Communication

Tumor Necrosis Factor Receptor Signaling

A DOMINANT NEGATIVE MUTATION SUPPRESSES THE ACTIVATION OF THE 55-KDA TUMOR NECROSIS FACTOR RECEPTOR*

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To investigate the signaling mechanism of the 55-kDa tumor necrosis factor (TNF) receptor a functional transfection based assay was developed. The human 55-kDa TNF receptor, stably expressed in mouse L929 cells, was demonstrated to be activated specifically by agonist antibodies and to initiate a signal for cellular cytotoxicity. A deletion mutant of the human TNF receptor lacking most of the cytoplasmic domain was found to be completely defective in generating the signal for cytotoxicity. Additionally, expression of the truncated receptor substantially suppressed signaling by endogenous mouse TNF receptors in response to TNF, but not in response to specific anti-murine TNF receptor antibodies. These results suggest that aggregation of 55-kDa TNF receptor intracellular domains, which are not associated in the absence of ligand, is an important component of the signal for cellular toxicity. This work also provides an example of a dominant negative mutation in a transmembrane receptor that lacks a tyrosine kinase domain, and suggests a more general utility of dominant negative mutations in the investigation of cytokine receptor function.

Tumor necrosis factor (TNF) is a potent cytokine produced mainly by activated macrophages, T cells, mast cells, and some epithelial tumor cells (1–3). The large number of biological effects elicited by TNF include hemorrhagic necrosis of transplanted tumors, cell proliferation, cytotoxicity, inflammatory, immunoregulatory and antiviral responses, and an important role in endotoxic shock (4–8). The first step in the induction of these various cellular responses by TNF is the binding to specific cell surface receptors. TNF receptors have been detected on a wide variety of normal tissues and cell lines that are either sensitive or resistant to TNF (9–12). Two distinct TNF receptors of approximately 55 kDa (TNF-R1) and 75 kDa (TNF-R2) have now been identified (13–16), and human and mouse cDNAs corresponding to both receptor types have been isolated and characterized (17–21).

A number of reports have described studies investigating the individual roles of the two TNF receptors. Both polyclonal and monoclonal antibodies directed against human TNF-R1 have been shown to behave as receptor agonists and elicit several TNF activities such as cytotoxicity, fibroblast proliferation, resistance to chlamydiae, and synthesis of prostaglandin E2 (15, 22, 23). In addition, polyclonal antibodies to both murine TNF-R1 and murine TNF-R2 have been developed, and each shown to behave as specific receptor agonists and induce a subset of murine TNF activities (24). Importantly, studies with the murine receptor agonist antibodies have demonstrated that signals from these receptors are not redundant, but rather that each signals distinct TNF activities. The murine TNF-R1 was shown to be responsible for signaling cytotoxicity and the induction of several genes, while the murine TNF-R2 was shown to be capable of signaling proliferation of primary thymocytes and a cytotoxic T cell line (24).

Although the signaling mechanism of neither TNF receptor is understood, several studies have suggested that cross-linking of receptor extracellular domains by either TNF or agonist antibodies is an important feature of the activation process of TNF-R1 (15, 25, 26). To examine further the signaling mechanism and structure-function relationships of the TNF receptors, it will be necessary to develop model systems in which the signal from transfected wild type and mutant receptors can be specifically assayed. A potential problem in the development of a functional assay for the 55-kDa TNF receptor is that its near ubiquitous expression in mammalian cell lines will make it difficult to distinguish between signals from transfected receptors and signals from endogenous receptors. In an attempt to circumvent this problem, we have chosen to express the human 55-kDa TNF receptor in the highly TNF-sensitive murine cell line L929, and then specifically activate the transfected human TNF receptor with species-specific agonist antibodies.

MATERIALS AND METHODS

Reagents—Recombinant human TNF (hTNF) (specific activity > 107 units/mg) was provided by the Genentech manufacturing group. The rabbit anti-murine TNF-R1 antibodies were identical to those described previously (24). The rabbit anti-human TNF-R1 antibodies were generated against the soluble extracellular domain of hTNF-R1 (26). The titer of the anti-hTNF-R1 was 1:50,000 as quantitated by a direct antigen-coated enzyme-linked immunosorbent assay. Monoclonal antibody 984 against human TNF-R1 (Genentech, Inc.) was produced as described previously (26) and inhibits the binding of TNF to human TNF-R1.1

Establishment of L929 Clones Expressing Human 55-kDa TNF Receptors—A plasmid containing a deletion mutant of the human 55-kDa TNF receptor was generated from plasmid pRK-TNF-R1 (17) by replacement of sequences between the HindIII and MluI sites with an oligonucleotide containing an in-frame stop codon. Verification of correctly modified cDNAs was determined by double-strand sequencing. This subcloning procedure results in an in-frame stop codon immediately following Phe235. The cDNAs encoding the human 55-kDa TNF receptor (17) and the truncated deletion mutant (see above) were cloned into the Rous sarcoma virus long terminal repeat (27) expression vector pRIS4 (Clai-BamHI polylinker sites). To increase translation of the expressed mRNAs, an upstream ATG codon (17–21).

1 Louis A. Tartaglia, R. F. Weber, and D. V. Goeddel, unpublished data.

2 B. M. Fendly, unpublished data.

3 C. Gornall, unpublished data.
was removed from the 5'-untranslated region during the subcloning procedure. The expression vectors encoding the intact and truncated 55-kDa TNF receptors were introduced into mouse L929 cells by electroporation. 5 × 10^6 cells in 1.0 ml were co-transfected with 0.5 µg of Scod-digested pRK.neo and 20 µg of Scod-digested TNF receptor expression vector. Cells were plated into 15-cm plates and after 2 days selected in media containing 600 µg/ml G418. After 12 days, individual G418-resistant clones were picked and expanded. To examine the expression of human TNF-R1, cells were incubated on ice for 60 min with 100 µg/ml anti-human TNF-R1 mAb 884 (see above) in phosphate-buffered saline containing 2% fetal bovine serum. The cells were then washed and stained with PE-conjugated goat anti-mouse immunoglobulins (Caltag) and analyzed on an Epics Elite instrument (Coulter).

**L929 Cytotoxicity Assay**—L929 cells (2 × 10^6 cells/well) were seeded into 96-well microtiter plates in 100 µl of media (low glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 1% L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin (Gibco Laboratories)) and incubated 24 h at 37 °C in a 5% CO₂ atmosphere. Medium was then brought to 10 µg/ml cycloheximide, and the anti-TNF-R antibodies or hTNF added to the wells and serially diluted. The plates were incubated for an additional 16 h (or the indicated time period) and the viable cells stained with 20% methanol containing 0.5% crystal violet. The dye was eluted with 0.1 M sodium citrate, 0.1 M citric acid, and 50% ethanol and absorbance measured at 540 nm.

**RESULTS**

Expression of Wild Type and Truncated Human 55-kDa TNF Receptors in Mouse L929 Cells—The cDNAs encoding the wild type human 55-kDa TNF receptor (17) and a mutant receptor deleted for the majority of the intracellular domain (182 of 221 amino acids) were placed downstream of the Rous sarcoma virus long terminal repeat in the expression vector pRK.neo. The receptor constructs were then co-transfected with the plasmid pRK.neo into the murine cell line L929. After selection with G418, individual clones expressing the neomycin resistance gene were picked and analyzed by flow cytometry for expression of the human 55-kDa TNF receptor. Several L929 clones expressing the intact human TNF-R1 were identified, and all showed a roughly similar level of expression. The expression profile of one representative clone is shown in Fig. 1A. Several clones expressing the truncated TNF-R1 were also identified, and the level of expression between different clones varied markedly. The expression profiles for two clones are shown in Fig. 1B.

We have previously reported that the murine L929 cell line is highly sensitive to polyclonal antibodies against murine TNF-R1 (homolog of the human TNF-R1). We have also developed polyclonal antibodies against human TNF-R1 and have found that they induce cytotoxicity in a number of human cell lines, but exhibit no activity in murine cell lines. To determine if human TNF-R1 expressed in murine L929 cells could be specifically activated, three L929 clones expressing human TNF-R1 were treated with the anti-human TNF-R1 antibodies. All three clones showed a strong cytotoxic reaction in response to the anti-human TNF-R1 antibodies (Table 1). No significant killing by these antibodies was observed in either the L929 parent or G418-resistant clones not expressing human TNF-R1 on their surface. These results demonstrate that the human 55-kDa TNF receptor can initiate a signal for cytotoxicity in a murine cell line.

To determine if the intracellular domain of TNF-R1 is required for signaling cytotoxicity, three independent clones expressing the truncated human TNF receptors were examined for a cytotoxic effect upon treatment with the anti-human TNF-R1 antibodies. The three clones chosen for this analysis expressed the truncated human TNF-R1 at levels similar to (L929.hR1Δ19), slightly higher than (L929.hR1Δ4), or much higher than (L929.hR1Δ16) clones expressing the full-length human TNF-R1 (Fig. 1B). None of the three clones expressing the truncated human TNF-R1 were killed in response to the anti-human TNF-R1 antibodies (Table 1), indicating a requirement for the TNF-R1 intracellular domain in the generation of this signal.

**Suppression of Endogenous TNF Receptor Signaling by Co-expression of Truncated 55-kDa Receptor**—In previous reports we showed that hTNF will bind with high affinity to human 55-kDa TNF receptor (17), soluble extracellular domain of the human 55-kDa TNF receptor (26), and murine TNF-R1

![Flow cytometric analysis of hTNF-R1 expressing transfectants. A, control cell line (L929.neo1) and a representative cell line expressing wild type hTNF-R1 (L929.hR1.17). B, control cell line (L929.neo2) and cell lines expressing truncated hTNF-R1 (L929.hR1Δ.4 and L929.hR1Δ.16). Cells were stained with anti-hTNF-R1 mAb 884 and PE-conjugated goat anti-mouse immunoglobulins. Ten thousand cells were analyzed per sample. Histograms show relative cell number (y-axis) versus log PE-fluorescence (x-axis).](image)

**TABLE I**

| Clone     | Viability % |
|-----------|-------------|
| L929      | 96 ± 3      |
| L929.neo1 | 99 ± 5      |
| L929.neo2 | 97 ± 3      |
| L929.hR1.2| 2.7 ± 1.2   |
| L929.hR1.15| 2.3 ± 1.2  |
| L929.hR1.17| 1.7 ± 0.5  |
| L929.hR1Δ.4| 99 ± 4   |
| L929.hR1Δ.16| 102 ± 8  |
| L929.hR1Δ.19| 97 ± 2   |

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8. L. A. Tartaglia, G. H. W. Wong, R. F. Weber, and D. V. Goeddel, unpublished data.
The data shown are the mean of three experiments. Materials and Methods. The data shown are the mean of three experiments.

Cytoxicity assays on L929 clones expressing the truncated 55-kDa receptor revealed a significant reduction in hTNF sensitivity which correlated with the expression level of the truncated receptor. A comparison of the hTNF sensitivity between clone L929.hR1A.16, clone L929.hR1A.4, (which have approximately 7-fold and 3-fold overexpression of truncated receptors compared with murine TNF-R1, respectively; data not shown), and a control G418-resistant L929 clone is shown in Fig. 2. Approximately 100-fold higher concentrations of TNF are required to induce cytotoxicity in the L929 clones expressing the truncated TNF-R1 than are required in the control clone. The increased hTNF resistance of clones expressing truncated TNF-R1 is not due to depletion of free TNF in the media by the truncated receptors, since significant resistance is still observed at 250 ng/ml (5 nM) hTNF, a concentration that is 1000-fold higher than the concentration of TNF receptors. In addition, longer TNF exposure times are required for cytotoxicity in L929.hR1A.16, even in the presence of a saturating concentration (50 nM) of TNF (Fig. 3). To control for the possibility that co-expression of truncated human TNF-R1 reduced TNF sensitivity by a mechanism other than cross-linking with endogenous murine TNF-R1 (such as down regulation of endogenous receptors), an anti-human TNF-R1 monoclonal antibody was used to inhibit the binding of TNF to the truncated human TNF-R1, yet still allow binding of TNF to the endogenous murine TNF-R1. Under these conditions the TNF sensitivity of clone L929.hR1A.16 approaches control levels (Figs. 2 and 3).

55-kDa TNF Receptors Are Not Aggregated in the Absence of TNF—The dominant negative effect of the truncated 55-kDa TNF receptors supports previous arguments that the cross-linking of TNF receptors is an important step in the generation of an intracellular signal. To determine whether the 55-kDa TNF receptors are associated in the absence of a ligand, we tested whether the truncated human 55-kDa receptor would interfere with signaling in response to a ligand that only recognizes the endogenous murine receptors. Fig. 4 shows a comparison of the sensitivity of clone L929.hR1A.16 and control clone L929.neo2 to agonist anti-mTNF-R1 antibodies (24). The anti-mTNF-R1 cytotoxicity dose-response curve is unaffected by co-expression of the truncated human 55-kDa receptor. These results suggest that the vast majority of 55-kDa TNF receptors on the cell surface are not aggregated in the absence of ligand, but rather undergo an induced physical association upon TNF binding.

Discussion

The recent cloning of the 55-kDa (TNF-R1) and 75-kDa (TNF-R2) TNF receptors has made them accessible to a molecular genetic analysis of their mechanisms of action and structure function relationships. However, model systems must first be developed where functional signals from transfected TNF receptors can be easily and specifically assayed. We have stably expressed the human 55-kDa TNF receptor in the highly TNF sensitive murine cell line L929. Treatment of these stable clones with species-specific agonist antibodies against the human 55-kDa TNF receptor resulted in nearly complete cytotoxicity, demonstrating that human TNF-R1 can be specifically activated in a murine cell and is compatible with the endogenous murine signal transduction apparatus.

To determine whether the signal for cytotoxicity is propagated through the intracellular domain of TNF-R1 or a signaling component interacting with only the extracellular domain, a truncated receptor was also tested for its ability to mediate cytotoxicity. This truncated receptor, deleted for 182 of its 221 amino acid intracellular domain, was devoid of any detectable signaling activity, suggesting that sequences within this region are required for the generation of the cytotoxic signal. Further mutant analysis with this functional transfected receptor should provide a means to identify sequences within the intracellular domain that initiate the signal for cytotoxicity.

Several studies have provided evidence that the cross-linking of 55-kDa TNF receptors is an important component of
nant negative mutations, TNF-R1 is a member of a recently described protein family where several transmembrane receptors might be susceptible to dominant negative mutations. Although it is difficult to predict which of the known receptors possess intracellular tyrosine kinase domains (30–33). Such dominant negative mutations have been exploited both in vitro (31, 32) and in vivo (30) to explore the mechanism and function of receptor-tyrosine kinases.

Although to our knowledge the only transmembrane receptors for which a dominant negative mutation has been described are tyrosine kinases, the strong evidence supporting oligomerization induced signaling of TNF-R1 prompted us to look for such an effect. Expression of a TNF-R1 with a defective intracellular domain resulted in very substantive suppression of endogenous TNF-R1 signaling. A shift of approximately 2 logs in the dose response was observed even in the highly TNF-sensitive mouse cell assay, which normally shows complete cytotoxicity at very low receptor occupancy (TNF concentrations <10-fold below the Kc).

The dominant negative effect of the truncated human TNF-R1 is not simply a result of depleting TNF from the assay supernatants since significant effects are still observed in large TNF excess. Additionally, expression of the truncated receptor has not resulted in down regulation of the endogenous murine receptors or downstream signaling components since cells expressing the truncated human TNF-R1 showed normal sensitivity to anti-murine TNF-R1 agonist antibodies. In addition, inhibiting the binding of TNF to the truncated receptors restores TNF sensitivity.

The normal sensitivity of L929 cells expressing the truncated TNF-R1 to anti-murine TNF-R1 antibodies also suggests that stable receptor oligomers do not preexist on the cell surface. If the defective receptors were preassociated with functional endogenous murine TNF-R1, they should have resulted in reduced sensitivity to the agonist antibodies in the same manner in which receptor signaling was suppressed in response to TNF. It would therefore appear that stable TNF-R1 oligomer formation only occurs upon the binding of TNF.

These results demonstrate that co-expression of defective human 55-kDa TNF receptors can result in a ligand-selective suppression of the corresponding endogenous murine receptors. This dominant negative effect should provide a useful tool for in vitro studies of TNF receptor function, since TNF receptors could be made unresponsive to endogenous TNF, yet still be activated by exogenously supplied agonist antibodies. In addition, the strong dominant negative effect observed with this type of transmembrane receptor implies a more general significance of cytokine receptor dominant negative mutations. Although it is difficult to predict which of the many transmembrane receptors might be susceptible to dominant negative mutations, TNF-R1 is a member of a recently identified and expanding family of transmembrane receptors.

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