Induction of tumor necrosis factor expression and resistance in a human breast tumor cell line

(cytokine/cytotoxicity/autocrine mechanisms)

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ABSTRACT Tumor necrosis factor (TNF) is a polypeptide cytokine that is cytotoxic to some but not all tumor cells. The basis for resistance to the cytotoxic effects of this agent remains unclear. We have studied the development of TNF resistance in human ZR-75-1 breast carcinoma cells. ZR-75-1 cells have undetectable levels of TNF RNA and protein. However, TNF transcripts are transiently induced in these cells by exposure to recombinant human TNF. This induction of TNF RNA is associated with production of TNF-like protein in cell lysates and culture supernatants. Stable resistance to TNF-induced cytotoxicity develops when ZR-75-1 cells are exposed to increased concentrations of TNF. The TNF-resistant cell, designated ZR-75-1R, continuously express TNF transcripts and a TNF-like protein. Furthermore, ZR-75-1R cell supernatants contain cytotoxic activity that is abrogated by polyclonal antibody against TNF. The ZR-75-1R cells also possess TNF receptors that are occupied or down-regulated by the TNF-like protein. These findings thus suggest that (i) TNF induces TNF transcripts and production of a TNF-like protein in ZR-75-1 cells and (ii) resistance to TNF-induced cytotoxicity is associated with stable TNF expression.

Tumor necrosis factor (TNF) is a polypeptide cytokine that exerts a wide variety of biological effects (1). TNF was originally identified by its ability to cause hemorrhagic necrosis in subcutaneous murine tumors (2). Subsequent studies showed that TNF is cytotoxic to certain murine and human tumors, both in vitro and in vivo (1–3).

The cytotoxic effects of TNF appear to be cell cycle-dependent. TNF causes accumulation of cells in G1 and G2 phases and cytolytic in late stages of mitosis (4). This cytolytic effect is enhanced by inhibitors of RNA and protein synthesis (5, 6). Tumor cells sensitive and resistant to TNF-induced cytotoxicity have similar numbers of cell surface receptors. Furthermore, both sensitive and resistant cells internalize and degrade TNF after receptor binding (7). Thus, cell surface receptors appear to be necessary but not sufficient for TNF cytotoxicity (8–10). The basis for the sensitivity or resistance of transformed cells to TNF remains unknown.

Exposure of TNF-sensitive L-929 mouse fibroblasts to TNF results in the development of stable TNF resistance (11). Paradoxically, this TNF-resistant subline produces a TNF-like protein that is cytotoxic to the parent L-929 cell line. We recently demonstrated that certain human epithelial tumor cell lines inherently resistant to TNF also produce both TNF mRNA and protein (unpublished work). These findings suggested that production of TNF may be associated with TNF resistance. The present studies investigated this relationship in human breast carcinoma cells sensitive to the cytotoxic effects of TNF in vitro. The results show that TNF treatment induces both TNF expression and resistance to TNF cytotoxicity.

METHODS

Cell Culture. The ZR-75-1 human breast carcinoma cells were obtained from the American Type Culture Collection and grown in RPMI 1640 medium with 10% heat-inactivated fetal bovine serum, 100 units of penicillin per ml, 100 μg of streptomycin per ml, 0.3 mg of L-glutamine per ml, 0.02 mM Hepes, and 1 μg of insulin per ml. The ZR-75-1 cells were sequentially exposed to human recombinant TNF (PAC-4D provided by Asahi Chemical Industry America at 1, 10, and 100 units/ml) (13). Sensitivity to TNF was assayed in a 96-well culture plate. Ten thousand ZR-75-1 cells were plated into each well and incubated for 5–7 days with increasing concentrations of TNF. The cells were fixed with 2.5% glutaraldehyde, washed, and stained with 0.05% methylene blue for 5 min. The plates were then washed and the dye was released with 0.33 M HCl. The absorbance of each well at 600 nm was determined on an automated plate reader (Bio-Rad). TNF cytotoxicity was determined as described (11).

RNA Blot Hybridization Analysis. Total cellular RNA was collected from the ZR-75-1 cells by the guanidinium isothiocyanate/cesium chloride method (14). The purified RNA (20 μg) was analyzed by electrophoresis in 1% agarose/6% formaldehyde gels followed by blot transfer to nitrocellulose paper. The human TNF cDNA (15) was nick-translated to a specific activity of ~5 × 106 cpm/μg. Hybridization conditions were as described (16).

Metabolic Labeling of TNF. ZR-75-1 cells were incubated overnight in leukemia-free RPMI 1640 medium with [4,5-3H]leucine (16 μCi/ml of medium; 140.2 Ci/mmol, New England Nuclear; 1 Ci = 37 GBq). The cells were lysed in 1% Triton X-100/0.1% NaDodSO4/0.15 M NaCl/50 mM Tris-HCl, pH 7.4/2 mM phenylmethylsulfonyl fluoride/1 mM EDTA. After centrifugation at 15,000 rpm (16,000 × g) for 5 min at 4°C, the lysate was incubated for 90 min at 4°C with 50 μl of a 50% (wt/vol) suspension of rabbit polyclonal anti-TNF antibody attached to Sepharose beads. Tissue culture supernatant was similarly incubated with the antibody-bead conjugate. The antibody-Sepharose beads were removed by centrifugation and washed five times with lysis buffer and then twice with phosphate-buffered saline (Dulbecco A, KC Biological, Lenoxa, KS). The immunocentrates were then analyzed by electrophoresis in NaDodSO4/7.5% polyacrylamide slab gels and autoradiography.

Measurement of TNF Levels. TNF levels were determined by analysis in a previously described (12) enzyme-linked immunosorbent assay (ELISA) that utilizes two monoclonal antibodies reactive with distinct epitopes on the TNF mole-

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Abbreviations: TNF, tumor necrosis factor; 125I-TNF, 125I-labeled TNF.
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Biologic activity was determined in an L-cell cytotoxicity assay (11) standardized with recombinant TNF. One unit in the ELISA is defined as the amount required for 50% cytotoxicity of the L-929 cells without actinomycin D.

**Cellular Binding of [125I]-Labeled TNF ([125I]-TNF).** TNF was labeled with [125I] by the Iodogen method (17) and purified by gel filtration. The labeled protein consisted of a single band at 17 kDa after NaDodSO4/polyacrylamide gel electrophoresis and autoradiography. Specific activity of the [125I]-TNF was 3 × 10^5 cpn/pmol. Binding assays were performed in triplicate on monolayers of ZR-75-1 cells (5 × 10^5 per well) in 24-well cluster plates. The cells were incubated with 100 pM [125I]-TNF for 30 min at 37°C. Similar studies were performed in the presence of either rabbit polyclonal anti-TNF IgG or normal rabbit IgG. The cells were then washed three times with ice-cold phosphate-buffered saline, lysed with 2% NaDodSO4, and monitored for radioactivity. Specific binding was determined by the difference between total binding and “nonspecific binding” in the presence of a 100-fold excess of unlabeled TNF.

**RESULTS**

We previously studied 14 human epithelial tumor cell lines for sensitivity to TNF-induced cytotoxicity (unpublished work). Eleven lines were resistant to TNF as defined by an IC50 of >1000 units/ml. In contrast, three sensitive human breast tumor lines—BT-20, MCF-7, and ZR-75-1—had IC50 values <10 units/ml. The ZR-75-1 cell line was particularly sensitive to TNF cytolysis, with an IC50 <1.0 unit/ml. These cells were used in the present studies.

Since both inherent and acquired resistance to TNF-induced cytotoxicity have been associated with TNF production, we examined the effect of TNF exposure on expression of TNF at the RNA and protein levels. TNF transcripts were undetectable in total RNA from untreated ZR-75-1 cells (Fig. 1A). In contrast, the TNF cDNA probe hybridized to a 1.6-kilobase transcript that was detectable in cells treated with TNF for 1 hr. The level of TNF RNA reached a maximum at 3 hr and then declined to undetectable levels by 12 hr (Fig. 1A). These transcripts were undetectable following similar exposures to TNF but in the presence of a neutralizing monoclonal antibody (data not shown).

In order to determine whether these TNF transcripts were associated with production of TNF protein, control and TNF-treated ZR-75-1 cells were incubated with [3H]leucine. Cell lysates and culture media were then assayed by immunoprecipitation with rabbit polyclonal antibodies reactive with human TNF. The lysate of TNF-treated cells contained a 17-kDa and a 45- to 50-kDa immunoreactive protein (Fig. 1B). Furthermore, a labeled 17-kDa TNF-like protein was detected in the culture supernatants of the TNF-treated ZR-75-1 cells but not of the control cells (Fig. 1C). Thus, treatment of ZR-75-1 cells with TNF appeared to induce production of both TNF RNA and TNF protein.

The ZR-75-1 cells were next exposed to increasing concentrations of TNF. After four passages, a subline was selected that grew in the presence of 10 units of TNF per ml. However, these cells reverted to the TNF-sensitive phenotype after removal of TNF selection. Additional stepwise increases to a TNF concentration of 100 units/ml resulted in the selection of stable TNF-resistant ZR-75-1 cells. This subline, designated ZR-75-1R, was resistant to TNF concentrations of 10^6 units/ml and has maintained this phenotype for more than 6 months in the absence of TNF.

The ZR-75-1R cells were studied by blot hybridization analysis for the presence of TNF transcripts. The TNF cDNA probe hybridized with both a 1.6-kilobase transcript and a larger RNA species not detected in the TNF-treated ZR-75-1 cells (Fig. 2A). Moreover, exposure of the ZR-75-1R cells to TNF at 100 units/ml for 3 hr had no detectable effect on either of these transcripts. The expression of TNF at the RNA level was also associated with production of a TNF-like protein. A 17-kDa protein was immunoprecipitated from ZR-75-1R control supernatant (Fig. 2B). Moreover, ZR-75-1R supernatant had detectable TNF-like reactivity in the ELISA and contained activity that was cytotoxic to mouse L-929 cells (data not shown). This cytotoxicity was abrogated by treatment with neutralizing antibody against TNF.

The development of ZR-75-1 cell resistance to the cytoxic effects of TNF may have been related to the loss of cell membrane TNF receptors. We therefore studied the interaction of [125I]-TNF with the surface of ZR-75-1 and ZR-75-1R cells. Analysis of the radioiodinated TNF by NaDodSO4/polyacrylamide gel electrophoresis and autoradiography revealed one band at 17 kDa. Binding of the [125I]-TNF to 10^6 cells was assayed for 1 hr at 37°C. Specific binding was detectable on the sensitive ZR-75-1 cells but not on the resistant ZR-75-1R cells (Fig. 3). The failure to detect binding of [125I]-TNF to the resistant cells may have been attributable to receptors already occupied or internalized with TNF. Therefore, ZR-75-1R cells were treated with polyclonal anti-RNA antibody for 15 hr and then assayed for [125I]-TNF binding. Under these experimental conditions, binding of [125I]-TNF was detectable on the ZR-75-1R cells. Similar
results were obtained after a 1-hr treatment with the polyclonal anti-TNF antibody. In contrast, treatment of these cells with an equivalent amount of nonspecific IgG had no effect on TNF binding (Fig. 3). These results suggest that the ZR-75-1R cells retained detectable TNF receptors.

**DISCUSSION**

The results show that treatment of ZR-75-1 human breast carcinoma cells with recombinant human TNF induces expression of the TNF gene at both the RNA and protein levels. This induction of TNF expression by TNF was abrogated by neutralizing antibody against this cytokine, thus adding support to the specificity of this effect. The TNF transcripts were induced by 1–3 hr of TNF exposure and then subsequently declined to undetectable levels. Identification of the mechanisms responsible for this regulation of TNF expression will require further study. However, the induction of TNF expression by TNF does not appear to be unique to ZR-75-1 cells. Indeed, we have found that treatment of human peripheral blood monocytes with TNF also induces TNF transcripts (unpublished data). Moreover, TNF apparently acts as both an immunomodulator and a mediator of monocyte tumoricidal activity (18).

TNF is cytotoxic or cytostatic to some but not all tumor cell lines (2, 3). The basis for resistance to the cytotoxic effects of this agent remains unclear. Previous studies have demonstrated that cytotoxins, like TNF, induce certain changes in cells that result in resistance to associated cytotoxic effects (19). This resistance is blocked by inhibition of protein synthesis (19). Similar studies have demonstrated that inhibitors of RNA synthesis enhance the cytotoxic effects of TNF (5), thus suggesting that induction of specific genes at the RNA and protein levels may be involved in the resistance mechanism. Although TNF has been shown to affect gene expression in a variety of different cells (20–25), the induction of TNF expression by TNF may in some way be associated with the development of resistance to the cytolytic effects of this agent.

Mouse L-929 cells selected for resistance to TNF produce a cytotoxic factor with biochemical and immunologic characteristics of TNF (10). The identification of TNF in these cells of fibroblastic origin suggested that nonhematopoietic cells were also capable of expressing this gene. These results further suggested that TNF expression is associated with resistance to TNF-induced cytotoxicity. Studies in our laboratory have demonstrated that TNF is expressed at the RNA and protein levels in certain human epithelial tumor cell lines (unpublished work), thus confirming that nonhematopoietic cells have the capacity for TNF expression. Indeed, TNF transcripts were detected in each of eight human epithelial tumor lines examined after treatment with cycloheximide, indicating that TNF RNA levels are negatively regulated in these epithelial cells by a labile protein. Epithelial tumor cells constitutively producing TNF were resistant to the cytotoxic effects of this agent, but not all resistant lines had detectable TNF RNA in the absence of cycloheximide. These findings prompted further studies on the development of stable TNF resistance in the sensitive ZR-75-1 line.

Resistance to the cytotoxic effects of TNF can be induced in the ZR-75-1 cells by exposure to increasing TNF concentrations. This TNF-resistant phenotype was initially unstable in the absence of TNF. However, further treatment with TNF resulted in a subline (ZR-75-1R) that has remained stably resistant to concentrations of TNF 100-fold higher than that used for induction of resistance. The ZR-75-1R cells express detectable levels of the 1.6-kilobase TNF transcript. A larger transcript that hybridized to the TNF cDNA probe was also detected in the ZR-75-1R cells but not in the TNF-treated ZR-75-1 cells. This transcript may represent a precursor of the mature 1.6-kilobase TNF RNA. The expression of TNF RNA in the ZR-75-1R cells was not associated with detectable amplification of the TNF gene (data not shown), suggesting that the level of RNA is regulated by an increase in transcriptional rate or by stabilization of the transcript.

The ZR-75-1R cells were shown to produce a 17-kDa TNF-like protein, which was detectable in cell culture supernatants. Moreover, the ZR-75-1R culture supernatants contained activity that was detectable in the TNF ELISA and was cytotoxic to mouse L-929 cells. This cytotoxic activity
was abrogated by neutralizing antibody against TNF, thus adding further evidence that the ZR-75-1R cells produce a TNF-like protein. The induction of TNF expression and TNF resistance are therefore also associated in the ZR-75-1R cells. However, the present findings do not demonstrate a direct relationship between these two parameters. Finally, TNF receptors are detectable on ZR-75-1R cells, although these binding sites are occupied or internalized in the absence of the polyclonal antibody against TNF. Thus, TNF-resistant cells may have low or undetectable levels of 125I-TNF ligand binding due to production of TNF-like proteins.

TNF is currently under clinical trial as an antitumor agent. Although these phase I trials are too early to determine efficacy, previous studies suggest that certain human epithelial tumor cells are inherently resistant to the cytotoxic effects of this agent (ref. 3 and unpublished work). Furthermore, certain human epithelial tumors may be capable of constitutively producing a TNF-like protein that, considering the relationship between TNF and cachectin, may contribute to the cachexia of malignancy (26). Finally, the present results suggest that treatment of human epithelial tumors with TNF may ultimately lead to the development of TNF resistance and the production of a TNF-like protein. Although the available experimental evidence is derived from in vitro studies, the effective clinical use of TNF may require further insights regarding this development of TNF resistance and approaches to reverse the TNF-resistant phenotype.

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