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Endogenous Tumor Necrosis Factor Exerts Its Protective Function Intracellularly against the Cytotoxicity of Exogenous Tumor Necrosis Factor

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ABSTRACT

Based on the findings that expression of endogenous tumor necrosis factor (enTNF), which is not present in TNF-susceptible cells, was generally observed in TNF-resistant cells and that TNF gene transfection gives rise to TNF resistance, the assumption was made that enTNF may be a protective protein against the cytotoxicity of exogenous TNF. However, it remains unknown whether the protection by enTNF is exerted in an intracellular or extracellular (autocrine) manner. We therefore transfected a nonsecretory human TNF gene (pTNF Δ pro) into highly TNF-sensitive mouse tumorigenic fibroblasts (L-M cells) and investigated their TNF susceptibility. The transfectants expressed enTNF which was not secreted into the medium and acquired an appreciable degree of resistance to exogenous TNF. A significant increase in the manganous superoxide dismutase level was also noted in the transfectants. These findings suggest that enTNF exerts its protective function intracellularly by inducing manganous superoxide dismutase production.

INTRODUCTION

TNF[2] is known to possess potent antitumor activity against tumor cells both *in vivo* (1–4) and *in vitro* (5–7). This antitumor activity is exerted through mechanisms involving receptor-mediated signal transduction (8–13). Some cell lines, however, are not sensitive or are minimally sensitive to TNF even though they have appreciable numbers of receptors on their surface (7). Furthermore, a TNF-resistant subline can be established by repeated long-term culture of sensitive cells in the presence of TNF (9). These results suggest that the cytotoxic effect of TNF is affected by both intrinsic and acquired resistance, although the mechanisms by which cells become resistant to TNF are still surrounded by considerable controversy.

Because the cells which produce and secrete cytocidal cytokines do not injure themselves (14), it may be reasonable to assume that those endogenous cytokines themselves play a role in the acquisition of self-protection (resistance). We previously found that TNF-sensitive cells do not express enTNF at either the transcriptional or the translational level, while enTNF expression is induced in TNF-sensitive cells by pretreatment with TNF, and that enTNF is expressed constitutively in TNF-resistant tumor cells (14). Furthermore, we have shown that enTNF is one of the proteins which protects against the cytotoxicity of exogenous TNF, by introducing resistance into TNF-sensitive cells with the transfection of a TNF expression vector or an antisense TNF mRNA expression vector (15). However, it is still unknown whether enTNF exerts its protective function in a intracellular or extracellular (autocrine) manner.

In the present study, we transfected a nonsecretory human TNF gene into L-M cells (highly TNF-sensitive mouse tumorigenic fibroblasts; American Type Culture Collection CCL 1, 2) and investigated whether or not its product acts as a protective factor against TNF cytotoxicity.

MATERIALS AND METHODS

TNF and Its Antibody. rhTNF, produced in *Escherichia coli* and purified, and rabbit anti-rhTNF polyclonal antibody, which neutralized the rhTNF activity, were generously provided by Asahi Chemical Industry Co., Ltd. (Tokyo, Japan). The TNF had a specific activity of 2.3×10^6 units/mg protein and, as the trimer, a molecular mass of 51 kilodaltons (16).

Nonsecretory-type Human TNF Expression Vector. pcDV-TNF (15), pSV2neo (17), and pHNtac4 (16) were donated by courtesy of Asahi Chemical Industry Co., Ltd. The nonsecretory human TNF gene (pTNF Δ pro) was constructed by the trimolecular ligation reaction of the *Pst*I-*Bam*HI fragment containing a SV40 early promoter, the *Pst*I-*Hind*III fragment containing a SV40 polyadenylation site and pBR322 replication origins of pcDV-TNF, and the 0.6-kilobase *Xho*II-*Hind*III fragment encoding the mature human TNF of pHNtac4.

E. coli HB101 was transformed by this ligation mixture. The structure of pTNF Δ pro was verified by restriction enzyme digestion of DNA isolated from transformants. The structures of pTNF Δ pro and pcDV-TNF are shown in Fig. 1.

Cell Culture and Transfection. Cotransfection into L-M cells of pTNF Δ pro and pSV2neo was performed by the lipofection method as described earlier (15). Briefly, 5×10^5 cells plated in a 35-mm dish were washed twice with 3 ml of Opti-MEM I reduced serum medium (Gibco, Grand Island, NY) and immersed in 3 ml of Opti-MEM I reduced serum medium. The cells were cultured for 24 h at 37°C after the addition of 100 μl of plasmid-lipofectin reagent (BRL, Gaithersburg, MD) mixture containing 20 μg of pTNF Δ pro and 1.5 μg of pSV2neo, and then 3 ml of Eagle's MEM supplemented with 20% fetal bovine serum were added. After 48 h of cultivation in this medium, G418-resistant cells were selected by further cultivation in MEM medium supplemented with 10% fetal bovine serum and 300 μg/ml of G418 for 14 days.

***In Vitro* Assay for Cytotoxic Action.** Cells (1×10^4 cells/100 μl) were added to the wells of the 96-well microculture plate (Falcon) and incubated at 37°C for 4 h in 5% CO_2. Then, rhTNF (1 to 1×10^3 units/100 μl) was added and followed by incubation at 37°C for 48 h in 5% CO_2. Cytotoxic activity was then assayed by the dye uptake method as described earlier (8).

Determination of Intracellular MnSOD Activity. MnSOD activity was assayed by the nitroblue tetrazolium method as described by Oberley and Spitz (18). Protein concentrations were determined by the Bio-Rad (Richmond, CA) protein assay. MnSOD activity was expressed in units per mg protein.

Measurement of TNF Concentration in Conditioned Media. The amount of TNF in the conditioned media of cells transfected with pTNF Δ pro and pcDV-TNF, which is the secretory-type TNF expression vector reported previously (15), was determined by enzyme-linked immunosorbent assay (19) using a monoclonal antibody against rhTNF, with peroxidase serving as the enzyme.

Western Blotting. Cells were solubilized with 1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, and 20 mM Tris-HCl (pH 7.4) and centrifuged (10,000 × *g* for 10 min). Supernatants were then subjected

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[2] The abbreviations used are: TNF, tumor-necrosis factor; enTNF, endogenous TNF; rhTNF, recombinant human TNF; MnSOD, manganous superoxide dismutase; MEM, minimal essential medium; SV40, simian virus 40.

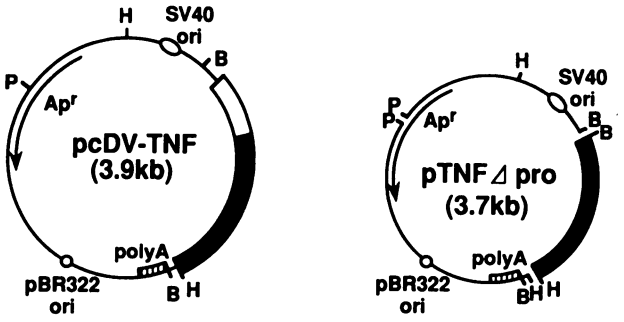

Fig. 1. TNF expression plasmid maps. The human TNF gene coding sequences are shown as a *thick line*. □, leader sequence; ■, mature human TNF sequence; *SV40 ori*, the region of SV40 origin containing the SV40 early promotor; *polyA*, SV40 polyadenylation site; *Ap^r*, ampicillin resistance gene; *B*, *Bam*HI; *H*, *Hin*dIII; *P*, *Pst*I; *kb*, kilobases.

to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (20). After the electrophoresis, fractionated protein was transferred to polyvinylidine difluoride membrane (Millipore) in 20% methanol, 48 mM Tris, 39 mM glycine, and 1.3 mM sodium dodecyl sulfate (pH 9.2) by using Trans-Blot SD Cell (Bio-Rad). Membranes were incubated for 3 h at room temperature with 3 μg/ml of ^{125}I-anti-rhTNF polyclonal antibody which was radiolabeled using the Enzymobead reagent (Bio-Rad). Membranes were then washed with phosphate-buffered saline (pH 7.4) 10 times and subsequently exposed to Kodak X-Omat film as above.

Binding Assay. Radioiodination of rhTNF was performed by the method of Bolton and Hunter (21) to obtain ^{125}I-labeled rhTNF with a specific activity of 6.9×10^5 cpm/μg. The binding assay was carried out in 12-well tissue-culture cluster plates (Costar) containing approximately 5×10^5 cells. After the plate had been washed three times with Eagle's MEM containing 0.1% bovine serum albumin, cells were incubated with various concentrations of ^{125}I-rhTNF and unlabeled rhTNF for 1 h at 4°C. Cells were washed again three times with Eagle's MEM containing 0.1% bovine serum albumin and were then solubilized in 0.5 M NaOH, and their radioactivity was determined with a gamma counter. The number of rhTNF receptors per cell and the dissociation constant (K_D) were determined by Scatchard plot analysis.

RESULTS

Induction of enTNF by Transfection of pTNF Δ pro to L-M Cells. L-M cells that are highly sensitive to TNF and express no constitutive enTNF were transfected with pTNF Δ pro (Fig. 1) by lipofection. The transfection frequency of L-M cells for pTNF Δ pro was approximately 10^{-4}. Twenty G418-resistant clones were obtained, and of these two clones, L-M (pTNF Δ pro) F10 and L-M (pTNF Δ pro) A3, were used in the subsequent experiments.

In order to examine the presence of enTNF, lysates of F10, A3, and L-M cells were analyzed by Western blotting. As shown in Fig. 2, protein reactive to anti-rhTNF antibody was clearly identified in both clones, while in L-M cells this protein was not detectable. No TNF activity as measured by enzyme-linked immunosorbent assay was detected in the conditioned media of either clone, while pcDV-TNF-transfected L-M cells did produce and secrete biological active TNF into the media (Fig. 3).

Induction of TNF Resistance by Transfection of pTNF Δ pro to L-M Cells. The susceptibilities of L-M and L-M (pTNF Δ pro) cells to exogenous TNF were assessed by exposing these cells to TNF ($0.1–10^3$ units/ml) for 48 h. As shown in Fig. 4, the 50% inhibition values of L-M (pTNF Δ pro) cell lines F10 and A3 increased 10.7- and 12.0-fold, respectively, as compared to those of control L-M cells.

Effects of Transfecting pTNF Δ pro to L-M Cells on Cell Growth. In order to examine the effect of transfection procedure on cell growth, growth curves of L-M and L-M (pTNF Δ pro) cells were compared *in vitro* (Fig. 5). L-M and L-M (pTNF Δ pro) cells both grew about 16-fold during culture for 84 h. No significant difference was observed in their growth curves (Fig. 5). Thus no apparent effect of transfection on cell growth was confirmed.

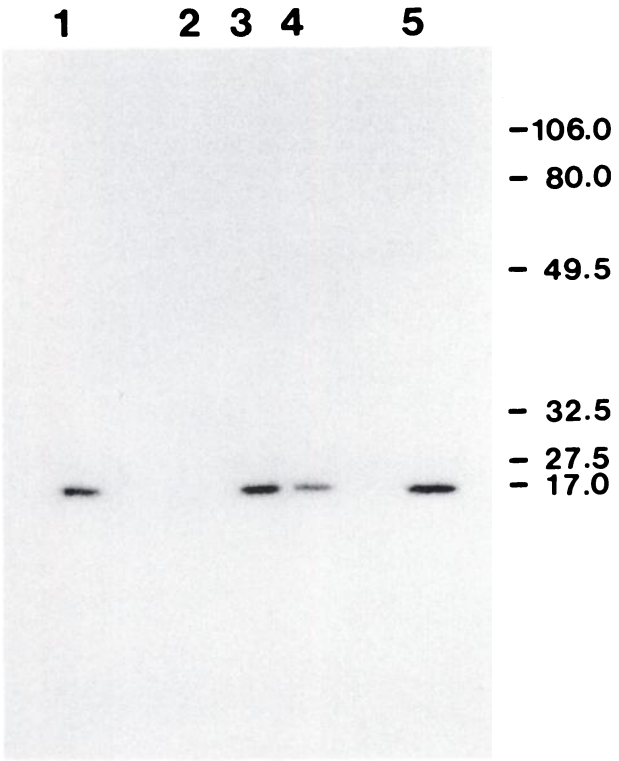

Fig. 2. Western blot analysis of enTNF in L-M cells and pTNF Δ pro-transfected clones. Cell extracts from each cell line were subjected to 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidine difluoride membranes. An immunoblot was developed using a ^{125}I-labeled anti-rhTNF polyclonal antibody (rabbit). The membrane was exposed to Kodak X-Omat film for 48 h at −70°C. *Right ordinate*, molecular weight in thousands. *Lane 1*, rhTNF; *Lane 2*, L-M; *Lane 3*, L-M (pTNF Δ pro) F10; *Lane 4*, L-M (pTNF Δ pro) A3; *Lane 5*, L-M (pcDV-TNF).

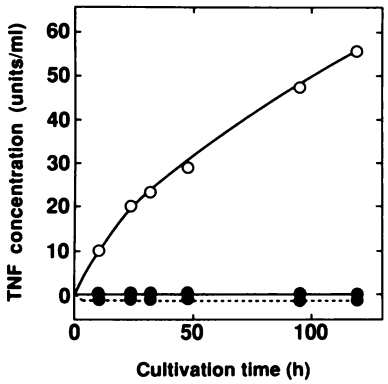

Fig. 3. Production of human TNF in pcDV-TNF- and pTNF Δ pro-transfected clones. Cells (1×10^6) were cultivated in 15 ml of Eagle's MEM containing 10% fetal bovine serum at 37°C. TNF concentrations in the conditioned medium at the indicated times were determined by enzyme-linked immunosorbent assay. Data shown were confirmed in three separate experiments. O—O, L-M (pcDV-TNF); ●—●, (pTNF Δ pro) F10; ●- -●, L-M (pTNF Δ pro) A3.

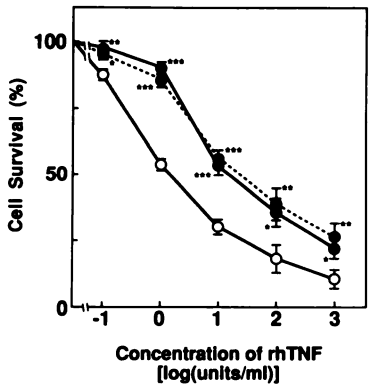

Fig. 4. Cytotoxic activity of rhTNF against pTNF Δ pro-transfected clones. Cells (1×10^5 cells/ml) were cultured with rhTNF ($0.1–10^3$ units/ml) for 48 h, and their susceptibility to the cytotoxic activity of TNF was determined by the dye uptake method. O—O, L-M cells; ●—●, L-M (pTNF Δ pro) F10; ●- - -●, L-M (pTNF Δ pro) A3. *Points*, mean of triplicate values; *bars*, SD. *, $P < 0.02$; **, $P < 0.01$; ***, $P < 0.001$ by Student's t test.

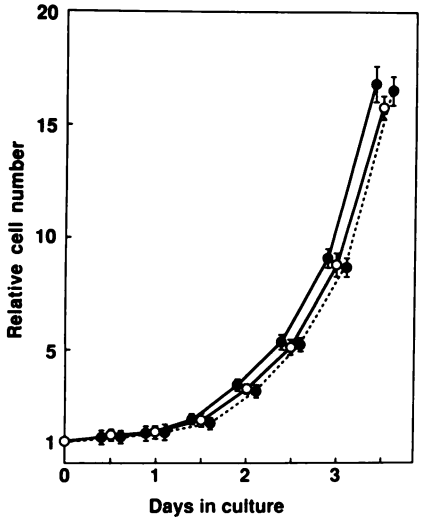

Fig. 5. L-M cell growth curve and pTNF Δ pro-transfected clones. Cells (5×10^3) were added to the wells of a 96-well microculture plate and incubated for 12, 24, 36, 48, 60, 72, or 84 h. The number of susceptible cells was then assessed by the dye uptake method. *Points*, determined from six separate wells; *bars*, SD. O—O, L-M cells; ●—●, L-M (pTNF Δ pro) F10; ●- - -●, L-M (pTNF Δ pro) A3.

Number of Cell Surface TNF Receptors on L-M and L-M (pTNF Δ pro) Cells. The number of TNF receptors on the L-M, L-M (pTNF Δ pro) F10, and A3 cells, as determined by Scatchard plot analysis, was, respectively, 1.31, 1.32, and 1.39 $\times 10^4$/cell (Table 1). The dissociation constants for the binding in those cells were almost the same, on the order of 10^{-10} M. Thus, the transfection of pTNF Δ pro appeared to have no significant effect on either the number of cell surface receptors or their binding affinity.

Changes in Intracellular MnSOD Levels with Transfection by pTNF Δ pro. MnSOD is the oxygen radical scavenger known to be a resistance factor against TNF cytotoxicity (22–24). Therefore, we investigated the changes of MnSOD activity in L-M cells and L-M (pTNF Δ pro) cells. As shown in Table 2, a 3.1- and 3.4-fold increase in MnSOD levels was noted in the transfectant as compared to control L-M cells.

DISCUSSION

We have already reported that the cells transfected with TNF expression vector acquire TNF resistance (15, 24) and that its protective function is exerted by inducing MnSOD production (24). These observations were further recently confirmed by Vanhaesebroeck *et al.* (25), who transfected several secretory types of TNF expression vectors encoding M_r 17,000 mature TNF, biologically inactive M_r 26,000 TNF proform, and the M_r 9,000 membrane-bound presequence part of M_r 26,000 proform to TNF-sensitive murine L929 cells and reported that biologically active TNF is essential for protection against the toxicity of exogenous TNF.

However, it is still unknown whether the protection by enTNF is exerted in an intracellular or extracellular (autocrine) manner. In the present study, therefore, we transfected the nonsecretory human TNF gene (pTNF Δ pro) to L-M cells and investigated their TNF susceptibility. The L-M (pTNF Δ pro) cells expressing enTNF which was not secreted into the medium acquired 10.7- to 12.0-fold resistance to exogenous TNF.

These findings suggest that enTNF exerts its protective function intracellularly. There are a number of possibilities concerning the mechanism whereby TNF resistance is produced by enTNF, including (*a*) enTNF acting to counteract cytocidal signal transduction by exogenous TNF; and (*b*) enTNF transmitting a signal intracellularly to induce the production of a defensive substance.

We have reported previously that oxygen radicals are at least partly involved in tumor cell killing by TNF (26, 27). MnSOD acts intracellularly as an oxygen radical scavenger, and its presence correlates positively with resistance to TNF (22–24). Furthermore, by increasing or decreasing MnSOD activity through gene transfection experiments, TNF cytotoxicity is, respectively, reduced or increased (22). On the other hand, since MnSOD is induced when cells are treated with exogenous TNF (23), it is still unknown whether enTNF can induce MnSOD production intracellularly.

In the present study, we measured a 3.1- and 3.4-fold increase in the MnSOD level in transfectants carrying the nonsecretory human TNF gene. These findings suggest that an increase in intracellular MnSOD induced by enTNF is one mechanism by which resistance to the cytocidal effect of exogenous TNF is conferred.

Table 1 *TNF receptors on the surface of L-M cells and pTNF Δ pro-transfected clones*

Strain	TNF receptor[a] No./cell ($\times 10^4$)	TNF receptor[a] K_D ($\times 10^{-10}$ M)
L-M	1.31	7.73
pTNF Δ pro F10	1.32	6.02
pTNF Δ pro A3	1.39	7.72

[a] The number of TNF receptors and affinity of TNF binding to its receptor (K_D) were determined as described in "Materials and Methods."

Table 2 *Induction of MnSOD in pTNF Δ pro-transfected clones*

The cells (2×10^6/ml), in 0.05 M potassium phosphate buffer (pH 7.8), were sonicated for 1 min. The resulting solution was centrifuged at 3000 rpm for 5 min to obtain the cell lysates. MnSOD activities and protein concentrations of the cell lysates were determined by the nitroblue tetrazolium method and the Bio-Rad protein assay, respectively.

Strain	MnSOD activity (units/mg protein)[a]
L-M	6.62 ± 0.94
pTNF Δ pro F10	22.19 ± 2.60[b]
pTNF Δ pro A3	20.40 ± 3.13[b]

[a] Mean ± SD of six separate experiments.
[b] Values significantly different ($P < 0.001$) from the corresponding value in L-M cells.

Browder *et al.* (28) reported that endogenously produced interleukin 3 interacts with its receptor and mediates signal transduction. This view is consistent with that of Keating and Williams (29), who reported that autocrine activation of platelet-derived growth factor receptors in v-*sis*-transformed cells occurs in intracellular compartments. Although our results demonstrate that no significant difference was observed in cell surface receptor number and binding strength between transfectants and L-M cells, there is still the possibility that enTNF interacts with an intracellular receptor (8), thereby counteracting signal transduction from outside the cell. In these instances, it is unclear why intracellular receptors for TNF mediate only the protection signal, although cell surface receptors transduce both signals for protection and killing.

It is also conceivable that enTNF is biologically active intracellularly even when not bound to any receptor.

The mechanism by which MnSOD production is induced by enTNF remains to be investigated further.

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