂), the positive wells were scored using an inverted microscope, and for selected cell concentrations, the number of cells in each clone was determined.

For calculation of clone frequencies, the data were plotted as the log of the fraction of nonresponding wells versus the number of cells plated per well. Linear regression analysis was applied to the data to calculate the best slope.\(^{(26)}\) Confidence intervals around the slope were calculated using a form of the Student’s t test. Conformance of the data to linearity was tested by using a χ² analysis: a χ² derived-probability P > .05 indicating single-hit curves, thus demonstrating activity on a single limiting cell type.

Average clone size was calculated as the ratio of (total cell number enumerated in 120 wells seeded at 1.5 cells/well)/(number of responding cells present in the 120 wells observed).\(^{(28)}\) Calculation of average clone size was done on triplicates of 120 wells.

RESULTS

TNFα potentiates IL-3-induced proliferation of CD34+ HPC. The effects of TNFα on CSF-induced proliferation of CD34+ HPC were examined in a liquid culture system. Results shown in Fig 1A indicate that TNFα (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 TNFα...
cultures when compared with IL-3 cultures. After 4 days and 8 days, approximately two- and threefold as many viable cells, respectively, were recovered when TNFα was added to cultures. The TNFα-induced enhancement of IL-3-mediated cell proliferation is dose dependent (Fig 2), with maximal stimulation observed at approximately 2.5 ng/mL TNFα. Interestingly, a combination of 2.5 to 25 ng/mL TNFα with a half maximal dose of IL-3 (0.25 ng/mL) results in a proliferative response higher than that obtained with an optimal dose of IL-3 used alone (2.5 ng/mL).

TNFα and β enhance the proliferation of CD34+ HPC induced by IL-3 and GM-CSF, but inhibit that induced by G-CSF. As GM-CSF and, to a lesser extent, G-CSF are also able to induce the proliferation of CD34+ HPC,22,23,24 we wondered whether TNFα would also enhance cell proliferation induced by these factors. Thus, CD34+ HPC were cultured with optimal concentrations of various factors used alone or in dual combinations. Data shown in Table 1 indicate that TNFα is able to enhance the GM-CSF-dependent proliferation of CD34+ HPC, and, as observed with IL-3, TNFα approximately doubles the level of 3H-TdR incorporation. The recovery of viable cells is also enhanced (data not shown). In contrast, TNFα inhibits the G-CSF-dependent proliferation of CD34+ HPC. Finally, TNFβ displays the same activities as TNFα on CSF-induced proliferation of CD34+ HPC.

IL-3 does not induce the generation of cells proliferating in response to TNFα. 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,22,23 we wondered whether IL-3 would induce the appearance of a cell population proliferating in response to TNFα alone. Thus, CD34+ HPC were precultured for various periods of time with IL-3, and proliferative responsiveness to TNFα and CSFs was subsequently assayed as measured by 3H-TdR incorporation in secondary liquid cultures. Results shown in Fig 3 indicate that TNFα 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 TNFα (data not shown).

TNFα increases the frequency and size of IL-3 responsive clones. We next addressed whether the potentiating effect of TNFα 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 TNFα 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 TNFα or GM-CSF and TNFα 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 TNFα. The number of growing clones was determined after 6 days of culture. As shown in Fig 4, TNFα increases the frequency of IL-3 responding cells (1 of 3.2 cells generates clones in the presence of IL-3 and TNFα, whereas 1 of 4.5 cells generates clones in the presence of IL-3 alone). The combination of IL-3 and TNFα generates 20% to 40% more clones than IL-3 alone (three separate experiments). The fact that the potentiating effect of TNFα is seen at very low cell concentration (0.5 cells per well) and the linear slope of the frequency analysis curves demonstrates that TNFα 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 TNFα 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 TNFα (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).

**Table 1. Effects of TNFs in Combination With CSFs on the Proliferation of CD34+ HPC in Liquid Culture**

<table>
<thead>
<tr>
<th>Factors</th>
<th>Medium</th>
<th>TNFα</th>
<th>TNFβ</th>
<th>IL-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Values show 3H-TdR uptake (CPM x 10^−3 ± SD) obtained from one representative experiment of three and expressed as mean ± SD of triplicate wells. Proliferation was measured at day 6; TNFs and all CSFs were used at saturating concentrations, as described in Materials and Methods.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium</td>
<td>3.5 ± 1.0</td>
<td>5.5 ± 2.8</td>
<td>4.5 ± 0.6</td>
<td>53.8 ± 6.8</td>
</tr>
<tr>
<td>IL-3</td>
<td>53.6 ± 6.8</td>
<td>95.2 ± 11.4</td>
<td>91.7 ± 7.2</td>
<td>53.6 ± 6.8</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>20.2 ± 2.5</td>
<td>41.5 ± 4.2</td>
<td>40.9 ± 6.8</td>
<td>66.1 ± 3.8</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>10.5 ± 1.9</td>
<td>5.1 ± 2.1</td>
<td>4.9 ± 0.4</td>
<td>63.0 ± 3.8</td>
</tr>
</tbody>
</table>

**Fig 2.** Dose dependence of the potentiating effect of TNFα on IL-3–induced CD34+ HPC proliferation. CD34+ HPC (5 x 10^5) were cultured in TNFα (—■—), TNFα + 25 ng/mL IL-3 (—▲—), TNFα + 2.5 ng/mL IL-3 (—□—), TNFα + 0.25 ng/mL IL-3 (—Δ—), or TNFα + 0.025 ng/mL IL-3 (—×—). 3H-TdR uptake was measured on day 6 of culture.
TNFα POTENTIATES PROLIFERATION OF CD34+ CELLS

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 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>
</tr>
<tr>
<td></td>
<td>0</td>
<td>245</td>
<td>141</td>
</tr>
<tr>
<td></td>
<td>TNFα</td>
<td>0</td>
<td>335</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>343</td>
<td>251</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>Medium</td>
<td>4</td>
<td>240</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>222</td>
<td>134</td>
</tr>
<tr>
<td></td>
<td>TNFα</td>
<td>7</td>
<td>310</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>356</td>
<td>276</td>
</tr>
</tbody>
</table>

Duplicate dishes plated with 5 x 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 (1201 were seeded at 13.5 cells per well, (2401 at 4.5 cells per well, and (3601 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.
CD34' committed progenitors. Thus, the potentiating and antipotential progenitors, while G-CSF acts on a subpopulation in liquid or semisolid cultures with parameters comparable with IL-3 alone (data not shown). TNFα was also shown to antagonistic effects of TNFα on CSF-induced CD34+ HPC by G-CSF. In this context, we and other investigators have previously shown that GM-CSF and IL-3 recruit largely overlapping CD34+ cell populations, including multipotential progenitors, while G-CSF acts on a subpopulation of CD34+ committed progenitors. Thus, the potentiating and antagonistic effects of TNFα on CSF-induced CD34+ HPC proliferation may be related to the heterogeneity of CSF responding target cells, which remain to be phenotypically defined.

TNFα has generally been described as an inhibitor of in vitro hematopoiesis.8,16 However, such studies reported that the degree of inhibition mediated by TNFα was variable4,16 and depended on the cellular composition of the bone marrow fractions tested and on the type of conditioned medium used as the source of CSFs. These inhibitory 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.4,10,16 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.3,32 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.3,17 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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Fig 5. TNFα increases the size of IL-3-dependent clones. Clone size distribution obtained in limiting dilution experiments done with CD34+ HPC seeded at 1.5 cells per well in the presence of IL-3 (■) or IL-3 + TNFα (□). After 6 days of culture, the size of each clone was determined by counting using an inverted microscope. TNFα or medium alone did not induce the development of any clones (≤8 cells per well). The clones were classified into sections of arbitrarily defined numbers. The numbers plotted correspond to the mean of triplicates of 120 wells observed. The average clone size calculated on triplicates of 120 wells were (A): Experiment 1, 34 ± 3 for IL-3 and 9 ± 4 for IL-3 + TNFα; and (B) Experiment 2, 32 ± 4 for IL-3 and 53 ± 2 for IL-3 + TNFα.


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