

TNF receptor-associated factor-2 binding site is involved in TNFR75-dependent enhancement of TNFR55-induced cell death

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ABSTRACT

TNF receptor-55 is the main mediator of TNF-induced apoptosis. TNF receptor-75-dependent induction or enhancement of cytotoxicity has been explained by intracellular signaling, "ligand passing", or induction of endogenous TNF. To study the function of human TNF receptor-75 (hTR75) and the interaction between human TNF receptor-55 (hTR55) and hTR75 in hTNF α -induced cytotoxicity, HEp-2 cells were transfected with bicistronic expression vector of hTR75 and its deletion mutants genes. hTNF α -induced cytotoxicity was determined by crystal violet colorimetric method. The expression of hTR75 and its deletion mutants in HEp-2 cells was demonstrated by RT-PCR and indirect ELISA. We found that the overexpressed hTR75 could significantly increase the susceptibility of HEp-2 cells to hTNF α which especially required TRAF2 binding site. hTR75 could not only mediate partial hTNF α -induced cytotoxicity independently but also fulfill an accessory role in enhancing or synergizing hTR55-mediated cytotoxicity.

Key words: *hTNF α , hTNF receptors, TRAF2, cytotoxicity, signal transduction.*

INTRODUCTION

Tumor necrosis factor alpha (TNF α) is a pleiotropic cytokine that is mainly produced by activated macrophages and lymphocytes[1]. TNF α initiates inflammatory, immune regulatory and pathophysiologic responses by binding to two distinct cell surface receptors of TR55 (55 kDa) and TR75 (75 kDa) [2]. Both receptors belong to TNF receptor superfamily because they share 28% homology and contain 4 conserved cysteine-rich subdomain in their extracellular regions[3]. It is the unique structural features of receptors that allow them to recognize TNF with specificity. The ligand binding properties of TR55 and TR75 have been extensively studied. In a recent report the rapid kinetics of TR75 association and dissociation have been taken as a basis to postulate a model termed "ligand passing" in which the ligand bound to TR75 may be passed over to TR55 to enhance TR55 signaling[4].

The intracellular domains of the two TNF receptors have no sequence homology, suggesting distinct biological function[2]. Deletion mutagenesis of TR55 revealed that a death domain[5], which consists of about 80 amino acids residues located to the C-terminal portion of the protein's intracellular region, is responsible for death induction and NF- κ B activation. Intracellular region of TR75 does not possess the death domain or any other domains with intrinsic catalytic activity. An adapter protein, TRAF2 (TNFR-associated factor-2), was found to function as the actual signal transducer in TR75-mediated signaling[6]. The results also indicate that a C-terminal region of 78 amino acids within the cytoplasmic domain of hTNFR-75 comprising amino acids 346-423 is required for signal transduction and TRAF2 binding. Hsu H et al.[7] have shown that interaction of the death domain protein TRADD with TRAF2 is critically involved in TNFR55-dependent activation of NF- κ B.

Thus, the ligand-induced interaction between TR55 and TR75 may have important implications

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in TNF signaling. In order to study the function of human TR75 in hTNF α -induced cytotoxicity in HEp-2 cells, we investigated the cytotoxic effects of the wild-type hTNF α and its mutants (R32WS86T-hTNF α and D143F-hTNF α) on the HEp-2 cells which had been transfected by TR75 gene, TR75 Δ -1 gene (deletion of TRAF2 binding site) or TR75 Δ -2 gene (deletion of intercellular domain) compared with the HEp-2 cells.

MATERIALS AND METHODS

Reagents and kits

Restriction endonucleases, AMV reverse transcriptase, Tag DNA polymerase, dNTP and RNasin were from Roche or Promega; Geneticin (G-418), TRIzol Reagent, Trypsin and Dulbecco's modified Eagle medium (DMEM) were from GIBCO BRL; New-born bovine serum (NCS) was from Hangzhou Sijiqin Biotechnology Company; McAbs to hTR55 or hTR75 were from R and D Systems, UK; HRP-labeled goat anti mouse IgG, cycloheximide (CHX) were from Sigma; Standard MW markers of nucleic acid were from Takara company; Wild-type hTNF α and its mutants (R32WS86T-hTNF α and D143F-hTNF α) were expressed in E.coli and purified in our lab.

Plasmid and cell line

pAD-CMV5-hTR75 (containing hTR75 cDNA) were provided by Dr. Kr?nke M; Expression vector TWOIRES was constructed in our lab; human larynx carcinoma-derived cell line, HEp-2 (Aarhus University, Denmark) was cultured in DMEM containing 10% NCS at 37°C in 95% air + 5% CO₂.

Recombinant Vectors Constructions, Expression and Identification

hTR75 Δ -1 (Δ 346-439) and hTR75 Δ -2 (Δ 276-461) coding regions were amplified by PCR using P1 and P2, P1 and P3 primers respectively.

P1: 5' -GAATGGATCCACCGGTATGGCGCCCGTCGCCGT-3'
P2: 5' -GATCACGCGTCTCGAGTTAGCTCCCGGTGCTGGC-3'
P3: 5' -GATCACGCGTCTCGAGTTATTCTCTCTGCAGGCA-3'

The bicistronic expression vector[8] with EMCV IRES, containing hTR75 gene or its mutants (hTR75 Δ -1 and hTR75 Δ -2) and neoR gene, was constructed and introduced into HEp-2 cells by electroporation. Cells (5×10^6 in 1.0 ml) were transfected with 10 μ g of the plasmid (376 V, 1080 μ F). The next day, G418 was added to a final concentration of 800 mg/ml. About two weeks later, individual colonies were picked up and expanded. Expression of hTR75 and its mutants was monitored by RT-PCR. RNA was prepared by TRIzol Reagent from 2×10^6 cells. After reverse transcription reaction, PCR was carried out with sense primer (CCGCCCAGGTGGCATTAC) and antisense primer (ATACTCGAGTGCCCTGGGGCCA) for hTR75; sense primer P1 and antisense primer P2 for hTR75 Δ -1; sense primer P1 and antisense primer P3 for hTR75 Δ -2. The expression of hTR75

also examined by ELISA. Cells (4×10^4 /well) were seeded into 96-well microtiter plates in 100 μ l of medium and incubated overnight. The cells were then fixed by 0.25% glutaraldehyde, and expression of hTR75 was detected by McAbs to hTR75 and anti-mouse IgG-HRP.

Competitive binding activity of hTNF α with receptors[9]

Wild-type hTNF α was coated in 96-well microtiter plates at 0.2 ~ 1 μ g/well in 100 μ l carbonate buffer (pH 9.6) and incubated overnight at 4°C. After washing and blocking, hTNF α mutein R32W-S86T-hTNF α or D143F-hTNF α were diluted serially in medium containing hsTR55-preS1 or hsTR75-preS1 fusion receptors, and added to the well. After incubation for 1 ~ 2 h at 37°C, HRP labeled anti-HBsAg preS1 McAbs were added. TMB was used as substrate, and optic density was measured at 570 nm.

Cytotoxicity assay

HEp-2, HEp-2-TR75, HEp-2-TR75 Δ -1 and HEp-2-TR75 Δ -2 cells were seeded into 96-well microtiter plates at 4×10^4 /well in 100 μ l medium and allowed to grow for 22 ~ 24 h. Cycloheximide was added to final concentration of 20 mg/l, and wild-type hTNF α or its muteins was added to the wells in serial dilution. The plates were incubated at 37°C. At 16 h, the viable cells were stained with staining buffer (22.3% ethanol containing 0.5% crystal violet, 8% methanol, 7 g/l NaCl) for 1 ~ 2 h. The dye was eluted with 33% citric acid, and absorbance was measured at 595 nm.

RESULTS

Characterization of recombinant hTNF α muteins

Structure-function studies of hTNF α showed that Asp143 is the key residue for binding to hTR55 while Arg32 and Ser86 are to hTR75[17]. In order to understand the accurate cellular responses mediated by hTR55 or hTR75, the hTR75-specific mutant R32W-S86T-hTNF α and the hTR55-specific mutant D143F-hTNF α were expressed in E.coli and purified. Their exclusive specificity for hTR55 or hTR75 was confirmed by the receptor competitive

Tab 1. Competitive binding assays of hTNF α and its mutant to hTR55 and hTR75

Mutants	Relative binding ability (Mutants/wt) (%)	
	hTR55	hTR75
Wt-hTNF α	100	100
R32W-S86T-hTNF α	8.9	< 0.26
D143F-hTNF α	< 0.098	5.5

binding assay[9] (Tab 1).

Expression of *hTR75* in *HEp-2* cells

The expression of *hTR75* in *HEp-2* cells at transcription and translation levels were analyzed by RT-PCR (Fig 1A) and indirect ELISA (Fig 1B), respectively.

Cytotoxicity assay of *HEp-2* and *HEp-2-TR75* cells

As showed in Fig 2 and Tab 2, for *HEp-2* cells, R32W-S86T-*hTNFα* presented a little lower cytotoxicity compared with wild-type *hTNFα*, and D143F-*hTNFα* was not cytotoxic in the presence of cycloheximide. Compared with *HEp-2* cells, *HEp-2-TR75* cells increased their sensitivity to wild-type *hTNFα* more than 10^5 folds while *HEp-2-TR75Δ-*

1 (deletion of TRAF2 binding site) and *HEp-2-TR75Δ-2* (deletion of intercellular region) cells increased their sensitivity to wild-type *hTNFα* about 100 fold. Meanwhile, *HEp-2-TR75* cells increased their sensitivity to the *hTR75*-specific mutant R32W-S86T-*hTNFα* about 100 folds after being treated 12 h together with cycloheximide. However, R32W-S86T-*hTNFα* presented the similar cytotoxicity on *HEp-2-TR75Δ-1*, *HEp-2-TR75Δ-2* and *HEp-2* cells. We also found that Wt *hTNFα* presented similar cytotoxicity on *HEp-2-TR75D-1* and *HEp-2-TR75Δ-2* cells, so did its mutant R32W-S86T-*hTNFα*. Furthermore, cytotoxicity by TR55-specific mutant D143F-*hTNFα* was observed on *HEp-2-TR75* cells in high concentration while D143F-*hTNFα* was not

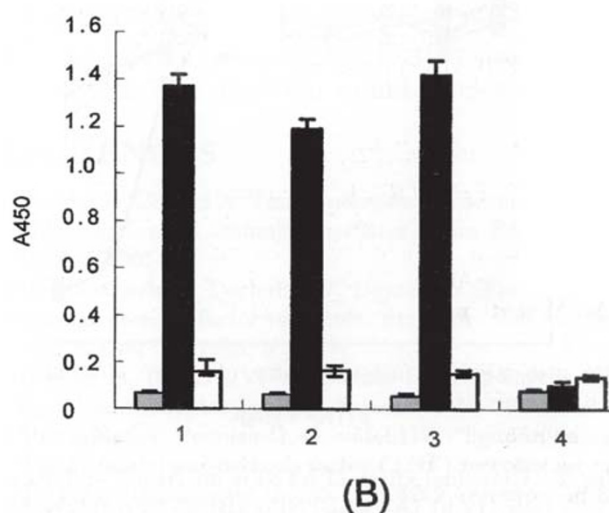
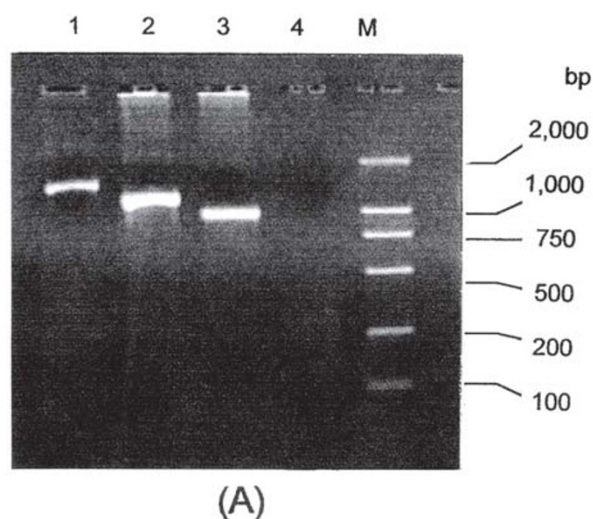


Fig 1. Identification of *HEp-2* cells transfected by *hTNFR75* and its mutants (A) RT-PCR analysis; (B) Indirect ELISA 1, *HEp-2-TR75* cells; 2, *HEp-2-TR75Δ-1* cells; 3, *HEp-2-TR75Δ-2* cells; 4, *HEp-2* cells; M, PCR Marker; ◆, Anti-*hTR55*; ■, Anti-*hTR75*; □, control.

Tab 2. Cytotoxic activity of *hTNFα* and its mutants' on *HEp-2*, *HEp-2-TR75*, *HEp-2-TR75Δ-1* and *HEp-2-TR75Δ-2* cells

	EC50 (g/L)		
	Wt <i>hTNFα</i>	R32WS86T- <i>hTNFα</i>	D143F- <i>hTNFα</i>
<i>HEp-2</i>	$2.24 \pm 0.139 \times 10^{-6}$	$1.58 \pm 0.016 \times 10^{-5}$	$< 10^{-2}$
<i>HEp-2-TR75D-1</i>	$3.1 \pm 0.161 \times 10^{-8}$	$1.26 \pm 0.117 \times 10^{-5}$	$< 10^{-2}$
<i>HEp-2-TR75D-2</i>	$4.68 \pm 0.427 \times 10^{-8}$	$2.0 \pm 0.077 \times 10^{-5}$	$< 10^{-2}$
<i>HEp-2-TR75</i>	$> 10^{-11}$	1.0×10^{-7}	$3.16 \pm 0.025 \times 10^{-4}$

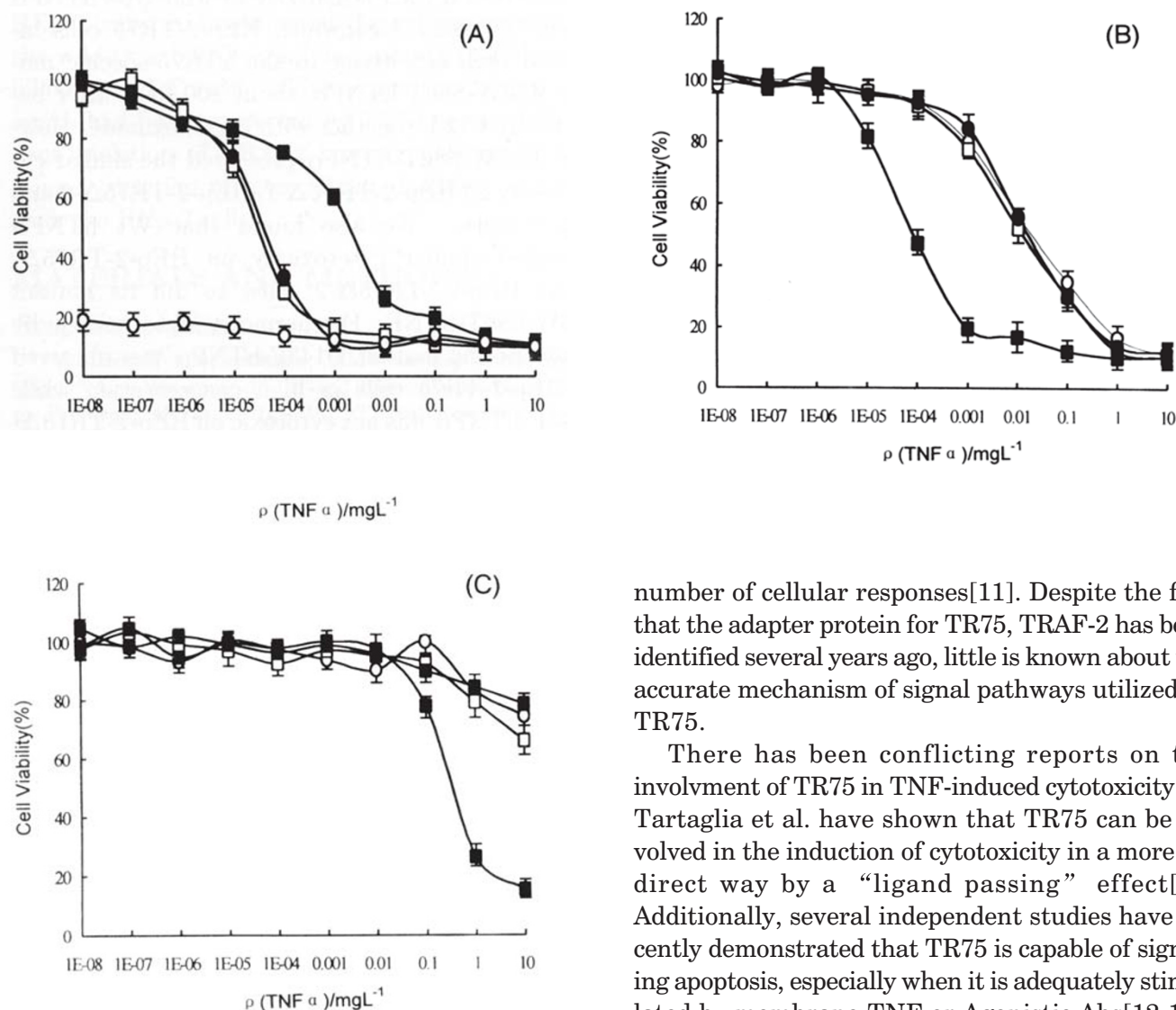


Fig 2. Cytocidal effect of hTNF α on HEp-2 and HEp-2-TR75 cells (12 h) (A) wt-hTNF α (B) hTNF α -R32W-S86T (C) hTNF α -D143F. -○-, HEp-2; -□-, HEp-2-TR75- Δ -1; -●-, HEp-2-TR75 Δ -2; -■-, HEp-2-TR75

cytotoxic on HEp-2-TR75 Δ -1 and HEp-2-TR75 Δ -2 cells.

DISCUSSION

Both TNF receptors, TR55 and TR75, are expressed in the majority of cell types and tissues. However, it is established that TR55 is necessary and sufficient for TNF α -induced cellular responses [10] through interacting with several adapter proteins, such as TRADD, FADD, RIP, etc. In contrast, TR75 appears to mediate only a limited

number of cellular responses[11]. Despite the fact that the adapter protein for TR75, TRAF-2 has been identified several years ago, little is known about the accurate mechanism of signal pathways utilized by TR75.

There has been conflicting reports on the involvement of TR75 in TNF-induced cytotoxicity[4]. Tartaglia et al. have shown that TR75 can be involved in the induction of cytotoxicity in a more indirect way by a "ligand passing" effect[4]. Additionally, several independent studies have recently demonstrated that TR75 is capable of signaling apoptosis, especially when it is adequately stimulated by membrane TNF or Agonistic Abs[12-15]. Costimulation of both receptors resulted in additive effects, meaning that probably both receptors initiate this response by use of the same signal transducer. In fact, it has been shown that TRAF2 is necessary for TR75 as well as for TR55-mediated activation of NF- κ B[7],[16].

HEp-2, a human larynx carcinoma-derived cell line, only expresses human TR55 and is susceptible to the cytotoxic action of hTNF α in the presence of cycloheximide [17]. This made it an ideal model system for studying hTNF α signal pathways. We found that overexpressed hTR75 not only independently mediate cytotoxicity, but also play an accessory role in enhancing or synergizing hTR55-mediated cytotoxicity. However, the cytocidal effect curve of HEp-2-TR75 cells shows that the hTR75-mediated

cytotoxicity requires high concentrations of both ligand and receptor. It is obviously different from that mediated by hTR55. The exact mechanism of this phenomenon is far from well understood. It is possible that hTR75 clustering after binding to hTNF α is far weaker than hTR55 clustering because the intracellular domain of hTR75 lacks the death domain, which has a strong tendency to self-associate[18]. Furthermore, Grell M[19] recently reported that the affinity of binding to hTNF α and the stability of hTNF α -receptor complex for hTR55 are remarkably higher than those for hTR75 under physiological condition (37°C). We think the difference on ligand-receptor binding characteristics between hTR55 and hTR75 may also explain the differential behavior of the two receptors.

Our data also show the hTR75-dependent enhancement of hTR55-induced cytotoxicity. In the Fig 2 and Tab 2 showed that TR75 can enhance the hTR55-mediated cytotoxicity intracellular and extracellular. Because compared with HEP-2 cells, HEP-2-TR75 cells increased their susceptibility to wild-type hTNF α more than 10^5 fold whereas HEP-2-TR75 Δ -2 (expressing TR75 deletion of intercellular domain) increased their susceptibility about 100 fold. On one hand, the “ligand passing” model could explain why HEP-2-TR75 cells are more susceptible by far to the cytotoxic action of wild-type hTNF α than its muteins. On the other hand, the evidence that hTNF α -induced cytotoxicity to HEP-2-TR75 cells was much higher than that to HEP-2-TR75 Δ -2 cells (expressing TR75 deletion of intercellular domain) shows that TR75 enhance the TR55-mediated cell death obviously though its intracellular domain. We also found that HEP-2-TR75 cells increased their susceptibility to R32W-S86T-hTNF α about 100 folds compared with HEP-2 cells. It is possible that unliganded hTR75 could also synergize hTR55-mediated cytotoxicity through its intracellular domain. Our results also showed that TRAF2 binding site was required by TR75-dependent enhancement of TR55-mediated cytotoxicity for both wild-type hTNF α and its mutant R32W-S86T-hTNF α presented similar cytotoxicity on HEP-2-TR75 Δ -1 (expressing TR75 deletion of TRAF2 binding site) and HEP-2-TR75 Δ -2 cells. It is indicated that the TRAF2 binding site plays important role in the cross talk of TR55 and TR75 intracellular.

It has been shown recently that the network of TNF receptors-induced signal transduction is composed of two main parts, each involving a distinct major protein-binding motif that prompts homophilic protein interaction[20]. One part involves several docking proteins, including FADD, TRADD, RIP, and RAIDD, which bind to each other as well as to TR55 and CD95 through a DD motif found both in the docking proteins and in the receptors. The other part is centered around a group of adapter molecules that share a protein-binding motif called the TRAF domain. The two parts of the network are linked through association of the TRAF domain in the adapter proteins TRAF2 with the regions upstream of the DD in the adapter proteins TRADD and RIP [21], [22]. Hsu H[7] reported that TRADD could also interact with TRAF2 and RIP, and thus stimulates pathways leading to activation of NF- κ B. Therefore, TRAF2 might play a key role in TR55 and TR75 interaction. Now we proved that TRAF2 is also involved in the TR75-dependent enhancement of TR55-mediated cell death. Thus when the above and related questions are further clarified, our knowledge of TNF function would be clearer.

REFERENCES

- [1] Beutler B, Cerami A. Tumor necrosis, cachexia, shock and inflammation: A common mediator. *Ann Rev Biochem* 1988; **57**:505-18.
- [2] Vandenabeele P, Declercq W, Beyaert R, Fiers W. Two tumour necrosis factor receptors: Structure and function. *Trends Cell Bio* 1995; **5**:392-9.
- [3] Beutler B, Hufel CV. Unraveling function in the TNF ligand and receptor families. *Science* 1994; **264**:667-9.
- [4] Tartaglia LA, Pennica D, Goeddel DV. “ligand passing” : The 75-kD tumor necrosis factor (TNF) receptor recruits TNF for signaling by the 55-kDa TNF receptor. *J Biol Chem* 1993; **268**:18542-8.
- [5] Tartaglia LA, Ayres TM, Wong GHW, Goeddel DV. A novel domain within the 55 kD TNF receptor signals cell death. *Cell* 1993 **74**:845-53.
- [6] Rothe M, Wong SC, Henzel WJ, Goeddel DV. A novel family of putative signal transducers associated with the cytoplasmic domain of the 75 kDa tumor necrosis factor receptor. *Cell* 1994; **78**:681-92.
- [7] Hsu H, Shu HB, Pan MG, Goeddel DV. TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. *Cell* 1996; **84**:299-308.
- [8] Fang J, Zhu B, Wang DB, Chen CQ, Duch MR, Pedersen FS. Overexpressed human TNF Receptor-75 can independently mediate hTNF α -induced cytotoxicity in BHK-21 Cells. *Acta Biochimica et Biophysica Sinica* 1999; **31**(6):

- 637-42.
- [9] Fang J, Lu F, Chen CQ. Expression and application of hsTR55/Tr75-preS1 fusion receptors. *J Cell Mol Immunol*, 2001; **17**(3):203-9.
- [10] Tartaglia LA, Goeddel DV. Two TNF receptors. *Immunol Today* 1992; **13**:151-3.
- [11] Baker SJ, Reddy EP. Modulation of life and death by the TNF receptor superfamily. *Oncogene* 1998; **17**:3261-70.
- [12] Grell M, P Scheurich, A Meager et al. TR60 and TR80 tumor necrosis factor (TNF)-receptors can indepently mediate cytolysis. *Lymphokine Cytokine Res* 1993; **12**: 143-8. [13] Grell M, Douni E, Wajant H, et al. Related Articles The transmembrane form of tumor necrosis factor is the prime activating ligand of the 80 kDa tumor necrosis factor receptor. *Cell* 1995; **83**:793-802.
- [14] Medvedev AE, Sundan A, Espevik T. Related Articles Involvement of the tumor necrosis factor receptor p75 in mediating cytotoxicity and gene regulating activities. *Eur J Immunol* 1994; **24**:2842-9.
- [15] Bigda J, Beletsky I, Brakebusch C, Varfolomeev Y, Engelmann H, Bigda J, Holtmann H, Wallach D. Related Articles Dual role of the p75 tumor necrosis factor (TNF) receptor in TNF cytotoxicity. *J Exp Med* 1994; **180**:445-60.
- [16] Rothe M, VM Dixit, DV Goeddel. TRAF2-mediated activation of NF-kB by TNF receptor 2 and CD40. *Science* 1995; **269**:1424-7.
- [17] Brockhaus M, Schoenfeld HJ, Schlaeger EJ, Hunziker W, Lesslauer W, Loetscher H. Identification of two types of tumor necrosis factor receptors on human cell lines by monoclonal antibodies. *Proc Natl Acad Sci USA* 1990; **87**: 3127-31.
- [18] Haridas V, Darnay BG, Natarajan K, Heller R, Aggarwal BB. Overexpression of the p80 TNF receptor leads to TNF-dependent apoptosis, nuclear factor-kB activation, and c-Jun kinase activation. *J Immunol* 1998; **160**:3152-62.
- [19] Grell M, Wajant H, Zimmermann G, Scheurich P. The type 1 receptor (CD120a) is the high-affinity receptor for soluble tumor necrosis factor. *Proc Natl Acad Sci USA* 1998; **95**:570-5.
- [20] D Wallach, EE Varfolomeev, NL Malinin et al. Tumor necrosis factor receptor and Fas signaling mechanism. *Annu Rev Immunol* 1999; **17**:331-67.
- [21] Hsu H, Huang J, Shu H-B et al. TNF-dependent recruitment of the protein kinase RIP to the TNF receptor-1 signaling complex. *Immunity* 1996; **4**:387-96.
- [22] Takeuchi M, Rothe M, Goeddel DV. Anatomy of TRAF2. Distinct domains for nuclear-kB activation and association with tumor necrosis factor signaling proteins. *J Biol Chem* 1996; **271**:19935-42.