Induction of Synthesis of Tumor Necrosis Factor in Human and Murine Cell Lines by Exogenous Recombinant Human Tumor Necrosis Factor

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ABSTRACT

Treatment of sensitive human myosarcoma cells (KYM-S) with exogenous tumor necrosis factor (r-TNF) resulted in the production of TNF by the cells. The newly synthesized cellular TNF was identified immunologically on Western blots and as a single 1.8-kilobase band on Northern blots. TNF synthesis began within 2 h of administration of the exogenous TNF in a dose-dependent manner. r-TNF also induced TNF synthesis in mouse tumorigenic fibroblasts (L-M).

Resistant sublines of these cells as well as TNF-nonsensitive human diploid fibroblasts possessed TNF mRNA without pretreatment, indicating an inverse correlation between levels of TNF expressed and sensitivity to the cytotoxic effects of exogenous TNF. It is conceivable that the newly synthesized cellular TNF functions in some protective manner to block cytotoxic effects of exogenous TNF.

INTRODUCTION

TNF is a macrophage-derived cytokine (1–5) which exerts diverse biological activities including antitumor effects (6–13), antiviral effects (14–16), growth promotion of normal diploid cells (13, 17), and inhibition of lipoprotein lipase (18). Among the well-characterized effects are those on tumors such as hemorrhagic necrosis or regression of transplanted tumors (1, 6, 7), suppression of pulmonary metastasis, and cytosis or cytotasis of transformed cell lines (10–12).

These antitumor activities are generally exerted through mechanisms involving receptor-mediated endocytosis (6, 17, 19, 20), suggesting that cell surface receptors are indispensable for the antitumor effects of TNF. Some cell lines, however, are not sensitive or poorly sensitive to TNF even though they have appreciable numbers of receptors on their cell surface (17, 19, 20). Furthermore, TNF-resistant sublines were established by long-term repeated culture of sensitive cells in the presence of TNF (17).

Although the mechanisms by which cells become refractory to TNF are presently obscure, early studies indicated the presence of putative intracellular counteracting proteins because when protein synthesis was suppressed by actinomycin D, tumor cells became more susceptible to TNF (21–24). Recently a direct correlation between inducibility of the gene encoding a β2-interferon-like protein (24) in TNF-treated human tumor cells and their resistance to the cytotoxic effect of TNF have been reported. More recently a TNF-like molecule was identified in the culture medium of a TNF-resistant clone established from TNF-sensitive murine tumorigenic fibroblasts (25). In addition, preliminarily we observed the increased levels of TNF mRNA in murine tumorigenic fibroblasts and human uterine carcinoma cell lines which were treated with exogenous TNF (23).

The induction of TNF mRNA in human breast tumor cell line by exogenous TNF was also reported by Spriggs et al. (26).

In the present study, we extended the previous observation to examine the relationship between the expression of intracellular TNF and their resistance to exogenous TNF.

MATERIALS AND METHODS

TNF and Its Antibody. Human recombinant TNF (r-TNF, 2.37 × 10^6 units/mg protein) (27) and its polyclonal antibody were provided by Asahi Chemical Industry Co., Ltd.

Cell Culture. Cell lines consisted of L-M (mouse tumorigenic fibroblast) cells, KYM-S (human myosarcoma) cells, HeLa (human uterine cervix cancer) cells, and HEL (human embryonic lung, normal diploid fibroblast) cells. L-R and KYM-R cells were TNF-resistant cells obtained by incubating L-M and KYM cells in the presence of gradually increasing concentrations of TNF (1–5 × 10^6 units/ml).

Incubations of L-M, L-R, and HEL cells were performed in Eagle's minimal essential medium supplemented with 10% fetal bovine serum (Flow Laboratories, North Ryde, Australia) and that of KYM-S and KYM-R cells in DM-160 medium and HeLa cells in RPMI 1640 supplemented with 10% fetal bovine serum at 37°C in a humidified 5% CO2 incubator.

In Vitro Assessment of Cytotoxicity. The cytotoxic effect of TNF on the various cells was determined by the inhibition of [3H]thymidine uptake (28) or by dye uptake assays (29).

Measurement of the Amount of TNF. The amount of TNF in the conditioned media of KYM, KYM-R, HeLa, and HEL cells were determined by the enzyme-linked immunosorbent assay (29) (Asahi Chemical Industry Co., Ltd.), using monoclonal antibody against human recombinant TNF, with peroxidase serving as enzyme. In this assay, the limit of detection was 0.1 unit/ml.

Binding Assay. Radiolabeling of r-TNF was performed by the method of Bolton and Hunter (30), to obtain [32P]-labeled r-TNF with a specific activity of 2.7 × 10^8 cpm/μg.

The binding assay for TNF receptors on the cell surface was performed as described previously (6, 9, 17). The number of TNF receptors per cell and the dissociation constant (Kd) were determined by Scatchard plot analysis (31).

DNA Probes. cDNA for human TNF was obtained by screening a human genomic library, using a cloned cDNA encoding a portion of rabbit TNF as a probe (27). The cDNA probe used in this paper encoded the complementary sequence of a specific region of TNF cDNA (33-mer). This TNF cDNA probe was labeled at its 5' end with γ-[32P]ATP (Amersham; 5 × 10^8 Ci/mmole) using T4 polynucleotide kinase (Takara) (32).

Dot Blot Hybridization of mRNA. mRNA was extracted from the cells by the guanidium isothiocyanate method (33) and oligodeoxynucleotide-midylate columns (type II; Collaborative Research) (34). mRNA were denatured with 7.4% formaldehyde and 50% formamide and then transferred to nitrocellulose membranes (Bio-Rad) using a microfiltration apparatus (Advantec) in 5x SSC, baked at 80°C for 2 h. Hybridizations with [32P]-labeled r-TNF cDNA probe were carried out at 42°C for 24 h in 50% formamide containing 1X Denhardt's solution (1X = 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin), 5X SSC, 20 mm sodium phosphate buffer (pH 7.0), 100 μg denatured salmon sperm DNA, and 1 × 10^6 cpm of [32P]-labeled r-TNF cDNA probe per ml. The membranes were then washed in 2X SSC/0.5% SDS for 5 min at room temperature, 2X SSC/0.1% SDS for 5 min at room temperature twice, and 0.1X SSC/0.1% SDS for 15 min.
SELF-INDUCTION OF TNF IN HUMAN AND MURINE CELL LINES

Scanning Procedure of Blotting. After the membranes were exposed to Kodak X-Omat film, the films were scanned by 2-wave chromato-scanner (Shimazu, CS-910) at A460 and the scanning areas were compared.

Northern Blot Hybridization of mRNA. mRNA extracted from cells were denatured with glyoxal and fractionated on 1.1% agarose gels containing 10 mM sodium phosphate buffer (pH 7.0) (35). Fractionated mRNA was then transferred to nylon membranes (Zeta-Probe; Bio-Rad) in 0.04 M Tris-acetate/0.001 M EDTA (pH 7.8) for 6 h at 4°C (electroblotting) and the membranes were baked and hybridized as above.

Immunoblotting. Procedures were those of Towbin et al (36). Cells were solubilized by 1% Triton X-100. 2 mM phenylmethylsulfonyl fluoride, sonicated, and centrifuged (10,000 x g, 10 min). Supernatants were then applied on to SDS-polyacrylamide gel electrophoresis (37). After the electrophoresis, fractionated protein was transferred to nitrocellulose membranes (Bio-Rad) in 20% methanol-Tris glycine buffer (pH 8.3) (electroblotting). Membranes were incubated for 3 h at room temperature with 10 µg of 125I-anti-r-TNF monoclonal antibody per ml which was radiolabeled using Enzymobead reagent (Bio-Rad). Membranes were then washed with phosphate-buffered saline (pH 7.4) 5 times and subsequently exposed to Kodak X-Omat film as above.

RESULTS

Western Blot Analysis of TNF in Human Myosarcoma Cells (KYM-S) Treated with r-TNF, in Its Resistant Subline (KYM-R), and in Human Diploid Fibroblasts (HEL). Lysates of TNF-sensitive human myosarcoma cells (KYM-S), which had been treated with TNF for 24 h (Fig. 1) were analyzed by Western blotting for the presence of intracellular TNF. As shown in Fig. 1, protein reactive to anti-r-TNF antibody was clearly identified in r-TNF-treated KYM-S cells while in nontreated KYM-S cells this protein was not detectable. The positive bands were also observed in TNF-insensitive cells, human diploid fibroblasts (HEL), and a TNF-resistant subline of KYM-S (KYM-R). The protein was slightly larger than that of the r-TNF.

Northern Blot Analysis of TNF in KYM-S, KYM-R, and HEL. To determine if the expression of TNF protein in TNF-treated KYM-S cells was indeed associated with the induction of TNF-mRNA, Northern blot analysis were performed (Fig. 2). A single and discrete band 1.8 kilobases long was observed in TNF-treated KYM-S cells. Corresponding bands were also identified in HEL and KYM-R but not in KYM-S that were not treated with TNF (Fig. 2).

Dot Blot Analysis of TNF mRNA in Mouse Tumorigenic Fibroblasts (L-M), r-TNF-treated LM, TNF-resistant LM (L-R), and Human Cervix Carcinoma (HeLa) Cells. Dot blot analysis of TNF-mRNA was extended to human cervix carcinoma cells (HeLa), a transformed human cell line with comparatively lower sensitivity to TNF than KYM-S. In the HeLa cells tumor cell TNF mRNA that was expressed in the absence of TNF was enhanced by treatment with exogenous TNF (Fig. 3). Species specificity of inducibility of TNF mRNA was also studied with murine tumorigenic fibroblasts (L-M), a cell line highly sensitive to TNF, and exposing them to human r-TNF (Fig. 4). TNF mRNA was expressed by treating the cells with r-TNF while cells not treated with r-TNF did not express any detectable TNF mRNA. A TNF-resistant cell line (L-R) had substantial amounts of hybridization without pretreatment by exogenous r-TNF (Fig. 3).

Concentration Dependence of Induction of TNF mRNA by Exogenous r-TNF in L-M Cells. Cytoplasmic extracts of L-M cells treated with different concentrations of r-TNF for 24 h were analyzed for the expression of TNF mRNA by dot blotting. As shown in Fig. 4, TNF mRNA increased as the concentration of exogenous TNF increased up to a dosage of 100 units/ml and thereafter reached a plateau.

Kinetcs of Induction of TNF mRNA by Exogenous r-TNF in L-M Cells. To measure the kinetics of induction of TNF mRNA by r-TNF, L-M cells were cultured with 100 units/ml of r-TNF for different periods of time. TNF mRNA started to accumulate after 3 h of incubation and continued to increase for 12 h (Fig. 5).

Relationship between Sensitivity to TNF, Number of TNF

![Western blot analysis of endogenous TNF in KYM, KYM-R, and HEL cells. Cell extracts from each cell were subjected to 15% polyacrylamide-SDS, gel electrophoresis and transferred to nitrocellulose filters. Immunoblot was developed by using a 125I-labeled anti-human recombinant TNF polyclonal antibody (rabbit). The nitro-cellulose filter was exposed to XAR-2 film for 48 h at -70°C. K, molecular weight in thousands.](image1)

![Northern blot analysis of KYM cells, KYM-R cells, and HEL cells. mRNA (10 µg) from each cell type was denatured with glyoxal and fractionated on a 1.1% agarose gel, transferred to nylon membrane, and hybridized with 1X-PJATP-labeled TNF cDNA probe. Autoradiography was for 7 days.](image2)
Receptors, and Expression of TNF-mRNA. Relationships between sensitivity to TNF to the number of TNF receptors, expression of TNF mRNA, and TNF activity in the cell culture supernatant of each cell line are summarized in Table 1.

Malignant cell lines with high sensitivity to TNF, KYM-S, and L-M had a high density of TNF receptors on their surface. In contrast to those with low sensitivity, HeLa had fewer receptors.

TNF-resistant cell lines, KYM-R and L-R, completely lacked TNF receptors. However, HEL cells which were also insensitive to TNF had appreciable numbers of TNF receptor. The TNF receptors complexed with ligand on HEL cells were internalized and transported to lysosomal compartments in a manner similar to that found in tumor cells (data not shown). Otherwise, no TNF activity was detected in the cell culture supernatant of TNF-resistant cell lines, KYM-R and L-R, which were refractory to TNF.

Table 1 Relationship between sensitivity to TNF, number of TNF receptors, and expression of TNF mRNA

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>TNF sensitivity (% of cytotoxicity)</th>
<th>TNF receptor/cell</th>
<th>Expression of TNF mRNA</th>
<th>TNF (ng/ml) in the conditioned medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-M</td>
<td>100</td>
<td>10,600</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L-R</td>
<td>ND*</td>
<td>ND</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>KYM-S</td>
<td>98.0</td>
<td>15,300</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>KYM-R</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>HeLa</td>
<td>4.8</td>
<td>1,200</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>HEL</td>
<td>ND</td>
<td>3,500</td>
<td>+</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Determined by dye uptake assay (TNF, 1 x 10^4 units/ml; 2 days of assay).

The amount of TNF was measured by enzyme-linked immunosorbent assay as described in "Materials and Methods."

KYM-S, KYM-R, HeLa, and HEL cells which were incubated for 24 h. Levels of expression of TNF mRNA closely correlated with the resistance of cells to exogenous r-TNF irrespective of the number of TNF receptors.

DISCUSSION

A protein reactive to anti-r-TNF antibody was produced in KYM-S cells which were pretreated with r-TNF. Since this protein was not present in KYM-S not treated with r-TNF and its molecular weight was slightly higher than that of r-TNF, it is not internalized r-TNF but appears to be newly synthesized, possibly a precursor of TNF. This notion was confirmed by the observation that TNF mRNA was expressed in TNF-treated KYM-S but not in untreated KYM-S, indicating that the induction probably occurred at a transcriptional level.

The induction of TNF mRNA was also observed in murine tumorigenic fibroblasts (L-M) treated with human r-TNF. TNF is known to exert its cytolytic activity in different species sharing a common receptor (17, 19, 20). Likewise there was no species barrier for the induction of TNF mRNA; TNF mRNA was induced in murine tumorigenic fibroblasts by human r-TNF.

In contrast to these TNF-sensitive cell lines, human normal diploid fibroblasts (HEL) and resistant clone of KYM-S or L-M which were refractory to TNF cytotoxicity had substantial amounts of endogenous TNF mRNA without r-TNF treatment. Furthermore HEL cells which are poorly sensitive to TNF also have TNF mRNA, although the levels of expression were slightly lower than those of refractory cells but were enhanced...
by exogenous r-TNF. These results, although the number of cell lines studied was limited, seem to indicate a reverse correlation between expression of TNF mRNA and sensitivity to TNF.

It has long been proposed that there must be a protein or proteins which are involved in the rescue of tumor cells from the cytotoxicity of TNF since actinomycin D or cycloheximide enhance the sensitivity of the tumor cells to TNF (21–24). Furthermore, we recently observed that normal diploid fibroblasts become sensitive to TNF by the treatment with actinomycin D, suggesting the presence of the rescue proteins in the normal cells (23). These earlier observations, taken together with the present results, suggest that the newly synthesized intracellular TNF in tumor cells or its precursor in normal diploid cells may be one of the rescue proteins.

The fact that the TNF-sensitive tumor cells treated with r-TNF eventually undergo cytolysis in spite of acquisition of intracellular TNF, may be explained by assuming that preexisting sufficient TNF may be required to overcome the cytotoxic attack by exogenous r-TNF. Mechanisms by which the refractoriness may be brought about by induction of TNF is presently not clear. Recently, Spriggs et al. (26) identified a TNF-like molecule in the culture medium of a TNF-resistant subline established from TNF-sensitive ZR-75-1 (human breast cancer) cells. However, it is clear that occupation of receptors by autocrine TNF is not likely because HEL cells, which are resistant to TNF and possess endogenous TNF, do have abundant TNF receptors. Another possible rescue protein, β2-interferon (25), was reported recently in resistant cell lines although it is not induced in TNF-sensitive cells. There may be other factors in addition to such as the lack of receptors, these rescue proteins that are involved in rendering the cells refractory to TNF.

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REFERENCES

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