Differential Biologic Effects Resulting From Bimodal Binding of Recombinant Human Tumor Necrosis Factor to Myeloid Leukemia Cells

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Human recombinant tumor necrosis factor (rTNF) bound to ML-1 and HL-60 human myeloid leukemia cells in a bimodal manner. Saturable high-affinity binding was maximal at ~3 nmol/L rTNF, whereas saturable low-affinity binding reached its maximum at ~30 nmol/L. As analyzed by computer program, the observed data fit a two-receptor site model, with $p < 0.05$. High-affinity binding concentrations of rTNF caused the differentiation of both cell lines, whereas low-affinity binding concentrations abolished this effect in a concentration-dependent manner. Thus, the type of biologic response elicited with rTNF in these cells is a function of the concentration at which the factor is applied. If generally applicable, this bimodal effect may require consideration when rTNF is to be used therapeutically.

Preparation of $^{125}$I-rTNF. rTNF was radiiodinated by the Bolton-Hunter method. Fifty microliters 0.1 mol/L sodium borate buffer, pH 8.4, was added to 40 μL phosphate-buffered saline (PBS) containing 1.96 mg rTNF; 32 μL of the mixture (0.7 mg rTNF) was reacted with 4 mCi Bolton-Hunter reagent (4,000 Ci/mmol; ICN Radiochemicals, Irvine, CA) for four hours at room temperature. The reaction was stopped by addition of 20 μL 1 mol/L glycine. The radioligand was separated from free iodine by filtration on a Sephadex G-10 column pretreated with bovine serum albumin (BSA, 2 mg/mL) and washed with PBS pH 7.5. $^{125}$I-rTNF was eluted with PBS in the void volume. The factor has a specific activity of 69 Ci/mmol protein, and on 10% polyacrylamide slab gel, gave a single labeled band of molecular weight (mol wt) 17,000, which retained the original biological activity >98%, as determined by the L-cell cytotoxicity assay described previously. For storage at −70°C, the factor was diluted with PBS containing 0.1% gelatin as carrier protein.

MATERIALS AND METHODS

Cell lines and culture conditions. HL-60, human promyelocytic leukemia cells, and ML-1, human myeloblastic leukemia cells, were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), as described previously. Tumor necrosis factor (TNF). Highly purified (>99.9%) human recombinant TNF (rTNF) was provided by Asahi Chemical, Tokyo. The specific activity of rTNF, as determined by cytotoxicity assay on LM-cells, was 2.18 × 10$^7$ U/mg. Therefore, 1 U/mL rTNF corresponds to 0.027 nmol/L of the factor.

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Preparation of $^{125}$I-rTNF binding assay. The binding assay was performed by a modification of a method described previously. $^{125}$I-rTNF was incubated for 30 minutes at 4°C with 5 × 10$^5$ ML-1 cells or with 2.5 × 10$^5$ HL-60 cells in 0.5 mL RPMI 1640 medium containing 10% fetal calf serum (FCS). After incubation, the cells were washed once and resuspended in 0.2 mL of the same medium free of rTNF. The cell suspension was layered onto 150 μL of a mixture of 20% olive oil and 80% di-n-butylphthalate, and was centrifuged for ten seconds at 9,000 g. The tip of the centrifuge tube containing the cell pellet was cut off, and the radioactivity of the pellet was measured in a γ counter. Nonspecific binding for each concentration of labeled rTNF was determined by incubating cell preparations with 10$^5$ U/mL unlabeled rTNF. Specific binding was obtained by subtracting nonspecific binding from total binding; nonspecific binding was determined for each input of labeled rTNF.

Assay of cell growth and differentiation. Cells were harvested from logarithmically growing cultures and were resuspended at a concentration of 3 × 10$^5$ cells/mL with the various concentrations of rTNF indicated. After three days of incubation at 37°C in a 5% CO$_2$ atmosphere, the cells were harvested, and viability, cell growth, and differentiation were determined. Viability was estimated by trypan
blue dye exclusion, cell growth was assayed by hemocytometer, and growth inhibition was determined as $1 - (\text{number of rTNF-treated cells/number of control cells}) \times 100\%$. The extent of differentiation was assessed by determining the percentage of NBT-positive cells obtained, using the procedure described previously. Monocyte/macrophage lineage-specific differentiation was confirmed by morphologic change and by nonspecific esterase activity. Therefore, all binding experiments were performed at 4°C. At 50 U/mL rTNF, nonspecific binding to ML-1 and HL-60 cells amounted to ~1% and ~3% of total binding, respectively, whereas at 500 U/mL, nonspecific binding amounted to 6% and 12%, respectively. At rTNF concentrations <100 U/mL (high affinity), 99% of the factor was elutable with pH 3 buffer (0.05 mol/L glycine, HCl/0.15 mol/L NaCl), indicating that no significant internalization occurred. In contrast, at concentrations >500 U/mL, 15% to 25% of the bound $[^{35}S]$rTNF resisted removal by this treatment. Resistance to the removal of a small (20%) fraction of rTNF at acid pH and 4°C was also observed in HeLa cells, but the significance of this refractivity remains unexplained.

As shown in Fig 1, $[^{35}S]$rTNF bound to ML-1 cells in a specific manner, the labeled ligand being displaced by an excess of ~100-fold of unlabeled rTNF. Equilibrium-specific binding of $[^{35}S]$rTNF to ML-1 cells showed a bimodal pattern (Fig 2), and a similar bimodal pattern was observed with HL-60 cells (not shown). This bimodal binding pattern is reflected in the curvilinear nature of the Scatchard plots shown in Fig 3 A and B. These binding data were analyzed by the ISIS-12 computer program developed by Thakur et al. The dissociation constants (kd) for high- and low-affinity binding were 0.22 and 6.3 nmol/L for ML-1 cells and 0.28 and 13.2 nmol/L for HL-60 cells. The number of high- and low-affinity receptors per cell were 4,500 and 2,730 for ML-1 and 2,910 and 2,870 for HL-60 cells. The goodness-of-fit of these data for two independent classes of receptor sites proved significant at $P < .05$ by the following statistical parameters: for ML-1—mean percentage of deviation (MPD) 0.78; SD of the percentage of deviation (SDMPD) 4.96; t test for bias (TB) 0.648; residual variance (RV) 31.05; root mean-square error (RMS error) 5.573; serial correlation (SC) 0.422; and $r$ test for significance (TS) 1.743. For HL-60, parameters were MPD —1.23, SDMPD 7.08, TB —0.673, RV 65.82, RMS error 8.113, SC 0.505, and TS 2.025.

To assess the effect that bimodal binding of rTNF exerts on cell growth and differentiation, increasing concentrations of the factor were added to cultures of ML-1 and HL-60 cells. At concentrations of 0.1 to 100 U/mL, rTNF inhibited the growth of these leukemic cells in a concentration-dependent manner (Fig 4A and B), and this inhibition was paralleled by a dose-dependent increase in the differentiated cell fraction. At rTNF concentrations >100 U/mL, both growth inhibition and differentiated cell fraction decreased in a dose-dependent manner. Viability of the HL-60 cells (Fig 4b) was not diminished significantly by any of the rTNF concentrations used, supporting our deduction that growth inhibition was the result of cell differentiation. In contrast, at a limited dose range of ~100 U/mL, rTNF exerted a cytotoxic effect on ML-1 cells (Fig 4A) reflected by a decrease in cell viability and in the number of mature cells. In ML-1 cell cultures treated with rTNF, a significant difference was observed between the extent of growth inhibition and the degree of cell differentiation. This difference probably was caused by differentiation-committed cells.
BIOLOGICAL EFFECTS OF BIMODAL TNF BINDING

Fig 3. Scatchard analysis of the binding data obtained with ML-1 (a) and with HL-60 cells (b).

Fig 4. Viability (○), growth inhibition (●) and differentiation (△) of ML-1 cells (a) and HL-60 cells (b) after incubation for three days with various concentrations of rTNF. Data are percentages of the values obtained without rTNF and represent means ± SD derived from at least three separate experiments.

which, on the day of assay, had not as yet expressed the differentiated phenotype.

DISCUSSION

The data we present show that rTNF binds to ML-1 and HL-60 human myeloid leukemia cells in a bimodal manner and the results, analyzed by computer program, favor a model involving two receptor sites. rTNF binding sites with two different affinities were previously shown to be present on human monocytes.4 The data also show that a close coincidence exists between binding affinity and the type of biological effect that results. High-affinity binding concentrations, up to ~100 U/mL, cause differentiation, whereas low-affinity binding concentrations abolish this effect. The concentrations of rTNF that provide half-maximal occupancy of the high- and low-affinity binding sites (kd) approximate the concentrations required for half-maximal biologic activity, supporting the indicated correlation between binding mode and biologic effect.

Given the results of the computer analysis, the divergent
biologic effects observed at high and low rTNF concentrations probably do not derive from negative cooperativity. However, the possibility exists that, at 37°C, high rTNF concentrations can cause receptor downregulation or desensitization.

Alternatively, the effects of rTNF may be mediated by growth and differentiation factors present in the culture medium, analogous to the differentiation-inducing activity of DNA-specific antineoplastic agents, which is dependent on natural differentiation factors contained in the serum. Thus, rTNF enhanced the binding of epidermal growth factor (EGF) to human FS-4 fibroblasts by increasing the number of EGF binding sites. EGF, in turn, limited the mitogenic response of osteoblastic bone cells to TGF-β and interfered with the antiproliferative effect of rTNF on human cervical carcinoma cells. This latter effect was not caused by downregulation of the TNF receptor or alteration of its affinity. Previously accrued data indicate that TNF and EGF activate identical or similar paths.

The differentiation-inducing activity of TNF had also been shown to depend on the presence of other effectors such as IL-1, and, in HL-60 cells, TNF shared binding sites with a differentiation-inducing factor, these sites being susceptible to downregulation by activation of protein kinase C.

Because bimodal binding was observed within 30 minutes at 4°C, it is unlikely that the phenomenon derived from induction of other regulatory molecules in the cells. Whatever the mechanism of its action, the bimodal biologic effect of rTNF may have to be taken into consideration when this factor is applied therapeutically.

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