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Tumor necrosis factor: Specific binding and internalization in sensitive and resistant cells

(receptors/interferon γ/lymphotoxin/chloroquine)

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ABSTRACT **Highly purified, *Escherichia coli*-derived recombinant human tumor necrosis factor (TNF) was labeled with ^{125}I and employed to determine receptor binding, internalization, and intracellular degradation in murine L929 cells (highly sensitive to the cytotoxic action of TNF) and in diploid human FS-4 cells (resistant to TNF cytotoxicity). ^{125}I-labeled TNF bound specifically to high-affinity receptors on both L929 and FS-4 cells. Scatchard analysis of the binding data indicated the presence of 2200 binding sites per L929 cell and 7500 binding sites per FS-4 cell. The calculated dissociation constants are 6.1×10^{-10} M and 3.2×10^{-10} M for L929 and FS-4 cells, respectively. In both L929 and FS-4 cells, incubation at 37°C resulted in a rapid internalization of the bulk of the cell-bound TNF, followed by the appearance of trichloroacetic acid-soluble ^{125}I radioactivity in the tissue culture medium, due to degradation of TNF. Degradation but not cellular uptake of TNF was inhibited in the presence of chloroquine (an inhibitor of lysosomal proteases) in both L929 and FS-4 cells, suggesting that degradation occurs intracellularly, probably within lysosomes. These results show that resistance of FS-4 cells to TNF cytotoxicity is not due to a lack of receptors or their inability to internalize and degrade TNF.**

Tumor necrosis factor (TNF) was originally described as a protein found in the serum of animals sensitized with *Bacillus* Calmette–Guerin or *Corynebacterium parvum* and challenged with bacterial endotoxin (1). TNF is functionally defined as a protein producing hemorrhagic necrosis of some tumors in experimental animals and cytolytic or cytostatic effects in tumor cells in culture. Since the major cellular source of TNF is the macrophage (1–6), TNF is thought to be a mediator of the cytotoxic activity of macrophages against tumor cells (7). Recently, human TNF was purified to homogeneity (8) and cDNA for human TNF was cloned, sequenced, and expressed in *Escherichia coli* (9–11). As a result of these advances, highly purified recombinant TNF has become available for experimental studies.

One of the most intriguing properties of TNF is the apparent selectivity of its cytotoxic and cytostatic activities. TNF is active against many types of tumor cells, whereas untransformed cells generally remain unaffected (1, 11). The mechanism of this selectivity as well as the mechanisms by which TNF exerts its cytolytic or cytostatic activity are unknown. The action of polypeptide hormones, growth factors, and cytokines is generally initiated by their binding to specific cellular receptors (12). This binding is usually followed by internalization of the receptor–ligand complex, through receptor-mediated endocytosis, and the eventual degradation of the ligand by lysosomal hydrolases (13).

In this study we examined the presence of high-affinity receptors on two cell lines—one highly sensitive (L929) and one completely resistant (FS-4) to the cytotoxic action of TNF. Specific high-affinity receptors were demonstrated on both cell lines. Neither the numbers of binding sites per cell nor the binding affinities of TNF for the receptors on the two cell lines could provide an explanation for the vastly different sensitivities of the two cell lines. Internalization and subsequent intracellular degradation also was demonstrated in both cell lines. Chloroquine, an inhibitor of lysosomal proteases (14), suppressed TNF degradation in both L929 and FS-4 cells. We conclude that the action of TNF involves binding to specific cell-surface receptors and receptor-mediated endocytosis. However, resistance of nonmalignant cells to the cytotoxic and cytostatic action of TNF is apparently not due to the absence of specific receptors or the failure of TNF to be internalized by such cells.

MATERIALS AND METHODS

Materials. Recombinant human TNF was produced in *E. coli*, purified to homogeneity (≥99%, based on $NaDodSO_4$/PAGE analysis), and supplied by the Suntory Institute for Biomedical Research (Osaka, Japan). The molecular weight of the *E. coli*-derived TNF was determined to be 34,000 by gel-permeation column chromatography. A single lot of TNF with a specific activity of 4.8×10^7 units/mg of protein was employed. Recombinant *E. coli*-derived human interferon γ (IFN-γ, specific activity 2.1×10^7 units/mg) was obtained from Biogen (Cambridge, MA). RPMI 1640 medium and Eagle's minimal essential medium (MEM) were purchased from GIBCO. Chloroquine and monensin were purchased from Sigma.

Cell Lines. L929 cells (alpha subline) were originally obtained from G. Granger (University of California at Irvine) and cultured in RPMI 1640 medium with 3% fetal bovine serum (FBS). The FS-4 diploid cell strain was established in this laboratory from a single human foreskin and cultured in MEM with 5% FBS.

Assay for TNF Activity. The biological activity of TNF was determined by a method developed for lymphotoxin by Spofford *et al.* (15), with modifications. Briefly, L929 cells were seeded at a density of 3×10^4 cells per well in 96-well plastic tissue culture plates in RPMI 1640 medium containing 3% FBS and mitomycin C (1 μg/ml). After 18–24 hr of incubation at 37°C in a humidified CO_2 incubator, 2-fold serial dilutions of the test samples were prepared in separate 96-well plates. Mitomycin C-containing medium from the L929 cultures was discarded and replaced with the dilutions of the test samples. After incubation for 18–24 hr at 40°C, the plates were examined microscopically for TNF-induced cytotoxicity. The highest dilution producing 50% cell lysis

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Abbreviations: TNF, tumor necrosis factor; ^{125}I-TNF, ^{125}I-labeled TNF; IFN, interferon.

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was taken as the endpoint of TNF activity. TNF activity is expressed in units/ml, based on a laboratory standard as described (16).

Radioiodination of TNF. Radioiodination was performed by the method of Bolton and Hunter (17, 18). Briefly, highly purified TNF was dialyzed against 1000 volumes of 100 mM sodium phosphate buffer (pH 8.0) and the retentate was concentrated 10-fold by Aquacide II (Calbiochem), to a final protein concentration of 6.7 mg/ml. Concentrated TNF (50 μl) was added to 1 mCi (37 MBq) of ^{125}I-labeled Bolton–Hunter reagent (monoiodinated; New England Nuclear) at 4°C. After 18 hr, glycine was added to a final concentration of 0.1 M to terminate the reaction. The iodinated sample was applied to a column of Biogel P-6DG (1 × 10 cm), equilibrated and developed at 25°C with 0.1 M Tris·HCl (pH 8.0) containing 0.25% (wt/vol) gelatin. Void-volume fractions were pooled and analyzed for TNF activity. Recovery of biological activity after iodination was between 80 and 100%. Analysis of radioiodinated TNF by $NaDodSO_4$/PAGE and autoradiography showed one major band at $M_r \approx 17{,}000$ (Fig. 1). This is in good agreement with the size predicted from the TNF cDNA sequence (9–11). On longer exposure of the gel, minor components could be detected with molecular weights of approximately 35,000 and 50,000, corresponding to the dimer and trimer, respectively (not shown). The specific activity of ^{125}I-labeled TNF (^{125}I-TNF) was determined to be 0.58 μCi/μg of protein.

Binding Assays. Binding assays were performed in confluent cultures in 60-mm plastic plates containing approximately 3×10^6 (L929) or 2×10^6 (FS-4) cells. After the removal of growth medium, cells were incubated with various concentrations of ^{125}I-TNF in 5 ml of RPMI 1640 medium containing 10% FBS. (This medium was chosen for the binding studies because it produced the least nonspecific binding.) When required, various concentrations of unlabeled TNF were added simultaneously with ^{125}I-TNF. After the appropriate incubation period at either 4° or 37°C, cells were washed four times with ice-cold RPMI 1640 medium containing 10% FBS and then solubilized in 0.1% $NaDodSO_4$ and radioactivity was determined in a gamma counter. Specific binding is defined as the difference between total binding and nonspecific binding in the presence of a 100-fold excess of unlabeled TNF. The data from saturation-binding experiments were entered in the Equilibrium Binding Data Analysis program on an IBM PC computer (19).

Estimation of Internalization and Degradation of Cell-Bound TNF. Ligand internalization and degradation were estimated by a procedure similar to that described by Costlow and Hample (20). Cultures of L929 and FS-4 cells were exposed to ^{125}I-TNF at 4°C for 2 hr. The cells were washed four times with ice-cold RPMI 1640 medium containing 10% FBS and then shifted to 37°C by adding prewarmed RPMI 1640 medium containing 10% FBS (2 ml) and further incubated at 37°C. At the times indicated, culture fluids were harvested, and the cells then were washed once with ice-cold phosphate-buffered saline (PBS) and incubated for 5 min at 4°C with 2 ml of 0.05 M glycine·HCl buffer (pH 3.0) containing 0.15 M NaCl. After removal of the glycine buffer, cells were washed twice with RPMI 1640 medium containing 10% FBS and solubilized in 0.1% $NaDodSO_4$. ^{125}I radioactivity found in the glycine buffer represented surface-bound TNF, and that found in solubilized cells gave a measure of the internalized intracellular TNF. To quantitate degradation of internalized TNF by the cells, trichloroacetic acid was added, to a final concentration of 10%, to the culture media collected before determination of cell-bound TNF; soluble counts were determined after removal of the precipitate by centrifugation at 1500 × *g* for 20 min.

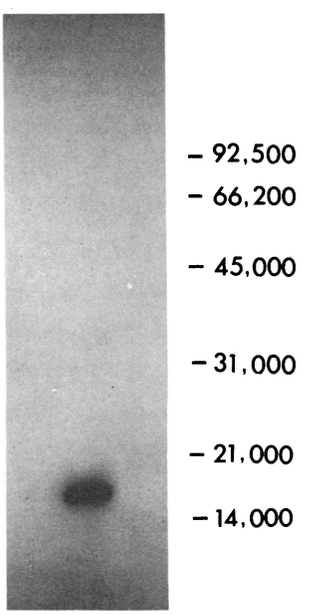

FIG. 1. Analysis of ^{125}I-TNF (1.5 ng, 35,000 cpm) by $NaDodSO_4$/10% PAGE and autoradiography. Positions of molecular weight markers are indicated at right.

RESULTS

TNF Action in L929 and FS-4 Cells. Murine L929 cells are highly susceptible to the cytotoxic action of TNF (1). TNF cytotoxicity is enhanced in L929 cells treated with mitomycin C (2) or in cells incubated at temperatures above 37°C (21). TNF titer in L929 cells treated with mitomycin C and incubated at 40°C was >1000 times higher than in cells not treated with mitomycin C and incubated at 37°C (Table 1). In contrast to the L929 cells, FS-4 cells showed no TNF-induced cytotoxicity even on prolonged incubation after exposure to mitomycin C and/or 40°C. The addition of human IFN-γ to FS-4 cells along with TNF also failed to induce signs of cytotoxicity. In many other cell lines, IFN-γ and TNF exert a synergistic cytotoxic action (ref. 22 and data not shown). Other experiments showed that TNF at concentrations up to 40,000 units/ml did not decrease the growth rate of FS-4 cells (data not shown). We conclude that FS-4 cells are completely refractory to the cytotoxic or cytostatic actions of human TNF.

Table 1. Comparison of TNF action in L929 and FS-4 cells

Group	Treatment*	Incubation temp., °C	TNF titer† × 10^{-3} L929	FS-4
1	None	37	32	<0.2
		40	6,400	<0.2
2	Mitomycin C	37	260	<0.2
		40	48,000	<0.2
3	IFN-γ	37	ND	<0.2
		40	ND	<0.2

ND, not done.

*L929 or FS-4 cells were seeded in 96-well microplates (30,000 cells per well) either in growth medium alone (groups 1 and 3) or in the presence of mitomycin C at 1 μg/ml (group 2). After 18 hr incubation at 37°C, the original medium was discarded and replaced with 2-fold dilutions of TNF. IFN-γ (100 units/ml) was added to cultures in group 3 at the same time as the TNF dilutions.

†Reciprocal of the highest dilution of TNF preparation causing lysis of 50% of the cell monolayer. TNF titer is based on readings taken either 24 or 48 hr after TNF addition in L929 cells incubated at 40°C or 37°C, respectively, and 96 hr after TNF addition in all FS-4 cultures.

Binding of TNF to L929 and FS-4 Cells. An analysis of the kinetics of TNF binding in preliminary experiments indicated that maximal specific binding at 4°C was reached between 1.5 and 2.5 hr after exposure of cells to ^{125}I-TNF. In contrast to 4°C, at 37°C the amount of total cell-bound radioactivity continued to increase for 3–4 hr and the total amount of cell-associated TNF reached higher levels (data not shown). The difference between the results at 4° and 37°C is likely to be due to the fact that although binding to receptors on the cell surface occurs at both 4° and 37°C, internalization can only take place at 37°C (see below).

TNF binding to L929 and FS-4 cells as a function of increasing concentrations of ^{125}I-TNF was analyzed at 4°C and 37°C 2 hr after the addition of TNF (Fig. 2). For both cell lines, specific binding approached saturation at TNF concentrations of 3–5 nM. Nonspecific binding at the highest TNF concentrations employed in this experiment represented 18% of total binding on L929 cells, and 7% on FS-4 cells. The Scatchard plot for the binding data at 4°C shows that the apparent dissociation constant in L929 cells is 6.1×10^{-10} M; and in FS-4 cells, 3.2×10^{-10} M. The average number of specific binding sites per cell, calculated from the Scatchard plot, is 2200 for L929 and 7500 for FS-4 cells.

Internalization and Degradation of Receptor-Bound ^{125}I-TNF. As shown with other protein ligands (23), elution of cell surface-bound TNF was found to be markedly pH dependent. While <5% of TNF bound to L929 cells at 4°C was eluted during a subsequent 5-min incubation at neutral pH, a 5 min treatment with pH 3 buffer (0.05 M glycine·HCl/0.15 M NaCl) released up to 90% of surface-bound TNF (data not shown). This technique, coupled with an analysis of the accumulation

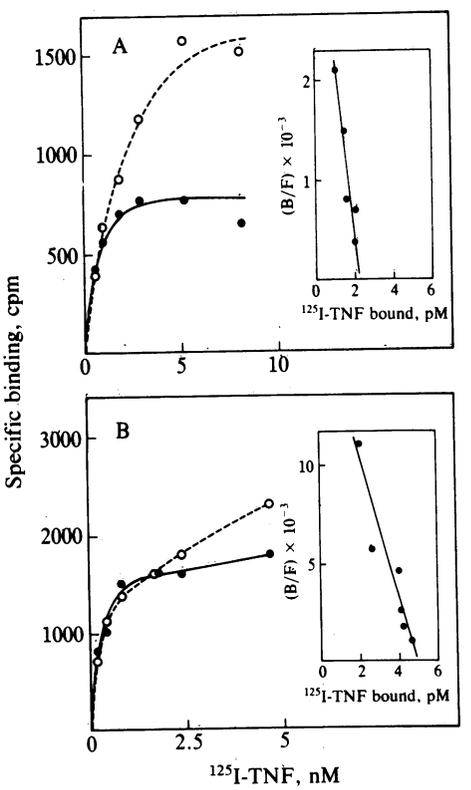

FIG. 2. Binding of ^{125}I-TNF to L929 (*A*) and FS-4 (*B*) cells. Cultures (3×10^6 L929 and 2×10^6 FS-4 cells per 60-mm plate) were incubated with increasing concentrations of ^{125}I-TNF in the presence or absence of a 100-fold excess of unlabeled TNF for 2 hr. Specific binding at 37°C (○---○) and at 4°C (●—●) was calculated by subtraction of nonspecific binding (in the presence of unlabeled TNF) from total binding. (*Insets*) Scatchard analyses of the binding data at 4°C. B/F, bound/free.

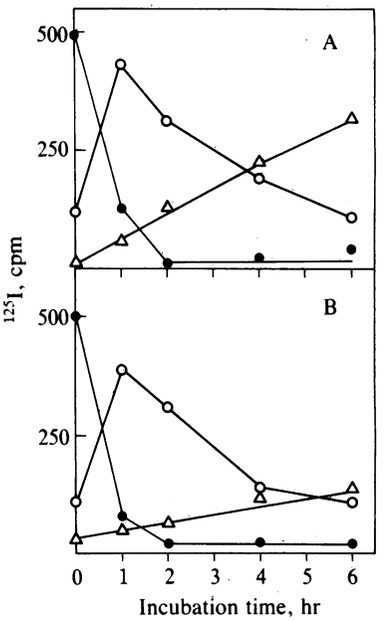

FIG. 3. Internalization and degradation of ^{125}I-TNF bound to L929 cells in the absence (*A*) or presence (*B*) of chloroquine. L929 cells were preincubated for 60 min at 37°C without or with chloroquine (0.1 mM). ^{125}I-TNF (15 pmol) was allowed to bind to L929 cells for 2 hr at 4°C. Thereafter cells were washed free of TNF and shifted to 37°C (at 0 hr). Treatment with chloroquine was continued throughout the duration of the experiment. Surface-bound (●), internalized (○), and degraded (△) ^{125}I-TNF were determined as described in *Materials and Methods*.

of Cl_3CCOOH-soluble ^{125}I radioactivity in the medium, allowed us to compare internalization and degradation of TNF in the sensitive and resistant cells.

In both L929 and FS-4 cells, cell-bound ^{125}I-TNF became resistant to release by pH 3.0 buffer after 1 or 2 hr at 37°C, which indicated that receptor-bound TNF had been internalized by the cell (Figs. 3*A* and 4*A*). The early rise of intracellular radioactivity (i.e., radioactivity resistant to acid

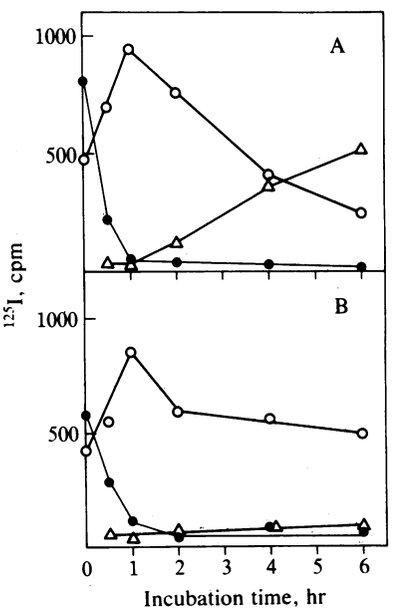

FIG. 4. Internalization and degradation of ^{125}I-TNF bound to FS-4 cells in the absence (*A*) or presence (*B*) of chloroquine. FS-4 cells were preincubated for 60 min at 37°C with or without chloroquine (0.2 mM). Other procedures and symbols are as in Fig. 3.

elution) was followed by a gradual decline. Cl_3CCOOH-soluble ^{125}I radioactivity was first detected in the medium after 1 or 2 hr and continued to increase until 6 hr. These results suggest that internalization and subsequent degradation of TNF occurred after binding to specific receptors at 37°C in both the TNF-sensitive and the TNF-insensitive cell line. No marked difference was seen in the kinetics of these events in the two cell lines, except that in FS-4 cells release of cell-bound TNF by pH 3.0 buffer at 0 time (i.e., immediately after 2 hr with ^{125}I-TNF at 4°C) was less efficient than in L929 cells. This difference might be related to the higher affinity of human TNF binding in FS-4 cells.

The effect of the lysosomotropic agent, chloroquine, on the binding, internalization, and degradation of TNF was also examined (Figs. 3*B* and 4*B*). Chloroquine had no effect on the internalization of ^{125}I-TNF in L929 cells and caused only a slight suppression of internalization in FS-4 cells at the later time points. In both cell lines, chloroquine caused a marked reduction in the appearance of Cl_3CCOOH-soluble ^{125}I in the medium. Monensin (0.02 mM) caused a similar inhibition of TNF degradation in L929 cells (data not shown).

DISCUSSION

Although the existence of TNF was reported 10 years ago (1), progress in the study of its mechanism of action was hampered by the lack of pure material. Recently, human TNF, obtained from the promyelocytic leukemia cell line HL-60, was purified to homogeneity (8). Amino acid sequence analysis of TNF revealed homology with another cytotoxic protein, termed lymphotoxin, purified from the lymphoblastoid cell line RPMI-1788 (24). Lymphotoxin was also described many years ago (25, 26) and, like TNF, it was known to be selectively cytotoxic for tumor cells. However, the relationship of these two proteins was not appreciated until their purification and, most recently, cloning and sequencing of the cDNAs for TNF (9–11) and lymphotoxin (27).

Availability of highly purified human TNF produced in *E. coli* by recombinant DNA technology has made it possible to study the initial stages of TNF action by methods used for many other classes of biologically active proteins. Polypeptide hormones, growth factors, and IFNs bind with a high affinity to specific surface receptors on target cells, and this binding is generally followed by receptor-mediated endocytosis (13, 18, 20, 23, 28). In the present study, we have shown that TNF action involves a similar pathway.

Saturation of receptors in L929 cells occurred at a TNF concentration of ≈100 ng/ml (4800 units/ml). An analysis of the data shown in Fig. 2 suggests that at a concentration of 1 unit/ml (which, by definition, is the TNF concentration required for 50% cell lysis), <1% of the available receptors are occupied; this would correspond to a mean occupancy of less than 20 receptors per cell in the equilibrium state at 4°C. Our results suggest the existence of a single class of TNF receptors. In view of the substantial structural homology between TNF and lymphotoxin and the similarities in their biological activities (9, 27), a common receptor may exist for these two cytotoxins.

Although our results demonstrate that receptor-mediated endocytosis and intracellular degradation occur after TNF binding to cell surface receptors, it is not clear whether these steps are required for biological activity of TNF. The continuous delivery of TNF, degradation products of TNF, and/or its receptors to intracellular targets might be responsible for the biological activity. In an earlier study, chloroquine was shown to reduce cytotoxicity of TNF preparations (5), but whether this reduction was related to chloroquine's action on the intracellular degradation of TNF remains to be determined. Alternatively, receptor occupancy might trigger a short-lived surface signal which could be repeatedly generated as internalized receptors are stripped of bound TNF and recycled back to the cell surface. In this case, internalization of TNF would not be a prerequisite for the biological response but would serve as a mechanism to increase the availability of cell surface receptors on prolonged exposure to TNF.

That the TNF-resistant FS-4 diploid cell line bound, internalized, and degraded TNF in a manner similar to that observed for the L929 line clearly shows that, although these events may be necessary for TNF action, they are not sufficient to result in cytotoxic or cytostatic activity. A comparison of five human tumor cell lines showed that their differences in sensitivity to the cytotoxic action of TNF also could not be explained by differences in TNF receptor numbers or their binding affinities (unpublished data). These data suggest that inhibition of tumor cell growth is not the only activity associated with TNF. Indeed, Beutler *et al.* (29) showed that TNF may be identical with a lipoprotein lipase inhibitor termed cachectin. In addition, recent data indicate that TNF can produce a marked stimulation of FS-4 cell growth (unpublished data).

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