In Vitro Interaction of Recombinant Tumor Necrosis Factorα and All-Trans–Retinoic Acid With Normal and Leukemic Hematopoietic Cells

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Both human recombinant tumor necrosis factor alpha (TNFα) and all-trans–retinoic acid (RA) inhibit the in vitro clonal growth of human myeloid leukemic cells. We investigated the in vitro interaction of TNFα and RA with normal and a variety of leukemic myeloid cells. With the promyelocytic HL-60 cells, TNFα (≥2.5 U/mL) in combination with RA synergistically inhibited clonal growth; TNFα at lower concentrations (≤1 U/mL) plus RA (10^{-10} mol/L) were antagonistic in their inhibition of growth. The ability of RA (10^{-8} mol/L) plus TNFα (2.5-5 U/mL) to enhance differentiation of HL-60 cells paralleled their ability to inhibit clonal growth of these cells. In addition, RA (10^{-8} to 10^{-7} mol/L) increased the number of TNFα receptors on HL-60 cells 1.3- to 1.7-fold without changing the affinity for the TNFα receptor. With the more immature KG-1 myeloblasts, concentrations of TNFα >10 U/mL synergistically interfered with RA to inhibit clonal growth; at lower concentrations of TNFα (<10 U/mL), RA appeared to inhibit the expected effect of TNFα. KG-1 cells were not induced to differentiate with either agent alone or in combination. With four of nine leukemic patients, TNFα in combination with RA (10^{-9} mol/L) inhibited leukemic clonal growth to a greater extent than each agent alone. No marked effect of the combined treatment was seen in two other patients. The RA reversed the inhibitory action of TNFα on normal human granulocyte-macrophage colony forming cells (GM-CFC) and on clonal growth of leukemic cells from three patients. Our study suggests that TNFα and RA interact in a complex manner with normal and leukemic hematopoietic cells.

MATERIALS AND METHODS

Chemicals. The RA was a generous gift from M. Sherman (Hoffmann-La Roche, Inc, Nutley, NJ). Stock solution of 2.5 × 10^{-10} mol/L RA was prepared in 100% ethanol and stored at −20°C under a nitrogen atmosphere for no longer than 14 days. Before use, dilutions were made in phosphate-buffered saline (PBS) to the final concentrations of 1 × 10^{-10} to 1 × 10^{-9} mol/L. The concentrations of ethanol (<0.04%) had no influence on colony formation in control plates as compared with culture plates not containing ethanol. All experiments were performed in subdued light. The TNFα was generously provided by Dr M. Shepard (Genentech Inc, San Francisco). The stock solution (1 mg corresponds to 3.7 × 10^{12} U; 1 U corresponds to 27 pg) was kept at 4°C in a sealed container. Before use, TNFα was diluted in alpha modified minimum essential medium (Flow Laboratories, Inc, Rockville, MD) plus 10% fetal calf serum (FCS) (Irving Scientific, Santa Ana, CA) to final concentrations of 10,000 to 0.5 U/mL.

Cell preparations. The human myeloid cell lines HL-60 (passage 35, mean doubling time 48 hours) and KG-1 (passage 30, mean doubling time 48 hours) were maintained in culture T flasks (Miles Lab, Naperville, IL) containing alpha medium and 10% FCS. The cells were incubated in a humidified atmosphere with 5% CO₂. The medium was changed twice a week. All experiments were performed in the logarithmic growth phase of the cells. Normal human bone marrow cells were obtained from informed, healthy volunteers. Likewise, peripheral blood or bone marrow samples from leukemia patients were obtained after informed consent. The bone marrow or peripheral blood cells were layered over Ficol-Hypaque (density 1.077 g/mL) (Sigma Chemical Co, St Louis) and centrifuged for 40 minutes × 450 g. The light density, mononuclear cell fraction was washed twice in PBS and resuspended in alpha medium plus 10% FCS. Leukemia cells from all patients with chronic myelogenous leukemia (CML) contained the Philadelphia chromosome.

Colony formation assay by two layer agar technique. The colony formation assay was performed as previously described. Briefly, an underlayer was plated in 1-mL portions in 35-mm petri dishes.
cells form colonies without CSF. The KG-l myeloblastic leukemia conditioned medium (CM) acetate esterase (ANAE) the cells were stained according to routine cell line (Mo) (Mo-CM) was used at concentrations of 1.5% to 2.5%. The overlayer (1-mL) contained cells, alpha medium, 16% FCS, antibiotics and 0.3% agar. The HL-60 promyelocytic leukemia cells form colonies without CSF. The KG-1 myeloblastic leukemia cells (early passage), normal human bone marrow and leukemic cells from patients required CSF for clonal growth. The culture dishes were incubated at 37°C in a humidified atmosphere with 7% CO₂ for ten to 13 days. Colonies (>40 cells) were counted using an inverted microscope.

**Cytochemistry.** For nitroblue tetrazolium reduction (NBT), a total of 1 x 10⁶ cells/mL were incubated in an equal volume of 1.25 mg NBT (Sigma), 17 mg bovine albumin (Sigma), and 10⁻¹⁰ mol/L 12-0-tetradecanoylphorbol 13-acetate (Consolidated Midland, New Rochelle, NY) in alpha medium plus 10% FCS for 30 minutes at 37°C. Cytospin slides of the cells were stained with safranin (Difco Laboratories, Inc. Detroit) and the number of cells containing reduced blue black formazan deposits was counted. For a-naphtyl acetate esterase (ANAE) the cells were stained according to routine hematologic methods, kindly performed by Dr F. Naeim (Department of Pathology, UCLA). Morphology was assessed by Giemsa staining. In each of the various experiments at least 200 cells were examined. Cell viability was >90% in all experimental procedures, as determined by trypan blue exclusion.

**Monoclonal antibodies, indirect immunofluorescence.** The monoclonal antibodies anti-κ-M3 and Y2FITC (mouse IgG2) were obtained from Becton-Dickinson (Mountain View, CA). Anti-κ-M3 reacts with 70% to 90% of normal human peripheral blood monocytes and has marginal reactivity with peripheral blood granulocytes. Y2FITC was used as isotypic control to define background staining. Analyses were performed on EPICS C flow cytometer (Coulter Electronics, Hialeah, FL), as described.

**Binding of 125I-TNFα by HL-60 cells.** The TNFα was iodinated by the iodogen method as described. Briefly, 10 μg of purified recombinant protein was incubated for ten minutes at 4°C in an iodogen (Pierce, Rockford, IL) coated glass tube with 1 μCi of 125I-TNFα (Amersham Corp., Arlington Heights, IL). Free iodide was separated from TNFα on a Sephadex G-25 column. The HL-60 cells were incubated with 125I-TNFα (10⁻¹⁰ to 9 x 10⁻¹⁰ cpn) at 23°C for 90 minutes in alpha-medium, 10% FCS, and 20 mol/L Hepes (Sigma), washed extensively and the cell pellets counted in a Beckman gamma-counter. Previous experiments by us showed that at 23°C the peak of specific binding was reached at 45 minutes. Nonspecific binding was determined by incubating the cells with a 100-fold excess of unlabeled TNFα. The specific activity of 125I-TNFα was determined with a self-displacement experiment in which KG-1 cells were incubated with 0.4 to 10 μg unlabeled TNFα in the presence of 4 x 10⁻⁵ to 5 x 10⁻⁵ cpn 125I-TNFα and found to be 4.2 x 10⁴ cpn/μg. Scatchard curves, the receptor number per cell and the affinity constants of binding were calculated with a computer program as described. Under reducing conditions, iodinated recombinant TNFα gave a single band of the approximate mol wt of 17,000 on a 12% SDS-PAGE gel. Under nonreducing conditions and longer exposure an additional faint band at ~34,000 daltons was observed (<2% of the intensity of the 17,000 band), indicating that little TNFα aggregates in vitro. Further, iodinated TNFα retained for over 2 months its biologic activity as measured by the inhibition of colony formation of HL-60 cells.

**Statistical methods.** Dose response curves were fit for each target (HL-60, KG-1 cells) and for each anti-tumor drug (RA, TNFα) by weighted least squares logistic regressions. At each percent inhibition, we computed a combination index (CI) for the additive (mutually exclusive binding) model using the method of Chou and Talalay. We computed a standard error for CI (SE(CI)) using the delta method. Drug combinations resulting in a given percent inhibition were said to be synergistic if CI + SE(CI) < 1, antagonistic if CI - SE(CI) > 1, and additive if CI + SE (CI) = 1. Cooperation between the drugs was also assessed by computing the fractional inhibitory concentration and its SE for each test concentration. These computations were based on the isobol method as described. The effect of adding RA to TNFα compared with TNFα alone in the leukemic patients was assessed by fitting a model linear in the logs of the data. Significance was determined by comparing the effects to its standard error, which results in normal z statistics.

**RESULTS**

We examined the effect of the combination of TNFα (0.5 to 100 U/mL) and RA (10⁻⁴, 10⁻⁹ mol/L) on clonal growth of the HL-60 promyelocytic cell line (Fig 1A). The TNFα and RA showed a dose-dependent interaction. Statistical analyses of the data (see Materials and Methods) showed that concentrations of TNFα of 2.5 U/mL in combination with RA (10⁻⁴, 10⁻⁹ mol/L) synergistically inhibited clonal growth of HL-60 cells. Evidence for an antagonistic interaction of TNFα and RA was observed at TNFα concentrations up to 1 U/mL when combined with 10⁻⁹ mol/L RA. Similar interpretations of the results were obtained when the isobol method of analysis was applied.

In an attempt to determine possible mechanisms of this enhanced inhibitory effect of TNFα and RA in HL-60 cells, several experiments were performed. The ability of TNFα plus RA to induce differentiation of HL-60 to mature cells was studied by examining the capability of these cells to reduce NBT (Fig 1B). At the concentrations tested (5 and 2.5 U/mL for six days), TNFα alone induced a mean 24% ±
6% (±SD) and 16% ± 4% (±SD) of the HL-60 cells to reduce NBT, respectively. The RA alone (10⁻⁴ mol/L) induced differentiation of a mean 34% ± 3% (±SD) of HL-60 cells. Exposure of HL-60 cells for six days to TNFα (5 U/mL) plus RA (10⁻⁴ mol/L) significantly (P < .001) increased the number of these cells that reduced NBT (mean 59% ± 8% [±SD]) compared with the effect of each agent alone (mean 24% ± 6% [±SD] and 34% ± 3% [±SD] for TNFα and RA, respectively). Absolute numbers of HL-60 cells of one representative experiment were (×10⁶/mL): TNFα and RA, respectively. Absolute numbers of HL-60 cells of one representative experiment were (×10⁶/mL): control, 1.2; TNFα 2.5 U/mL, 0.85; RA (10⁻⁴ mol/L), 0.75; TNF 5/µL plus RA, 0.4; TNF 2.5 U/mL plus RA, 0.5. Lower concentrations of TNFα (0.5, 1.0 U/mL) in combination with RA (10⁻⁴ mol/L) showed no increase in the number of NBT positive cells, as compared with cells treated with RA alone; and TNFα alone showed no effect on NBT reduction at these concentrations (data not shown).

To determine the pathway of differentiation, HL-60 cells were exposed to TNFα plus RA (5 U/mL and 10⁻⁴ mol/L × 6 days, respectively) or to each agent alone (Table 1). Morphologically, 17% of the cells treated with RA alone showed granulocytic like differentiation; TNFα in combination with RA increased the number of differentiated cells to 62% with 20% of the cells showing morphological features of granulocytic-like cells and 42% having metamyelocytic- or monocytic-like appearance. In the presence of TNFα alone, 12% of the cells showed monocytic-like differentiation. No adherence was observed in cells treated with either TNFα alone or TNFα plus RA. Cytochemical analyses revealed that exposure to TNFα plus RA increased the number of ANAE positive cells to 35%, as compared with 15% ANAE positive cells when treated with TNFα alone, respectively. Fifteen percent of these cells (cultured with TNFα plus RA) were morphologically differentiated but were ANAE negative. Cytofluorometric analyses showed that the combination of RA and TNFα increased the percentage of HL-60 cells expressing leu M3 (antigen expressed on monocytes but not granulocytes) to 24%, as compared with 15% and ±2% in cells treated with TNFα or RA, respectively. Together, these results suggest that HL-60 cells when cultured with RA plus TNFα differentiate towards a mixed population of monocytic- and granulocytic-like cells.

The effect of RA on the expression of the TNFα receptors by HL-60 cells was examined as measured by the binding of 125I-labeled recombinant human TNFα (Table 2 and Fig 2, for a representative Scatchard plot). The HL-60 cells were incubated for five days with several concentrations of RA (10⁻⁷ to 10⁻⁵ mol/L) and number of TNFα receptors per cell was determined. The wild type HL-60 cells expressed a single class of high affinity receptors (mean 776 ± 40 [±SD] receptors with a Kd binding affinity of 42 pmol/L). The RA increased expression of the number of TNFα receptors without a marked change in the affinity constants of binding of TNFα (1.3, 1.6, and 1.7-fold increase for cells cultured with 10⁻⁴ mol/L, 10⁻⁵ mol/L, and 10⁻⁶ mol/L RA, respectively as compared with wild-type HL-60 cells).

The effect of clonal growth of the combination of TNFα (1 to 1,000 U/mL) and RA (10⁻⁴ mol/L) was further studied using cells of the human myeloblastic line, KG-1, which are more immature than HL-60 cells and do not undergo differentiation in the presence of RA. The KG-1 cells were less sensitive than HL-60 cells to the inhibitory action of TNFα (50% clonal inhibition [ED₅₀] at 15 U/mL for KG-1 cells as compared with 3.5 U/mL for HL-60 cells). By contrast, KG-1 cells were more sensitive than HL-60 cells to inhibition of growth by RA (ED₅₀ 4 × 10⁻⁵ mol/L and 1 × 10⁻⁴ mol/L, respectively). The combination of TNFα and RA resulted in various effects on clonal growth of KG-1 cells. The TNFα showed synergism with RA (10⁻⁴ mol/L) in the inhibition of clonal growth only at concentrations ≥10 U/mL. At lower concentrations of TNFα (<10 U/mL), the two agents were antagonistic in their inhibition of clonal growth of KG-1 cells. The TNFα

<p>| Table 1. Differentiation of HL-60 Cultured With TNFα and RA |
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<table>
<thead>
<tr>
<th>Treatment (6 d)</th>
<th>RA (10⁻⁴ mol/L)</th>
<th>TNFα (5 U/mL)</th>
<th>No. of Cells (× 10⁶/mL)</th>
<th>Morphology (Percent Differentiated Cells)</th>
<th>ANAE (Percent Positive Cells)</th>
<th>Leu-M3 (Percent Positive Cells)</th>
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<td>–</td>
<td>–</td>
<td>1.4</td>
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<td>+</td>
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<td>0.9</td>
<td>17*</td>
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<tr>
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<td>+</td>
<td>+</td>
<td>0.7</td>
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HL-60 cells were cultured for six days with TNFα and/or RA and analyzed as described in Materials and Methods. Similar results were obtained in three independent experiments.

Abbreviations: ANAE, a-naphthylacetate esterase; RA, retinoic acid; TNFα, recombinant tumor necrosis factor α.

*Granulocytic-like cells.
†Monocytic-like cells.
‡Twenty percent granulocytic-like cells; 42% with metamyelocytic or monocytic features.
TNFα AND ALL-TRANS RA INHIBIT HMLC

Fig 2. Scatchard plot of specific TNFα binding to HL-60 cells. HL-60 cells were cultured for five days either in the presence of RA (10^{-8} mol/L) or in the absence of RA (control). 0.8 \times 10^6 cells were then incubated with 10,000 to 900,000 cpm of ^{125}I-TNFα for 90 minutes at 23°C. Correlation coefficients were R = 0.986 for the control samples, and R = 0.980 for the RA-treated samples.

(2.5, 5.0 U/mL) and RA (10^{-4} mol/L) alone or in combination were not able to induce differentiation of KG-1 cells as determined by morphology and NBT-reduction (data not shown).

Diverse responses on clonal growth of leukemic cells freshly obtained from patients were observed when combinations of TNFα (1 to 10,000 U/mL) and RA (10^{-7} mol/L) were tested (Fig 4). The diagnoses are listed in the legend of Fig 4. In six of nine patients (no. 1 through 6) both RA and TNFα alone inhibited clonal growth to various degrees. With patients no. 1 through 4, the TNFα in combination with RA showed on average a greater inhibition of leukemic clonal growth (P < 0.05) as compared with TNFα alone. In two patients (no. 5 and 6), RA appeared to reduce the effect of TNFα at some but not all TNFα concentrations. In three patients (no. 7 through 9) TNFα alone inhibited leukemic growth, whereas RA alone stimulated leukemic growth; and when combined with TNFα, RA markedly decreased (P < 0.05) the inhibitory action of TNFα.

Further studies investigated the effect of the combination of TNFα and RA on GM-CFC (Fig 5). Normal human myeloid progenitor cells were ~2,800 times less sensitive to the inhibitory action of TNFα than HL-60 cells (ED₅₀ at 10,000 U/mL and 3.5 U/mL TNFα, respectively) when CM from a human T lymphocyte cell line (Mo-CM) was used as a source of CSF. The RA alone (10^{-7} mol/L) enhanced GM-CFC formation ~1.6-fold. The addition of RA (10^{-7} mol/L) to the culture dishes containing TNFα (1 to 10,000 U/mL) either partially or completely reversed the inhibitory effect of TNFα on formation of GM-CFC. For example, at a concentration of 1,000 U/mL TNFα, a mean 40% ± 6% (±SD) of the GM-CFC were inhibited as compared with control plates; with the addition of 10^{-7} mol/L RA to plates containing 1,000 U/mL TNFα, GM-CFC formation was
nearly the same as control plates (mean 92% ± 6% [±SD] of control).

DISCUSSION

Our study shows that TNFα and RA interact in a complex manner with leukemic cell lines, leukemic cells from patients and normal human bone marrow cells. With HL-60 cells, TNFα (≥2.5 U/mL) and RA mainly synergistically inhibited clonal growth (Fig 1A). However, evidence for an antagonism was seen at lower concentrations of TNFα (≤1 U/mL) and RA (10⁻⁹ mol/L). At these concentrations, RA seemed to reduce the expected inhibition of TNFα on clonal growth inhibition of HL-60 cells. The more immature myeloblastic KG-1 cells were less sensitive than HL-60 cells to the inhibitory action of TNFα alone, and only higher concentrations of TNFα (≥10 U/mL) showed a synergistic interaction with RA (Fig 3). At <10 U/mL TNFα, antagonism was seen. A phase I trial with TNFα in cancer patients showed that after intravenous injection of 100 µg/mol/L, peak serum levels of 8,000 pg/mL (~300 U/mL) were reached with acceptable toxicity. Our study with HL-60 and KG-1 cells shows that TNFα had synergistic interactions with RA at concentrations of both TNFα and RA that can be achieved in vivo.

Recent studies demonstrated that TNFα induced HL-60 cells to differentiate towards monocytes/macrophages. The RA in vitro induced HL-60 and leukemic cells from some patients to differentiate towards granulocytes. The ability of the combination of TNFα and RA to enhance differentiation of HL-60 cells (Fig 1B) suggests that part of the synergism between TNFα and RA to inhibit clonal growth might be explained by their combined ability to increase the number of HL-60 cells that differentiate into mature nondividing cells. A previous study by us showed that TNFα preferentially inhibited growth of colonies containing granulocytes rather than macrophages or macrophage/granulocyte colonies. This suggested that the combination of TNFα and RA may have selectively enhanced differentiation along the granulocytic pathway. However, analyses by morphology, cytochemistry and antigen expression showed that the combined treatment with TNFα and RA resulted in differentiation of HL-60 cells along both the macrophage and granulocyte pathway (Table 1), confirming previously reported data.

The TNFα probably mediates its action through binding to specific receptors present on the surface of the cell. Normal and leukemic hematopoietic cells express receptors for TNFα. The RA (10⁻⁹ – 10⁻⁷ mol/L) moderately increased the number of TNFα receptors expressed on HL-60 cells (1.3- to 1.7-fold) as compared with wild-type HL-60 cells, but did not alter the binding affinity of the TNF receptors for their ligand in these cells (Fig 2, Table 2). Studies recently showed that γ-interferon (γ-IFN) increased the number of TNFα receptors on several human carcinoma cell lines, on murine L929 fibroblasts, and on the myeloid U937 cells. Similar to the combination of TNFα and RA, TNFα plus γ-IFN had an increased cytotoxic/cytostatic effect as compared with each agent alone on human carcinoma cell lines, myeloid leukemia cell lines and leukemic cells from patients, suggesting that increased cellular expression of the TNFα receptor may explain the enhanced responses. However, recent studies by us as well as others found no direct correlation between the number of receptors expressed on cells of various myeloid lines and their sensitivity to the growth inhibitory effects of TNFα. Furthermore, TNFα was effective in clonal growth inhibition at concentration well below the saturation of its receptor (<10% occupancy) together. These results suggest that expression of increased numbers of TNFα receptors induced by RA is unlikely to account for the greater sensitivity of HL-60 cells and that mechanisms distal to TNFα binding to its receptor might be responsible for the enhanced cytotoxic/cytostatic effect of TNFα in combination with RA.

Various types of responses were observed on clonal growth inhibition of leukemic cells from patients. Both RA and TNFα alone inhibited clonal growth of leukemic cells of the majority of the patients, in agreement with previous studies. However, RA alone also stimulated clonal growth of three of nine leukemic patients (no. 7 through 9). Two of the patients had CML in myeloid blast crisis (no. 7 and 8), and one had therapy-related AML (no. 9). Similarly, previous studies reported stimulation of leukemic clonal growth in selected patients. The combination of TNFα plus RA showed a greater clonal growth inhibition compared with TNFα alone in some patients (no. 1 through 4); whereas in other patients (no. 5 through 9), RA either reduced or even reversed the inhibitory effect of TNFα. This suggests that in selected patients, RA may not enhance the inhibitory effect of TNFα, but possibly may protect leukemic growth from the inhibitory action of TNFα.

Our study confirms previous studies that retinoids enhance in vitro clonal growth of normal human myeloid progenitor cells. The TNFα alone inhibited clonal growth of normal human GM-CFC only at much higher concentrations (ED₉₀ ~10,000 U/mL) as compared with leukemic clonal growth of both HL-60 cells (ED₉₀ 3.5 U/mL) and the majority of clonogenic leukemic cells harvested from patients (Fig 5). The combination of TNFα and RA had less growth inhibitory effect than TNFα alone on normal GM-CFC. This suggests that RA may have a protective effect on the TNFα-mediated inhibition of normal GM-CFC, and theoretically may allow higher concentrations of TNFα to be given without suppressing normal hematopoiesis.

Although RA inhibited in vitro clonal growth of myeloid leukemic cells of the majority of patients, in vivo RA usually had a therapeutic effect limited to patients with acute promyelocytic leukemia and selected patients with the myelodysplastic syndrome. Phase I and II trials with TNFα for AML are ongoing. In vivo animal studies are warranted to investigate further the complex interaction of TNFα and RA on normal and leukemic hematopoiesis and to help identify patients who may benefit from this combined treatment.

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