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Tumor Necrosis Factor Receptor (TNFR)-associated factor 2 (TRAF2) is not Involved in GM-CSF mRNA Induction and TNF-Mediated Cytotoxicity

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Tumor necrosis factor receptor (TNFR)-associated factor 2 (TRAF2) is known to act as a signal transducer that connects TNFR2 to its downstream effector functions such as proliferation of thymocytes, regulation of gene expression, and cell death. TRAF2 consists of largely two domains, the N-terminal half that contains a signal-emanating region and the C-terminal half that is responsible for binding to the intracellular region of TNFR2. In this study, we examined the possible roles of TRAF2 in granulocyte-macrophage colony-stimulating factor (GM-CSF) gene expression and cell death. A truncated mutant of TRAF2 (Δ2-263) that contains only a C-terminal half was generated, and transiently transfected to the A549 cell, a human lung cancer cell line, and L929 cell, a murine TNF-sensitive cell line. GM-CSF mRNA was induced in untransfected A549 cells both in dose- and time-dependent manner upon the exposure of TNF. However, neither the full length TRAF2 nor the mutant altered GM-CSF mRNA production regardless of the presence or absence of TNF. Furthermore, neither TRAF2 versions significantly changed the cytotoxic effect of TNF on L929 cells. These data suggest that TRAF2 may not be involved in the signal transduction pathway for GM-CSF gene induction and cell death mediated by TNF.

Key words: TNF, TRAF2, GM-CSF, cytotoxicity

TNF exerts its functions by binding two types of receptors, TNFR1 and TNFR2 (23). TNFR1 (p55) appears to be responsible for the generation of most activities involved in TNF-mediated cellular responses including cytotoxicity (4,5,26), host defense against pathogenic microorganisms (17), and induction of gene expression such as nitric oxide synthetase (iNOS), Mn-superoxide dismutase (Mn-SOD) (26) and A20 (22). In contrast TNFR2 (p75) mediates a few cellular events relatively confined to immune functions. These include proliferation of thymocytes and a murine cytotoxic T cell line CT6 (7,25,26), induction of GM-CSF mRNA in T cells (28), the inhibition of early hematopoiesis (11), and induction of apoptosis (8). The two receptors belong to the TNFR superfamily and share the conserved and repeating cysteine residues in the extracellular ligand binding domain, yet lack sequence homology between intracellular domains of both (21). Indeed, intracellular regions of TNFR1 and TNFR2 attract or activate distinct molecules leading to largely nonoverlapping TNF signaling (see below).

TRAF1 and TRAF2 have been identified as the first molecules that are able to bind the intracellular region of TNFR2. TRAF2 consists mainly of two domains: the N-terminal and the C-terminal half. The former contains a RING finger and a zinc finger, and is thought to activate TNFR2-mediated signal transduction. The C-terminal half is called TRAF domain and is responsible for protein oligomerization besides interaction of the TRAF protein with TNFR2 (19). The overexpression of TRAF2 results in NF-B activation in diverse cell types (9, 18, 27). The truncated TRAF lacking the N-terminal half has been shown to abrogate both NF-κB activation and proliferation of thymocytes, thus acting as a dominant negative inhibitor (18). On the other hand, TRADD binds to the intracellular region of TNFR1 to induce two biological activities of TNF, NF-κB activation and cell death in a variety of cell lines (10). TRADD is also able to bind TRAF2 (9). Transfection assay demonstrated that the role

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of TRAF2 in TNFR1 signaling is restricted to NF-κB activation, but not apoptosis (18). Thus the two events seem to be independent and distinct.

It is unknown whether TRAF2 is directly involved in GM-CSF mRNA induction although it was demonstrated that TRAF2 is capable of interaction with TNFR2 (19) which mediates GM-CSF mRNA induction (28). Futhermore, the role of TRAF2 in TNFR1-mediated apoptosis is still unclear since it was shown that TNFR2 overexpression induces cytotoxicity (8) and that TRAF2 induces antiapoptotic pathways in lymphocytes during TNF-induced apoptosis (13). It is, therefore, of interest to observe TRAF2 functions in those events. In this study we examined the effects of TRAF2 and a mutant TRAF2 (Δ2-263) on GM-CSF mRNA induction and also tested whether TRAF2 could alter the cytotoxic responsiveness of TNF-sensitive cells.

Materials and Methods

Materials

A549 cells and L929 cells were purchased from the ATCC and Korean Cell Line Bank (Seoul National Univ., Seoul, Korea), respectively. Recombinant human TNF was purchased from R&D systems (Minneapolis, USA), anti-Flag monoclonal antibody (M5), BCIP/NBT, MTT, aprotinin and PMSF were obtained from Sigma Chemical Co. (St. Louis, USA). Restriction enzymes were obtained from New England Biolabs (Beverly, USA). PCR primers were purchased from Bio-synthesis Inc. (Biosyn., Lewisville, USA). RPMI1640 medium, fetal bovine serum (FBS), and penicillin-streptomycin mixture were obtained from Gibco, BRL (Gaithersburg, USA).

Probes

A piece of the GM-CSF gene was amplified by a conventional polymerase chain reaction (PCR) using *Taq* polymerase (Boerinhger Mannheim, Mannheim, Germany). The upstream and downstream primers are CAAGCTTAAGGGCCCCTTGACCATG and TGGATCCGGGTCAGTGTGGCCCAGGG corresponding to 271-295 and 575-590, respectively, based on the GM-CSF mRNA sequence reported (29). The amplified product was subcloned to the *Bam* HI and *Hind* III sites of pGEM-3Zf (Promega, Madison, USA) for *in vitro* transcription. As a control, a fragment of the β-actin gene was also amplified by PCR and subcloned to the same vector. The primers for β-actin were the upstream primer, TAAGCTTAAGGAGAAGCTGTGCTACG, and the downstream primer, GGGATCCACGTCACACTTCAT.

Construction of a mutant TRAF2

The full length of TRAF2 with a N-terminal Flag epitope in the pRK5 mammalian expression vector was previously described (9) and kindly given by Dr. D. V. Goeddel (Turalik Inc. CA, USA). A truncated TRAF2 (Δ2-263) was generated by PCR using *Pfu* polymerase (Stratagene, La Jolla, USA). PCR primers are follows: the upstream primer is ATGTCGACATGGACTACAAGGACGACGATGACAAGGTGGGGCCAGAGCTACTC including a Flag epitope (DYKDDDDK) sequence, and the downstream primer is ATGCGGCCGCCTAGAGTCCTGTTAGGTC. PCR was performed for 30 cycles (1 min at 95°C, 1 min at 56°C, and 2 min at 72°C), and the resulting PCR product containing only the C-terminal TRAF do- main (Δ2-263) was digested and cloned into the *Sal* I and *Not* I sites of pRK5.

Transfection

The wild type and mutant TRAF2 (Δ2-263) constructs were transfected to A549 cells (6×10^6 cells/60-mm dish) that had been grown in RPMI1640 medium containing 10% FBS and 1% penicillin-streptomycin were transfected with the full length TRAF2 or the mutant TRAF2 using a Superfect transfection kit (Qiagen, Hilden, Germany), and the cultures were incubated for 40 h. The transfected cultures were stimulated with rhTNF for 2 h and harvested for RNA isolation and immunoblotting. For cytotoxicity assay, the cultured L929 cells (6×10^6 cells/60-mm dish) were transfected with these constructs, incubated for 30 h, and treated with trypsin. The aliquots of the transfected cells were collected for subsequent immunoblotting. Remaining cells were transferred to 96-well microplates at a concentration of 4×10^4 cells/well and incubated overnight.

Western blot

Cells were lysed with 200 μl of suspension buffer (0.1 M NaCl, 0.01 M Tris-Cl pH 7.6, 1 mM EDTA, 1 μg/ml aprotinin, and 100 μg/ml PMSF), and the lysate was incubated on ice for 1 h (20). After centrifugation of cells at 14,000 × rpm for 10 min, the supernatant was mixed with an equal volume of SDS gel-loading buffer (50 mM Tris-Cl, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol), and the mixture was heated at 95°C for 10 min and run on 10% SDS-polyacrylamide gel. The proteins were electrophoretically transferred to nitrocellulose membranes (Schleicher & Shuell, Keene, USA) at a constant volt of 60 V for 3 h according to Laemmli (12). The blot was incubated with 10 μg/mL of anti-Flag monoclonal antibody (M5) (Eastman Kodak, Rochester, USA) in

TBS at 4°C for 2 h. After washing, the membrane was incubated with alkaline phosphatase-conjugated goat anti-mouse antibody (ZYMED, SF, USA) (1 : 1000 dilution) and developed in the presence of BCIP/NBT solution.

RNA isolation

Total RNA was isolated from A549 cells. RNA isolation followed the procedure of Chomczynski and Sacchi (1) using Tri-reagent (MRC, Cincinnati, USA). Briefly, cells were washed three times and pelleted. RNA was extracted with guanidine isothiocyanate and phenol, and precipitated with ethanol.

In vitro transcription and RNase protection assay (RPA).

The recombinant plasmid harboring a piece of the GM-CSF gene or β–actin was linearized with *Hind*III for the production of antisense riboprobes. *In vitro* transcription was performed according to manufacturer's instruction (Ambion, Austin, TX). The reaction mixture in a final volume of 20 μl containing 1 μg of linearized plasmid DNA, 40 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 500 μM ATP, CTP, GTP, 12.5 μM [α–^{32}P] UTP (800 Ci/mmol, 40 mCi/ml) (Amersham, Buckinghamshire, UK), and 5 U of T7 RNA polymerase was incubated at 37°C for 1 h. After the mixture was treated with 1 μl of DNase (2 unit/μl) for removal of DNA, the radiolabeled probes were purified by electrophoresis in 5% polyacrylamide/8 M urea gels. The resulting transcripts were excised from the gel after x-ray film exposure for a few minutes, and eluted overnight at 37°C in 350 μl elution buffer containing 0.5 M NH_4OAc, 1 mM EDTA, and 0.1% SDS. RPA was performed with the RPAII kit (Ambion, Austin, USA) as previously described (3). Ten μg of total RNA from cells were hybridized with riboprobes (8×10^4 cpm) at 45°C overnight in 20 μl of 40 mM PIPES (pH 6.4), 80% deionized formamide, 400 mM NaOAc, and 1 mM EDTA. The hybridized mixture was then treated with RNase A/T1 (1:100 dilution in 200 μl of the RNase digestion buffer) at 37°C for 30 min, and analyzed by 5% polyacrylamide/8 M urea gel electrophoresis. The protected fragments of GM-CSF and the β-actin riboprobes are 313 and 230 nucleotides (nt) in length, respectively.

MTT assay

The cytotoxicity of transfected L929 cells to TNF was determined by a MTT assay as described previously (2). The transfected L929 cells were seeded in 96-well microplates at a concentration of 4×10^4 cells/100 μl/well in RPMI1640 medium containing 10% FBS and 1% penicillin-streptomycin, and allowed to grow overnight. The cultures were then changed with the same medium containing 5 μg/mL of actinomycin D-manitol (Sigma, St. Louis, USA), and serially-diluted rhTNF were added to each well. Triplicate cultures were set up for each diluent of rhTNF. After incubation for 16 h, cytotoxicity was assessed using the MTT cytotoxicity assay (6).

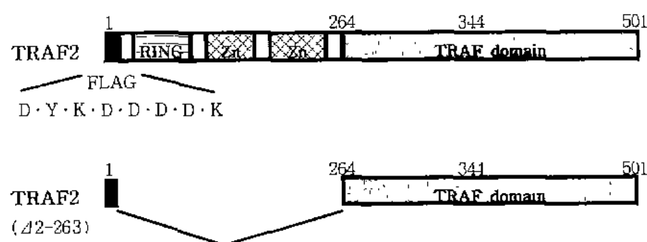

Fig. 1. Structure of TRAF2 and a truncated TRAF2 mutant. The truncated form of TRAF2 (Δ2-263) was generated by PCR as described in *Materials and Methods*. The Flag epitope (DYKDDDDK) was inserted to the N-terminal of the TRAF2 for subsequent immunoblot analysis.

Results and Discussion

Since it has been demonstrated that TNF mediates GM-CSF mRNA expression via TNFR2 (28) and TRAF2 serves as a signal transducer of TNFR2 (19), we wished to examine the role of TRAF2 in the cytokine gene expression. To this end, we generated a truncated version of TRAF2 (Δ2-263) by conventional PCR (Fig. 1). The truncated form of TRAF2 contains only the C-terminus, the region responsible for binding the cytoplasmic region of TNFR2. This form is known to be able to bind TNFR2, but can not transduce the signal due to lack of the N-terminal half, thus acting as a dominant negative inhibitor against activation of NF-κB, which is required for transcriptional activation of the GM-CSF gene (14,16). A Flag epitope was also inserted into the first two codons of the TRAF2 to confirm the expression of the constructs by subsequent immunoblot analysis. We examined whether TNF could induce GM-CSF mRNA expression in A549 cells by RPA analysis. A549 cells also responded to TNF to produce GM-CSF mRNA in a dose-response manner (Fig. 2a). The cytokine mRNA was not induced in unstimulated A549 cells, and was detectable as early as 1 h and attained peak level in 2 h upon TNF treatment (Fig. 2b). Optimal synthesis of GM-CSF mRNA is, therefore, detected when A549 cells are incubated with TNF 10 ng/ml for 2 h. This result is in agreement with the previous finding in which TNF induced GM-CSF mRNA expression in the same cells (15). A549 cells were transiently transfected with either the full length of TRAF2 or its truncated form (Δ2-263), and stimulated with TNF (10 ng/ml) for 2 h. The TRAF2 pro-

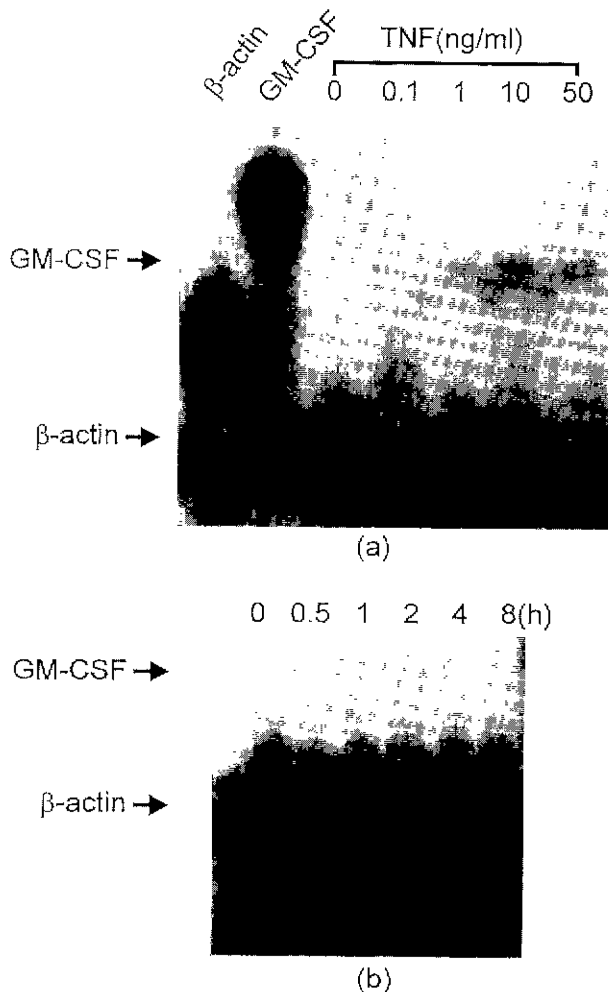

Fig. 2. GM-CSF mRNA expression in untransfected A549 cells : dose- and time-response studies. A549 cells (6 × 10^6 cells/60mm dish) were treated either with different concentrations of rhTNF for 2 h (a), or with rhTNF (10 ng/ml) for different periods of time (b). GM-CSF and β-actin mRNA levels were examined by RPA as described in *Materials and Methods*. Total RNA (10 μg) from each culture was isolated and subject to RPA analysis. The gel was exposed to X-Omat film overnight at −70°C.

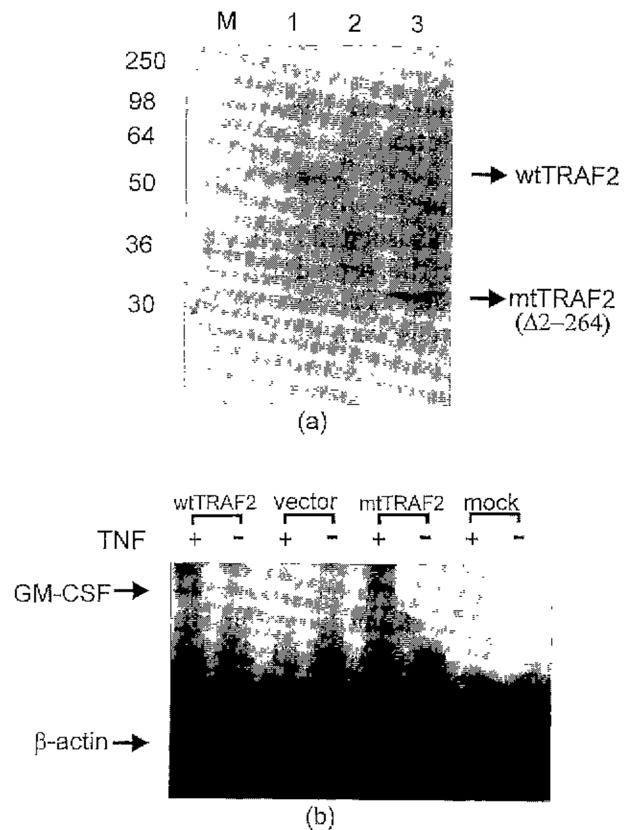

Fig. 3. The effect of TRAF2 expression on GM-CSF mRNA production in A549 cells. A549 cells (6 × 10^6 cells/60 mm dish) were transfected with either the full length of TRAF2 or the truncated form (Δ2-263), incubated for 30 h, and treated with rhTNF (10 ng/ml) for 2 h. The cell lysates were prepared from half the amount of the transfected cells and subject to immunoblot analysis (a). The total RNA (10 μg) was isolated from the rest of the cultures and subject to RPA analysis (b). Representative of two experiments.

tein and the mutant were easily detected as 56 and 32 kDa, respectively, by immunoblot analysis using an anti-Flag monoclonal antibody (M5) (Fig. 3a). Our result revealed that GM-CSF mRNA induction was observed only in transfected cultures upon exposure of TNF. The expression of the full length of TRAF2 did not increase GM-CSF mRNA induction nor did the truncated form (Δ2-263) block GM-CSF mRNA production by TNF (Fig. 3 b). A similar result were obtained in which TNF mRNA induction was not affected by TRAF2 expression (data not shown). This result was somewhat surprising because GM-CSF gene expression have been shown to be mediated via TNFR2 (28). The inability of the murine TRAF2 to modulate the cytokine gene expression does not appear to be due to species specificity since it was shown to binds human TNFR2 efficiently (18,19). The failure of TRAF2 to modulate GM-CSF mRNA induction reflects that NF-κB activation alone may not be sufficient for transcriptional activation of the GM-CSF gene since TRAF2 was clearly shown to activate NF-κB activation in the cell types tested.

TRAF2 binds TNFR1 indirectly via interaction with TRADD (9), which mediates TNFR1-induced apoptosis. Thus TRADD could compete with TNFR2 for TRAF2. We thought that TRAF2 expression could reduce the TRADD protein present in small amounts by direct association, thereby blocking the action of TRADD involved in cell death. We examined, therefore, whether TRAF2 expression could alter TNF-mediated cytotoxicity in murine L929 cell, a TNF-sensitive cell line. TRAF2 expression was observed in transfected L929 cells by immunoblot analysis (data not shown). Simultaneously, the transfected cells were

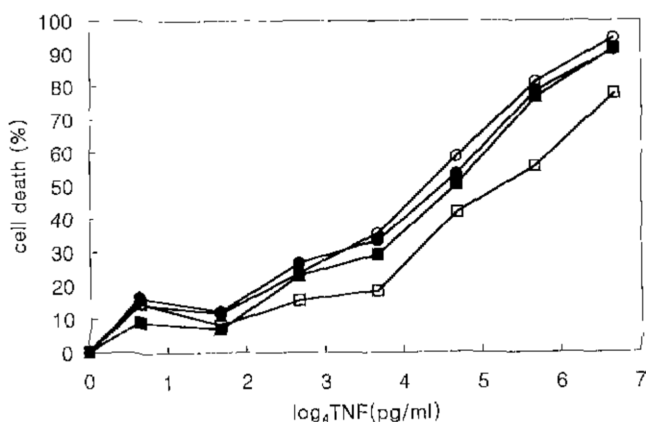

Fig. 4. The cytotoxic effect of TRAF2 expression on L929 cells. L929 cells (6×10^6 cells/60 mm dish) were transfected with TRAF2 (full length: ●–●) or a mutant TRAF2 (Δ2-263: ■–■) construct, incubated for 30 h, detached from the dish by trypsinization, transferred to 96 well microplates at a concentration of 4×10^4 cells/well, and incubated overnight. The transfected cells were exposed to serially diluted rhTNF and incubated in the presence of actinomycin D (5 μg/ml) for 16 h. Then a MTT assay was carried out. The data shown here represent one of four experiments. The symbols, □–□ and ○–○ indicate untransfected cells and vector, respectively.

transferred to a 96-well microplate, and cytotoxic response to TNF was measured in the presence of actinomycin D (5 μg/mL) using a MTT assay. Our result showed that TRAF2-transfected L929 cells displayed a comparable ED_{50} (608 ± 26.7 pg and 416 ± 35.71 pg of TNF for full length TRAF2 and for mutant TRAF2, respectively) (Fig. 4), confirming previous results that TRAF2 as well as the mutant does not affect TNF-induced apoptosis (18). Inability to affect cell death is not likely due to poor expression of TNFR2 in L929 cells because L929 cells are known to express similar levels of both receptors (24). In summary, we firstly demonstrate that TRAF2 may not be involved in TNF-mediated GM-CSF mRNA induction, and ensure the independence of TRAF2 to cytotoxicity.

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