Synergy Between Transforming Growth Factor-β and Tumor Necrosis Factor-α in the Induction of Monocytic Differentiation of Human Leukemic Cell Lines

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We examined the effect of transforming growth factor-β (TGF-β) alone and in combinations with other factors on the growth and differentiation of the human promyelocytic cell line HL60 and the human monoblastic cell line U937. Treatment with TGF-β alone did not significantly affect growth or differentiation of HL60 cells, while it significantly inhibited proliferation and induced monocytic differentiation of a small percentage of U937 cells. Combinations of TGF-β and tumor necrosis factor-α (TNF-α) acted in synergy to inhibit cell proliferation and to induce monocytic differentiation of both HL60 and U937 cells. In contrast, no synergy was observed when HL60 cells were treated with TGF-β in various combinations with interferon-α (IFN-α), interferon-γ (IFN-γ), and retinoic acid. Examination of TNF-α receptor expression on HL60 and U937 cells showed that these cell lines expressed comparable levels of high-affinity TNF-α binding sites. Treatment of HL60 and U937 cells with TGF-β did not induce significant changes in TNF-α receptor expression in either cell line. In contrast, HL60 cells expressed much lower levels of TGF-β receptors than did U937 cells. Treatment of both HL60 and U937 cells with TNF-α induced a dose-dependent increase in expression of TGF-β receptors, suggesting that the synergy between TNF-α and TGF-β may result, at least in part, from upregulation of TGF-β receptor expression by TNF-α.

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MATERIALS AND METHODS

Reagents. Bovine TGF-β1 and TGF-β2 were purified to homogeneity as described elsewhere. A polyclonal rabbit anti-TGF-β antiserum was purchased from R&D System (Minneapolis, MN). Human recombinant TNF-α (specific activity 2 x 10^9 U/mg) was a gift from Genzyme (Boston, MA). Human recombinant interferon-γ IFN-γ (specific activity 2.5 x 10^8 U/mg) was a gift from Biogen (Cambridge, MA), and human recombinant IFN-αA (specific activity 2 x 10^9 U/mg) was a gift from Dr P. Sorter of Hoffmann-LaRoche (Nutley, NJ). All trans-retinoic acid (RA, Sigma Chemical, St Louis, MO) was dissolved in dimethylsulfoxide (DMSO) and then diluted at least 10,000-fold into the culture medium so that the final concentration of DMSO was never greater than 0.001%. Nitroblue tetrazolium (NBT) and phorbol myristate acetate (PMA) were obtained from Sigma.

Cell cultures. The HL60 cell line (passage 29) was provided by Dr T. Breitman (National Institutes of Health, Bethesda, MD). HL60 and U937 cells were cultured in polystyrene tissue culture flasks in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), glutamine, penicillin, and streptomycin for HL60 cells and with 10% heat-inactivated FCS, glutamine, nonessential amino acids, HEPES, and gentamicin for U937 cells. For HL60 cells, passages 35 through 60 were used. Experiments were always initiated with exponentially growing cells. For thymidine uptake experiments, 2 x 10^4 cells/well were incubated in 200 μL complete medium in 96-well plates for 4 days at 37°C in a humidified atmosphere of 5% CO2 with the various treatments, as indicated. During the last 12 hours of incubation, 1 μCi of [3H]-thymidine (specific activity 40 to 60 Ci/mmol, Amer sham, Arlington Heights, IL) was added to each well. Cultures were harvested on glass-fiber filters, and [3H]-thymidine uptake was measured. For evaluation of morphology and cytochemistry, HL60 and...
U937 cells were seeded at $2 \times 10^5$ cells per well in 1 mL complete medium in 24-well plates. Cultures were incubated for 4 days and harvested. Cell number was determined with a hemocytometer. Viability, as measured by trypan blue dye exclusion, was always greater than 90%.

**Evaluation of cell differentiation.** NBT reduction was evaluated by incubating $10^5$ cells in 100 mL complete medium for 30 minutes at 37°C with an equal volume of 0.2% NBT in phosphate-buffered saline (PBS) with 200 ng/mL PMA added. The percentage of cells containing intracellular blue-black formazan deposits was then determined after at least 200 cells were counted. The presence of the monocyte-specific marker $^6 \alpha$-naphthyl acetate esterase (ANAE) was evaluated with a commercially available kit (Sigma); the percentage of positive cells was determined by counting at least 200 cells on cytospins counterstained with Wright's stain. Cell morphology was evaluated on Jenner-Giemsa-stained cytospins.

**Binding assays.** HL60 and U937 cells were seeded in tissue culture flasks at the same cell density used for differentiation experiments and incubated with various treatments for 24 hours as indicated. At the end of the incubation, cells were washed twice in PBS. For TNF-α binding assays, $5 \times 10^5$ cells were incubated for 3 hours at 4°C in 0.5 mL binding buffer (RPMI 1640 containing 0.5% bovine serum albumin (BSA), 25 mmol/L HEPES, pH 7.2) containing 0.25 ng $^{125}$I-TNF-α, without or with increasing amounts of unlabelled TNF-α (from 0.03 to 100 ng). $^{125}$I-TNF-α, labeled by the lactoperoxidase method, was provided by Drs Corrado Baglioni and Richard Smith (State University of New York at Albany, NY). The specific activity of the $^{125}$I-TNF-α was 109 μCi/μg. For TGF-β binding assays, $5 \times 10^5$ cells were incubated for 4 hours at 4°C in 0.5 mL binding medium (Eagle's Minimum Essential Medium (EMEM) with 0.5% BSA, 25 mmol/L HEPES, pH 7.2) containing 0.2 ng $^{125}$I-TGF-β (Biomedical Technologies, Stoughton, MA) without or with increasing amounts of unlabelled TGF-β1 (from 0.015 to 100 ng). The specific activity of the $^{125}$I-TGF-β varied between 100 and 140 μCi/μg depending on the preparation. At the end of the incubation, the cell-bound ligand was separated from the unbound ligand by centrifugation at 4°C through a 2:1 (vol/vol) mixture of dibuty1 phthalate and dinonyl phthalate (EM Science, Cherry Hill, NJ) as previously described. The cell pellet was counted in a γ-counter. Nonspecific binding was calculated from the plateau of the competition curve and was subtracted from total binding. Specific binding data were analyzed by the method of Scatchard.
cell proliferation was measured by counting cells in a hemacytometer (data not shown).

Because combinations of TNF-α and TGF-β strongly inhibited the growth of HL60 cells, we examined the effect of treatment with combinations of TGF-β and TNF-α on differentiation of HL60 cells. Similar to what occurred with cell proliferation, treatment with TGF-β1 alone did not induce significant differentiation (Fig 2). As previously reported, treatment with 5 ng/mL (100 U/mL) TNF-α alone induced monocytic differentiation of a small percentage of the cells.14 Addition of TGF-β1 resulted in a dose-dependent increase in the percentage of cells differentiated toward the monocytic lineage, as evaluated by the percentage of cells that reduced NBT and expressed ANAE, a marker of monocytic lineage (Fig 2), and that showed monocytic morphology (data not shown).

For better definition of the role of TGF-β in promotion of monocytic differentiation, we then tested whether TGF-β was also able to synergize with other known inducers of monocytic differentiation of HL60 cells. Like TNF-α, IFN-γ and to a lesser extent IFN-α can induce monocytic differentiation of HL60 cells.15 Moreover, although higher concentrations of RA alone induce differentiation of HL60 cells toward the neutrophilic lineage,16 10-8 mol/L RA strikingly enhances the ability of TNF-α, IFN-α, or IFN-γ to induce monocytic differentiation of HL60 cells.17,18 Therefore, we tested the effect of treatment with combinations of TGF-β1 and RA, IFN-α, IFN-γ, or TNF-α on differentiation of HL60 cells. Results of a representative experiment (Table 1) showed that TGF-β1 did not potentiate differentiation induced by IFN-α or IFN-γ alone or in combination with RA. In contrast, TGF-β1 enhanced monocytic differentiation of cells treated either with TNF-α alone or with a combination of RA and TNF-α.

To investigate whether the synergy between TGF-β and TNF-α in inducing monocytic differentiation could be generalized to other cell lines, we tested the effect of combinations of TGF-β1 and TNF-α on proliferation and differentiation of the monoblastic cell line U937. TGF-β1 alone inhibited cell proliferation, with maximal inhibition of approximately 70% (Fig 3), in agreement with previous observations.7 In addition, TGF-β acted synergistically with TNF-α to inhibit proliferation, as shown by the isobologram (Fig 3). Similar results were obtained when cell proliferation was evaluated by counting cells in a hemacytometer (data not shown). Similar to the effect observed on HL60 cells, treatment of U937 cells with TGF-β1 and TNF-α resulted in synergistic induction of monocytic differentiation, evaluated by the percentage of cells that reduced NBT (Fig 4) and that expressed mature monocytic morphology (data not shown).

Increase in TGF-β receptor expression on HL60 and U937 cells after treatment with TNF-α. To investigate a possible mechanism of the synergy between TNF-α and TGF-β, we examined the effect of treatment with TNF-α on TGF-β receptor expression and the effect of treatment with TGF-β on TNF-α receptor expression on HL60 and U937 cells.

Affinity labeling of many cell types with [125I]-TGF-β typically shows the presence of three cross-linked complexes with molecular weights (mol wt) of 65, 85, and >200 Kd.19,20 Examination of TGF-β receptor expression by affinity labeling of HL60 and U937 cells showed that both cell lines expressed detectable levels of TGF-β binding proteins (Fig 5). Untreated U937 cells appeared to have much higher levels of TGF-β receptors than untreated HL60 cells, how-

### Table 1. Effect of TGF-β1 Alone and Combined With Other Agents on Monocytic Differentiation of HL60 Cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TGF-β1 (10 ng/mL)</th>
<th>NBT</th>
<th>ANAE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>-</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>RA 10 nmol/L</td>
<td></td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>TNF-α 5 ng/mL</td>
<td></td>
<td>16</td>
<td>20</td>
</tr>
<tr>
<td>RA 10 nmol/L, TNF-α 5 ng/mL</td>
<td></td>
<td>62</td>
<td>52</td>
</tr>
<tr>
<td>IFN-γ 20 ng/mL</td>
<td></td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>RA 10 nmol/L, IFN-γ 20 ng/mL</td>
<td></td>
<td>54</td>
<td>48</td>
</tr>
<tr>
<td>IFN-α 25 ng/mL</td>
<td></td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>RA 10 nmol/L, IFN-α 25 ng/mL</td>
<td></td>
<td>51</td>
<td>48</td>
</tr>
<tr>
<td>IFN-γ 20 ng/mL, RA 10 nmol/L</td>
<td></td>
<td>49</td>
<td>46</td>
</tr>
</tbody>
</table>

Fig 2. Effect of treatment with TGF-β1 with or without TNF-α on differentiation of HL60 cells. After 4-day incubation with TGF-β1 with (△, O) or without (△, O) 5 ng/mL TNF-α, the percentage of cells reducing NBT (△, △1) and expressing ANAE (O, O) was evaluated. Results are the means of two to five experiments; bars representing SD.
SYNERGY BETWEEN TGF-β AND TNF-α

Fig 3. Effect of treatment with TGF-β1 and TNF-α on proliferation of U937 cells. After 4-day incubation with the indicated concentrations of TGF-β1 without (□) or with 0.125 ng/mL (○), 0.25 ng/mL (■), 0.5 ng/mL (▲), and 1 ng/mL (▲) TNF-α, cell proliferation was evaluated by [3H]-thymidine uptake. Results are the means of triplicate assays. Insert: isobologram of antiproliferative effect of TGF-β1 and TNF-α at 50% inhibition.

However. As shown in Fig 5, treatment of both cell lines with TNF-α for 24 hours induced a significant increase in expression of the three TGF-β receptor cross-linked complexes, especially the 65-Kd moiety. When treatment with TNF-α was prolonged to 72 hours, no further increase in expression of TGF-β binding proteins was observed (data not shown), indicating that maximal induction of TGF-β receptor expression by TNF-α occurred in the first 24 hours of treatment. Treatment of HL60 and U937 cells with TNF-α for 24 hours induced an increase in TGF-β specific binding of approximately four- to fivefold and twofold, respectively (data not shown). However, owing to high nonspecific binding we could not generate reliable values for the number of binding sites and the dissociation constant by Scatchard analysis of TGF-β binding data for these cells.

We also tested the effect of the treatment with other inducers of monocytic differentiation on expression of TGF-β receptors on HL60 cells. As shown in Fig 6, neither treatment with IFN-γ nor treatment with IFN-γ and RA for 24 hours significantly increased expression of TGF-β receptors on HL60 cells.

Examination of TNF-α receptor expression on HL60 and U937 cells showed the presence of a single class of binding sites with a dissociation constant of approximately $7 \times 10^{-11}$ mol/L, in agreement with previous observations. As shown in Table 2, treatment of HL60 and U937 cells with TGF-β had no significant effect on TNF-α receptor expression on either cell line.

DISCUSSION

We report that TGF-β potentiates the antiproliferative effect of TNF-α on both HL60 and U937 cell lines and strongly synergizes with TNF-α in inducing monocytic differentiation of these cell lines. To our knowledge, this is the first report that describes an effect of TGF-β on differentiation of myeloid leukemic cell lines, suggesting that TGF-β may be one of the signals that plays a role in monocytic differentiation of hematopoietic cells.

Both TGF-β1 and TGF-β2 synergized with TNF-α in inhibiting proliferation of HL60 cells. TGF-β1 appeared to be more potent than TGF-β2, however. Although this could result from some loss of biological activity of TGF-β2 during purification, it may reflect an actual difference in biological activity of these two molecules on hematopoietic cells or, alternatively, may result from preferential binding and inactivation of TGF-β2 by α2-macroglobulin present in the FCS.

In contrast to the synergistic effects of TGF-β and TNF-α on HL60 cells, no synergy was observed between TGF-β and IFN-α or IFN-γ, two other known inducers of monocytic differentiation of these cells. Moreover, TGF-β enhanced differentiation of cells treated with combination of RA and TNF-α, but not that of cells treated with combinations of RA and IFN-α or IFN-γ. Therefore, the ability of TGF-β to increase monocytic differentiation of HL60 cells appears,
under the conditions used, to be specific for the presence of TNF-α.

Our data suggest that, depending on the inducing stimuli, different biochemical and cellular events play a role in the commitment and subsequent differentiation of HL60 cells toward the monocytic lineage. The different effect of TGF-β in the presence of TNF-α compared with either IFN-α or IFN-γ could represent a useful experimental system to dissect the biochemical events involved in monocytic differentiation of leukemic and possibly normal hematopoietic cells.

With regard to the mechanism of the synergy between TNF-α and TGF-β in inducing monocytic differentiation of HL60 and U937 cells, treatment of both cell lines with

Table 2. Effect of Treatment With TGF-β on Expression of TNF-α Receptors on HL60 and U937 Cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>TGF-β (ng/mL)</th>
<th>Dissociation Constant (mol/L)</th>
<th>Receptors/Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL60</td>
<td>—</td>
<td>$6.8 \times 10^{-11}$</td>
<td>1,000</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>$6.7 \times 10^{-11}$</td>
<td>1,300</td>
</tr>
<tr>
<td>U937</td>
<td>—</td>
<td>$8.5 \times 10^{-11}$</td>
<td>1,800</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>$8.1 \times 10^{-11}$</td>
<td>1,600</td>
</tr>
</tbody>
</table>

Cells were treated for 24 hours with the indicated amounts of TGF-β, and the binding assays were performed as described in the Materials and Methods section. Data were analyzed by the method of Scatchard.
SYNERGY BETWEEN TGF-β AND TNF-α

TNF-α induced a significant increase in expression of TGF-β binding proteins. In contrast, we did not observe a significant effect of treatment with TGF-β on TNF-α receptor expression on either cell line. In affinity labeling experiments, multiple TGF-β binding proteins have been described. Although the biological significance of these multiple TGF-β binding proteins has not been established, recent evidence suggests that the 65-Kd TGF-β receptor cross-linked complex may represent binding of TGF-β to its functional receptor (reference 24 and Falk LA, Sill K, Keller JR, Ruscetti FW, submitted for publication). Treatment of both HL60 and U937 cells with TNF-α induced a significant increase in the 65-Kd TGF-β receptor cross-linked complex. There was also good correlation between the ability of a particular cytokine to increase TGF-β receptor expression and its ability to synergize with TGF-β in inducing monocytic differentiation of HL60 cells; TNF-α, but not IFN-γ, with or without RA, both significantly increased TGF-β receptor expression and synergized with TGF-β in inducing monocytic differentiation. These results suggest that the observed synergy between TNF-α and TGF-β may result, at least in part, from the TNF-α–induced increase in TGF-β receptor expression. Whether postreceptor events also play a role in the synergy between TNF-α and TGF-β in inducing monocytic differentiation has not been determined.

We observed that U937 cells, which are already committed to the monocytic lineage, express higher levels of TGF-β receptors than the less differentiated HL60 cells and that the level of TGF-β receptor expression correlates with the differing sensitivity of these two cell lines to treatment with TGF-β alone. Moreover, peripheral blood monocytes have been reported to have detectable levels of TGF-β receptors. However, there does not appear to be a generalized increase in TGF-β receptor expression during monocytic differentiation because treatment of HL60 cells with a combination of IFN-γ and RA, which very effectively induced monocytic differentiation, did not significantly increase expression of TGF-β binding proteins at the time point examined.

Finally, with regard to the role of TGF-β in regulation of hematopoiesis, TGF-β has been reported to inhibit formation of colonies from immature precursors. Based on these previous observations and our present data, we hypothesize that TGF-β may play a bifunctional role in hematopoiesis both by inhibiting proliferation of immature precursors and by promoting differentiation of late precursors committed to the monocytic lineage. In vivo, TGF-β may protect immature precursors from the effect of cell cycle-active drugs and concurrently induce differentiation of leukemic cells, and therefore have therapeutic application.

ACKNOWLEDGMENT

We thank Drs Corrado Baglioni and Richard Smith for radiolabeled TNF-α. We also thank Richard Dick for excellent technical assistance, Carla Hemp for manuscript preparation, and Des Joost J. Oppenheim, Dan L. Longo, Jonathan R. Keller, and Hsiang-fu Kung for critical review of the manuscript.

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