Tumor Necrosis Factor and Lymphotoxin Gene Expression in Human Tumor Cell Lines

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ABSTRACT

In this paper we show that eight of 17 tumor cell lines of various tissue origin constitutively express tumor necrosis factor (TNF) mRNA. Five of these eight cell lines concomitantly contained lymphotoxin (LT) mRNA. Of the remaining nine cell lines that lacked detectable TNF or LT gene expression, five could be induced by phorbol ester and/or cytokines to accumulate the respective mRNAs. While TNF mRNA was found not only in neoplastic hematopoietic cells, but also in cell lines derived from carcinomas, LT gene expression seemed to be restricted to lymphoid tumor cells. Tumor cells that expressed LT mRNA also secreted LT protein and proved to be resistant to the cytostatic/cytotoxic effects of their own protein product as well as to exogenous recombinant TNF and recombinant LT. In contrast, most of the cell lines containing TNF mRNA did not release TNF protein into the supernatants, indicating that TNF gene expression may be controlled predominantly at a posttranscriptional level. Thus, the presence of TNF mRNA does not necessarily reflect a TNF-resistant phenotype. Our findings demonstrate that TNF and/or LT mRNA expression is a rather common phenomenon in long-term cultured tumor cell lines and reveal that ectopic TNF and/or LT production by tumor cells may be involved in certain paraneoplastic syndromes as well as in tumorigenesis.

INTRODUCTION

TNF and LT (also called TNF-β) are two related cytokines produced by peripheral blood mononuclear cells in the course of microbial infections, neoplastic diseases, and autoimmune disorders (1,2). TNF and LT share 30% homology at the amino acid level (3–5), bind to the same cellular receptor, and exert very similar biological activities (6–11). Based on their immunomodulating activities (12–15) and cytotoxic effects on tumor cells, TNF and LT are felt to contribute to the host’s immune response against development of cancer. However, apart from its protective roles, TNF has been implicated in the pathogenesis of endotoxin shock (16) and some autoimmune disorders (17). For example, the ability of TNF to stimulate collagenase and prostaglandin E₂ production by synovial cells (18) may lead to the destruction of bone and cartilage in rheumatoid arthritis. In addition, TNF or LT activity has been detected in several other inflammatory disorders like scleroderma or multiple sclerosis (17), suggesting a possible involvement of TNF and LT in the pathogenesis of these diseases. Similarly, it is conceivable that TNF and LT produced autonomously or in response to specific tumor-related stimuli may be relevant to the pathogenesis of cancer. For example, the potent angiogenic activity of TNF (19, 20) might stimulate neovascularization, which is instrumental for tumor development in vivo. We were interested in determining whether tumor cells themselves are able to produce either TNF or LT. Unlike reactive TNF and LT production by macrophages or lymphocytes, which may be of either protective or pathological nature, TNF and LT production by tumor cells would underscore the possibility of pathological roles of these agents in tumorigenesis. In addition, demonstration of TNF or LT production in cancer patients will eventually help to predict primary TNF resistance.

In this paper we show by RNA blotting and immunoprecipitation analysis that, in many tumor cell lines, the TNF gene is constitutively expressed or can be induced by phorbol ester or cytokines. LT gene expression is less frequent and apparently restricted to tumor cells of lymphoid origin.

MATERIALS AND METHODS

Cell Lines. HTLV-I-infected cell lines HuT102, MT-2, and MT-4 were provided by Dr. Popovic, National Cancer Institute, Bethesda, MD, and Dr. Hunsmann, Göttingen, Federal Republic of Germany, respectively. All other cell lines used were obtained from American Type Culture Collection (Rockville, MD) and tested negative for Mycoplasma contamination. Cells were maintained under standard culture conditions as described (12). Sensitivity to TNF was assayed by [³²P]thymidine incorporation as described (11). Cells were cultured for various times with different concentrations (0.1 to 100 ng/ml) of rTNF. During the last 6 h of incubation, 0.5 µCi of [³²P]thymidine per culture was added. The amount of [³²P]thymidine incorporated into DNA was determined by liquid scintillation counting.

RNA Blotting Analysis. Total cellular RNA was isolated by the method of Chirgwin (21), size fractionated by formaldehyde/agarose gel electrophoresis, and transferred to nitrocellulose. Equivalent quantities of RNA transferred to nitrocellulose were controlled by ethidium bromide staining. Filters were hybridized to cDNA probes labeled with [³²P] by random priming (22) as described (23). The TNF cDNA probe used was a 800-base pair EcoRI fragment of clone X42-4 (24). The LT cDNA was a 950-base pair EcoRI fragment representing the coding region. HLA-B7-specific cDNA was obtained from S. Weissman, New Haven, CT.

TNF and LT Assay. The activity of TNF and LT was measured on actinomycin D-treated murine L929 cells as described (25). Cell lysis was determined by staining the cells with crystal violet (0.5%) in methanol-water (1:4, v/v), followed by automatic micro-enzyme-linked immunosorbent assay reading. The concentrations of TNF and LT for each sample were defined by comparison to a standard curve of serial dilutions of recombinant TNF or LT. The sensitivity of this assay is around 20 pg/ml. Cytotoxic activity remaining after neutralization with either mouse monoclonal anti-TNF (TNF-D; neutralizing capacity, 2.7 x 10⁵ units/ml) or rabbit anti-rLT antiserum (2.9 x 10⁵ neutralizing units/ml; kindly provided by Dr. G. Adolf, Vienna, Austria) was determined by incubating appropriate dilutions of the specific antibodies with the test samples for 4 h at 4°C prior to the assay.

Biosynthetic Labeling of Cells and Immunoprecipitation of TNF and LT. Cells were incubated for 16 h in methionine- and cysteine-free RPMI medium supplemented with 1% bovine serum albumin, [³⁵S]methionine (50 µCi/ml), and [³⁵S]cysteine (50 µCi/ml). Cell-free supernatants were preclained with nonimmunized rabbit serum and Protein A-Sepharose CL-4B (Pharmacia) in 10 mM Tris-HCl (pH 7.5) / 0.15 M NaCl/2 mM EDTA: 1% Nonidet P-40: 1 mM phenylmethylsulfonyl fluoride. Following centrifugation, the supernatants were incubated for 16 h at 4°C with either monoclonal anti-TNF or polyclonal anti-LT antibodies. Forty µl of a 50% suspension of Protein A-Sepharose CL-

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2 In partial fulfillment of their Ph.D. theses.
3 The abbreviations used are: TNF, tumor necrosis factor; CHX, cycloheximide; IFN, interferon; LT, lymphotoxin; rTNF, recombinant tumor necrosis factor; rLT, recombinant lymphotoxin; rIFN, recombinant interferon; cDNA, complementary DNA; SDS, sodium dodecyl sulfate; PMA, phorbol myristate acetate; HTLV, human T-lymphotropic virus.
TNF PRODUCTION BY HUMAN TUMOR CELLS

4B were added, and the samples were incubated with end-over-end mixing at room temperature for 2 h. The beads were then washed 4 times, and the immunoprecipitated material was analyzed on 5 to 20% gradient SDS-polyacrylamide gels according to Laemmli (26).

RESULTS

In order to evaluate the expression of TNF and LT genes in human tumor cells, we first analyzed for the presence of TNF and LT mRNA. The cells examined in this paper included three cell lines derived from HTLV-I-positive T-cell malignancies (HuT 102, MT-2, MT-4), seven non-virus-transformed, hematopoietic cell lines of lymphoid and nonlymphoid origin, and six cell lines derived from various solid tumors.

A typical experiment is illustrated in Fig. 1. Raji cells constitutively express LT mRNA, but very little TNF mRNA. TNF mRNA accumulation could be enhanced by either PMA or CHX, a protein synthesis inhibitor. In contrast, the human breast carcinoma cell line HTB26 lacks both TNF mRNA and LT mRNA (Fig. 2, Table 1), and neither PMA nor IFN-γ could induce TNF gene expression. We as well as others have previously shown that TNF itself is a potent inducer of TNF mRNA expression (25, 27). Indeed, in HTB26-cells, TNF mRNA was detected only after rTNF treatment. Thus, to investigate the inducibility of TNF mRNA synthesis, a panel of various tumor cells was treated with either PMA, CHX, IFN-γ, or rTNF. The results of the respective RNA blotting analyses are listed in Table 1. TNF mRNA was detected in 8 of 17 tumor cell lines; of the remaining 9 cell lines, 5 could be induced by PMA, cycloheximide, or TNF to accumulate TNF mRNA. LT mRNA expression appeared to be more tissue specific in that only 5 of the lymphoid cell lines constitutively contained this message. Jurkat cells could be induced by PMA to synthesize LT mRNA. LT mRNA was not detected in any of the nonlymphoid leukemic cell lines or cell lines derived from solid tumors.

Since all tumor cell lines investigated are growing well in the culture medium, the question arises, whether TNF or LT mRNA expression is associated with tumor cell resistance to the cytostatic or cytotoxic activity of exogenous TNF and LT. As shown in Table 1, LT mRNA expression, indeed, correlated with a TNF-resistant status. In contrast, the presence of TNF mRNA appears not to predict the TNF-responsive state. For example, the human breast carcinoma cell line MCF7 contains TNF mRNA, yet is growth inhibitable by treatment with exogenous rTNF. However, the presence of TNF mRNA does not necessarily warrant secretion of TNF protein. For example, in the mouse, posttranscriptional regulatory mechanisms of TNF gene expression have been established based on the finding that the secretion of TNF protein required external stimuli in addition to those inducing TNF mRNA synthesis (28). Thus, we next determined whether or not TNF and LT mRNA expression in human tumor cells correlated with the release of the respective cytokine into the culture supernatants.

The T-cell leukemia line HuT78 constitutively expresses both TNF and LT mRNA (Fig. 3). It should be noted that the TNF mRNA was more abundant than LT mRNA. Unlike HLA-A,B,C gene expression, the levels of both TNF and LT mRNA species could be enhanced by PMA treatment. Interestingly, untreated HuT78 cells released only LT, but not TNF, as revealed by immunoprecipitation studies using specific anti-TNF and anti-LT antibodies (Fig. 4). However, when biosynthetically labeled HuT78 cells were stimulated with PMA, the amount of immunoprecipitable LT increased, and they secreted newly synthesized TNF into the culture supernatant. The ab-

Fig. 1. Expression of LT and TNF mRNA in Raji cells. Total RNA was extracted from Raji cells left untreated (Lane 1) or cultured for 2 h and 4 h, respectively, in the presence of either PMA (10 ng/ml) (Lanes 2 and 3), cycloheximide (10 μg/ml) (Lanes 4 and 5), or a combination of both (Lanes 6 and 7). Twenty μg of RNA were then analyzed by gel electrophoresis, transfer to nitrocellulose, and hybridization to the indicated cDNA probes.

Fig. 2. Induction of TNF mRNA in breast carcinoma cell line HTB26. HTB26 cells were cultured in control medium (Lane 1), or incubated for 4 h in the presence of rTNF (10 ng/ml) (Lane 2), rIFN-γ (10 ng/ml) (Lane 3), a combination of both rTNF and rIFN-γ (Lane 4), or PMA (10 ng/ml) (Lane 5).
Clinical trials. The interest in TNF application to tumor treat-
ment stems from the observation that TNF can induce hemor-
rhagic necrosis when injected into tumor-bearing mice. The
results of the study described here, however, demonstrate that
13 of 17 human tumor cell lines investigated either constitut-
ively express TNF and/or LT mRNA or can be induced by
various stimuli to accumulate these messages. LT mRNA
expression correlated well with LT secretion by tumor cells and
thus may serve as a useful exclusion criterion to eliminate those
patients, who would clearly not respond to LT treatment.
Moreover, TNF and/or LT production points to possible roles
in the pathogenesis of neoplastic disease.

It has been shown previously that TNF gene expression is
not restricted to monocytes, but can also be induced in normal
lymphoid cells (29, 30). Moreover, continuous TNF production
has been demonstrated in fibroblasts and the breast carcinoma
cell line MCF7 selected for TNF resistance (27, 31). As shown
in this study the ability of TNF mRNA expression extends to
neoplastic lymphoid cell lines as well as cell lines derived from
solid tumors of multiple tissue lineages. Since only a few of
these TNF mRNA-positive cells release soluble TNF, tissue
specificity of TNF gene expression may reside in control me-
chanisms regulating TNF translation and/or secretion. Our
findings suggest that TNF, like other cytokines such as CSF-1 (32),
is translated into a pro-TNF molecule that is anchored in the
presence of TNF in the supernatants of untreated HuT78 cells
could be confirmed by biological assays for TNF and LT (Table
2). The cytotoxic activity in the supernatant of untreated HuT78
cells could be inhibited completely with anti-LT, but not anti-
TNF antibodies, indicating the absence of TNF protein. In
contrast, supernatants of induced HuT78 cells contained TNF
activity, which is evident from the reduction of cytotoxic activity
by anti-TNF antibodies.

To address this discrepancy of detectable TNF mRNA and
lack of TNF activity, we investigated HuT78 cells for mem-
brane-bound TNF. Using a cocktail of six distinct anti-TNF
antibodies, HuT78 cells were cleanly stained positive, when analyzed
by indirect immunofluorescence (Table 3). Following PMA
treatment, cell surface TNF expression decreased considerably,
suggesting that the TNF activity released by PMA-treated
HuT78 cells comprises not only newly synthesized TNF (Fig.
4), but also preformed, membrane-derived TNF. HuT102 was
the only tumor cell line investigated that constitutively secreted
TNF-like activity (Table 2), which was confirmed by immuno-
precipitation analysis (not shown). From the panel of cell lines
tested, only Raji, HL-60, and BT20 could be induced by PMA
to secrete TNF, while the other tumor cell lines expressing
TNF mRNA did not produce detectable levels of TNF. For
both HL-60 and HuT78 cells, residual cytotoxic activity was
observed after neutralization with a combination of anti-TNF
and anti-LT antibodies. Since the amounts of antibodies used
were sufficient to neutralize large quantities of TNF and LT
(e.g. in the HuT102 medium), these data suggest the presence of
cytotoxic factors distinct from either TNF or LT.

**DISCUSSION**

TNF is currently used as an anticancer agent in Phase I/II
clinical trials. The interest in TNF application to tumor treat-
ment stems from the observation that TNF can induce hemor-
RHINIA
membrane. Secretion of the soluble $M_s \approx 17,000$ TNF then apparently requires further stimuli, such as activators of protein kinase C.

Since only a small fraction of TNF mRNA-containing tumor cell lines was found to secrete TNF (Table 2), the demonstration of TNF mRNA does not necessarily allow conclusions as to the TNF-responsive state. This is underscored by the heterogenous sensitivity of TNF mRNA-positive tumor cell lines. Moreover, even a priori TNF-sensitive tumor cells may acquire a TNF-unresponsive state, when induced to secrete TNF. For example, HL-60 cells, which respond to exogenous TNF with growth arrest (23), are able to secrete biologically active TNF upon treatment with PMA. However, PMA downmodulates TNF binding capacity in HL-60 cells via phosphorylation of receptors by protein kinase C (33, 34). It seems as if the very stimulus that induces TNF secretion at the same time desensitizes TNF receptors of the producer cell itself, thereby rendering it unresponsive to the action of TNF. In the case of HL-60 cells, this coupling of TNF secretion and TNF receptor affinity down-modulation may explain why this a priori TNF-sensitive cell type does not respond to its own product. The results of our study show that, in order to link TNF gene expression with TNF resistance, one clearly has to assay at the protein level.

In contrast to TNF gene expression, LT mRNA levels correlate well with LT activity in the supernatants, indicating that LT gene expression may be primarily controlled at the level of mRNA transcription. Due to a common receptor system for TNF and LT (6-11), one would expect that tumor cells, which secrete LT, do not respond to either of these agents. Indeed, all of the tumor cells capable of LT mRNA expression proved cross-resistant to the cytotoxic/cytostatic action of TNF (Table 1). It will be interesting to examine whether LT mRNA demonstrated by in situ hybridization in tumor biopsies is consistently associated with the primary TNF-resistant state. The demonstration of LT mRNA in tumor cells may eventually help in predicting the tumor cell response phenotype to TNF.

TNF and LT production by tumor cells implicates a possible role of these cytokines in cancer pathogenesis, which can be easily deduced from some of the biological effects of these agents. For example, the secretion of lymphotoxin by human myeloma cells has recently been linked to osteoclastic bone destruction and hypercalcemia in patients with myeloma (35).
Similarly, constitutive LT production by HTLV-I-infected T-cells (Table 2) may explain lytic bone lesions and hypercalcemia frequently observed in adult T-cell leukemia (36). Finally, because of its potent angiogenic activity (19, 20), TNF may stimulate the growth of blood vessels, thereby promoting tumor development in vivo.

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