Effect of Gene Transfer of Tumor Necrosis Factor Receptors into Human Lung Carcinoma Cell Line

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The human lung adenocarcinoma cell line A549 is known to be resistant to tumor necrosis factor alpha (TNF-α)-mediated tumor cell lysis in spite of the expression of 55 kDa TNF receptor (TNF-R55) mRNA and its cell surface protein. In this study, we investigated the mechanism of TNF-α resistance and the role of two types of TNF receptors (TNF-R55 and TNF-R75 (75 kDa TNF receptor)). TNF-R55 or TNF-R75 cDNA was transfected into A549 cells. In addition, a C-terminal deletion mutant of TNF-R75 which lacks the intracellular domain of TNF-R75 was also transfected into A549 cells. We assessed the TNF-α-mediated tumor cell lysis of these transfected clones, and found that the cytotoxic effect increased in transfected clones highly expressing TNF-R55, but not in low-expression clones. As for TNF-R75, the cytotoxic effect of TNF-α was observed in TNF-R75-transfected clones even when expression was low. Furthermore, the cytotoxic effect was also observed in clones transfected with the deletion mutant of TNF-R75, as well as the complete TNF-R75. These results indicate that a certain level of expression of TNF-R55 is necessary for obtaining TNF-α-mediated tumor cell lysis in the absence of TNF-R75. On the other hand, the expression of TNF-R75 strongly induces TNF-α-mediated cytotoxicity through TNF-R55 in the absence of an intracellular signal via TNF-R75.

Key words: TNF-α — TNF receptor — Lung cancer — Cytotoxicity

Tumor necrosis factor alpha (TNF-α), a multifunctional cytokine, was originally described as a polypeptide inducing hemorrhagic necrosis of certain tumors *in vivo*.[1] TNF-α is cytostatic or cytotoxic *in vitro* against a variety of tumor cell lines,[2–4] and it interacts with a wide variety of cell types that express specific receptors on the cell surface. The cDNA for two types of TNF receptors having approximate molecular weights of 55 kDa (TNF-R55) and 75 kDa (TNF-R75) have been isolated.[5–9] Both receptors appear to be expressed in various tissues and to share a considerable homology in extracellular domains. However, the fact that the intracellular regions of the two TNF receptors are completely unrelated in sequence suggests the existence of distinct biological functions.[10] It has been reported that cytotoxicity, which is one of the most characteristic functions of TNF-α, is principally mediated through TNF-R55, which bears the intracellular sequences responsible for programmed cell death (death domain).[11–14] However, recent reports demonstrate that TNF-R75 not having the intracellular death domain also induces TNF-α-mediated tumor cell lysis in some tumor cell lines.[15, 16] Although the mechanism of TNF-α-mediated tumor cell lysis through TNF-R75 is not clear, some models have been proposed.[17–19] Human lung adenocarcinoma cell line A549 is resistant to TNF-α-mediated tumor cell lysis in spite of the expression of TNF-R55 mRNA and its cell

surface proteins.[7, 20, 21] The reason why TNF-resistant cells fail to respond to TNF-α even if they express TNF-R55 on their cell surface is unclear. In this study, we investigated the mechanism of TNF-α resistance and the contribution of TNF-R75 to the cytotoxic effect of TNF-α on A549 cells by transfecting TNF-R55 cDNA, TNF-R75 cDNA, or TNF-R75 deletion mutant cDNA into A549 cells.

MATERIALS AND METHODS

Cells The human lung adenocarcinoma cell line A549 was originally obtained from the Japanese Cancer Research Resources Bank and was maintained in Eagle's minimal essential medium supplemented with 1% nonessential amino acids, 1% L-glutamine, 1% sodium pyruvate, 1% penicillin-streptomycin, and 10% fetal calf serum (MEM/FCS). The human histiocytic lymphoma cell line U937 was obtained from the American Type Culture Collection and was maintained in RPMI 1640 supplemented with 1% nonessential amino acids, 1% L-glutamine, 1% sodium pyruvate, 1% penicillin-streptomycin, and 10% fetal calf serum (RPMI/FCS).
Probes Plasmids bearing the complete coding region of human TNF receptor (TNF-R55 or TNF-R75 cDNA in pUC19) were kindly provided by Dr. H. Loetscher (F. Hoffmann-La Roche Ltd., Basel, Switzerland). A 1.3 kb *Eco*RI-*Eco*RI fragment of the TNF-R55 cDNA was used

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as a TNF-R55 probe.[5–7] A 0.9 kb *Sac*I-*Sac*I fragment of the TNF-R75 cDNA was used as a TNF-R75 probe.[9, 10] Human β-actin probe was used as an internal control.[22]

Vector construction TNF-R55 and TNF-R75 cDNA were cloned in the pRC/RSV eukaryotic expression vector (Invitrogen, San Diego, CA) that contains Rous sarcoma virus long terminal repeat (RSV-LTR) and the neomycin-resistance gene (*Neo*) expressed under the SV40 promoter. For the construction of the TNF-R55 expression vector, TNF-R55 cDNA in pUC19 was digested with *Eco*RI. Because of the lack of an appropriate restriction site for digestion, two pieces of restriction fragment digested with *Eco*RI (1.2 kb and 0.9 kb) were cloned into Bluescript SK+ (Stratagene, La Jolla, CA), respectively. Complete TNF-R55 cDNA was then reconstituted in Bluescript by the partial digestion and ligation method.[23] The reconstituted TNF-R55 cDNA in Bluescript was blunted after digestion with *Not*I and *Xho*I, and inserted into the blunt-ended *Bst*XI site of the pRC/RSV eukaryotic expression vector. In the construction of the TNF-R75 expression vector, TNF-R75 cDNA was digested with *Eco*RI, blunted, and then inserted into the blunt-ended *Bst*XI site of pRC/RSV vector. The C-terminal deletion mutant of TNF-R75 (TNF-R75Del) was generated by digestion of TNF-R75 cDNA with *Eco*RI and *Bcl*I. The restriction fragment was then blunted and cloned into the blunt-ended *Bst*XI site of pRC/RSV expression vector. Thus, the TNF-R75Del mutant receptor consisted of extracellular and transmembrane domains, but lacked the intracellular domain of TNF-R75.

Transfection The expression vectors encoding the TNF-R55, TNF-R75, and C-terminal deletion mutant of TNF-R75 (TNF-R75Del) were transfected into A549 cells. Transfections were performed in 60 mm plates using 10 μg of plasmid DNA per plate by the calcium phosphate precipitation method.[23] After 6 h of exposure, cells were washed 3 times with MEM and added to 4 ml of complete medium. Forty hours after medium exchange, cells were selected in medium containing 800 μg/ml G418 (GIBCO, Grand Island, NY). After 2 weeks of selection, G418-resistant clones were selected randomly from the surviving colonies and used in the following experiments. The mock-transfected clone was established by transfecting the pRC/RSV plasmid without a cDNA insert, and used in the experiments as a control.

Flow cytometry The cells were incubated in phosphate-buffered saline (PBS) with 0.5 m*M* EDTA for 3 min at 37°C, then detached by vigorous pipetting and harvested into complete medium containing 10% FCS. This suspension was centrifuged at 1500 rpm for 3 min at 4°C, and the cells were resuspended (1×10^6 cells/150 μl) in PBS. To examine the expression of human TNF receptors, cells were incubated with monoclonal antibody (mAb) against TNF-R55 (10 μg/ml) (Austral Biologicals Co., San Ramon, CA) or mAb against TNF-R75 (10 μg/ml) (Genzyme, Cambridge, MA) for 30 min at 4°C. The cells were then washed and stained with fluorescein-conjugated goat anti-mouse IgG or goat anti-rat IgG diluted 1:200 in PBS for 30 min at 4°C. Fluorocytometric detection was done with a Coulter Epics XL equipped with an argon-ion laser (Coulter Electronics, Luton, UK).

RNA isolation and northern blot analysis Total RNA was isolated from cell lines by the guanidium thiocyanate-phenol-chloroform extraction method as described by Chomczynski and Sacchi.[24] For northern blotting, 20 μg of each RNA preparation was electrophoresed in a 1% agarose gel containing 1.1 *M* formaldehyde. The RNA was transferred to a positively charged nylon membrane (Boehringer Mannheim, Mannheim, Germany). After UV cross-linking, the filters were immersed in prehybridization buffer composed of 5×SSC (1×SSC is composed of 0.15 *M* NaCl and 15 m*M* sodium citrate), 50% formamide, 0.02% sodium dodecyl sulfate (SDS), 0.1% N-lauroylsarcosine, 2% (W/V) solution of blocking reagent for nucleic acid hybridization (Boehringer Mannheim), and 20 m*M* sodium maleate, pH 7.5 for 3 h at 42°C. Detection of mRNA with [α-^{32}P]dCTP-labeled (using the Multiprime labeling system: Amersham, Buckinghamshire, England) probe was carried out by hybridization in the same prehybridization buffer for 18 h at 42°C. Membranes were washed twice in a solution containing 2×SSC with 0.1% SDS for 15 min at 65°C, followed by washing once in 1×SSC with 0.1% SDS for 30 min at 65°C, and then twice in 0.1×SSC with 0.1% SDS for 15 min at room temperature. The integrated radioactivity of mRNA bands for TNF receptors was detected with a Bio-Image analyzer (BAS 2000, Fuji Photo Film Co., Tokyo).

TNF-α-mediated tumor cell lysis TNF-α-mediated tumor cell lysis was assessed by measuring cytotoxicity against a mock-transfected clone of A549 and TNF receptor gene-transfected clones of A549. Target cells were labeled with $Na_2[^{51}Cr]O_4$ for 2 h. Radiolabeled target cells (5×10^4 cells/well) were mixed with recombinant human TNF-α (1000 U/ml) in 96-well U-bottomed sterile microtiter plates in the presence of cycloheximide at the concentration of 1 μg/ml. After 18 h incubation at 37°C, 100 μl aliquots of supernatants was collected from the wells and counted in a gamma counter. Experimental release was determined from the amount of ^{51}Cr released by the target cells when incubated with TNF-α in the presence of cycloheximide. Spontaneous ^{51}Cr release was determined from target cells in the medium in the presence of 1 μg/ml cycloheximide. Total count was determined from the 1% NP-40 lysate of the target cells. The percentage of specific ^{51}Cr release was calculated as: {(experimental release−spontaneous release)/(total release−spontaneous release)}×100. All tests were performed in triplicate and mean values were calculated.[25]

RESULTS

Construction of expression vectors for TNF receptors and the mutant receptor To analyze the relation between the resistance to TNF-α-mediated tumor cell lysis and the expression of TNF receptors, we constructed expression vectors for two types of TNF receptor (TNF-R55 and TNF-R75) and a C-terminal deletion mutant of TNF-R75 (TNF-R75Del). The structures of the expression vectors are shown in Fig. 1. TNF-R55 or TNF-R75 cDNA was inserted into a multiple cloning site of the expression plasmid pRC/RSV, downstream of RSV-LTR and under its transcriptional control (Fig. 1, A and B). The C-terminal truncated TNF-R75 cDNA was also inserted into the pRC/RSV vector. The mutant receptor (TNF-R75Del) (from which 140 or more amino acids had been removed from the C-terminal end) consisted of extracellular domain and transmembrane domain sequences, but lacked the intracellular domain of TNF-R75 (Fig. 1C). The experiments using TNF-R75 mutant were based on the reports of Bigda et al.[18] and Rothe et al.[26] They showed that a C-terminal region of 78 amino acids within the cytoplasmic domain of TNF-R75, comprising amino acids 346–423, was required for signal transduction. Thus, we deleted the sequences coding from leucine 298 to serine 439 in the TNF-R75 gene.

Expression of TNF receptor genes and the mutant receptor gene in transfected clones Expression of genes from TNF-R55, TNF-R75 or TNF-R75Del cDNA was studied by RNA blot analysis using the *Eco*RI fragment of TNF-R55 extracellular domain sequences as a probe
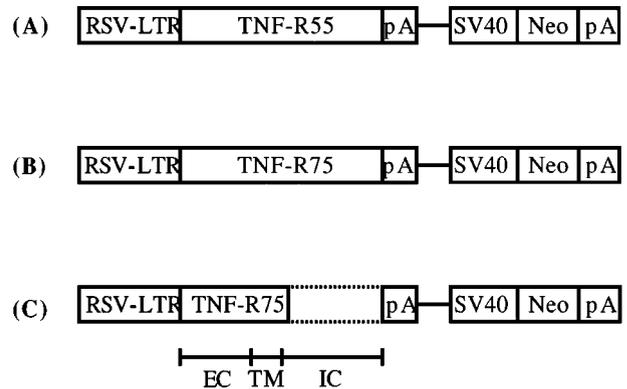

Fig. 1. Structure of the expression vectors. The full-length TNF-R55 (A) or TNF-R75 cDNA (B), or C-terminal truncated *TNF-R75* gene (C) was ligated into the *Bst*XI site of the pRC/RSV expression vector that includes the enhancer-promoter sequences from the Rous sarcoma virus long terminal repeat (RSV-LTR), a polyadenylation signal (pA), the SV40 origin for episomal replication (SV40 ori), and the gene that confers resistance to G418 (*Neo*). The dotted region indicates deleted sequences of TNF-R75 cDNA (C). EC, extracellular domain; TM, transmembrane domain; IC, intracellular domain.
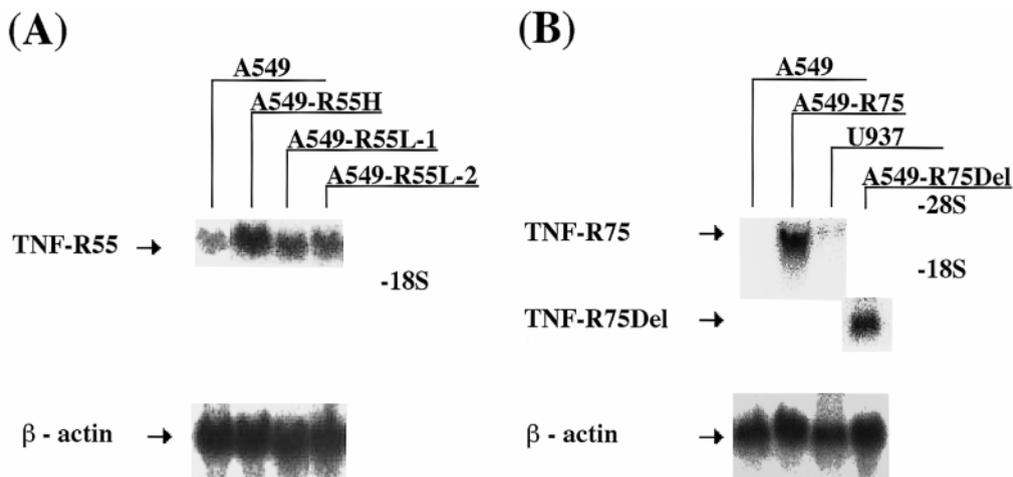

Fig. 2. Northern blot analysis of TNF receptor gene-transfected clones. Total cellular RNA was harvested and subjected to RNA blot analysis using the TNF-R55 probe (A, top panel) or the TNF-R75 probe (B, top panel). The filter was stripped and rehybridized with a probe derived from human β-actin cDNA (A and B, bottom panel) as a control for sample loading. RNA samples were derived from the following cells: lane A549, a cell line from human adenocarcinoma of the lung; lanes A549-R55L-1 and A549-R55L-2 (two representative clones of A549-R55L) and A549-R55H, *TNF-R55* gene-transfected clones from A549 cell line; lane A549-R75, a complete *TNF-R75* gene-transfected clone from A549 cell line; lane U937, a human histiocytic lymphoma cell line; lane A549-R75Del, a C-terminal truncated *TNF-R75* gene-transfected clone from A549 cell line. RNA from the U937 cell line was used as a positive control for TNF-R75 mRNA expression.

for *TNF-R55* gene expression, and the *Sac*I fragment of TNF-R75 extracellular domain sequences as a probe for *TNF-R75* and *TNF-R75Del* gene expression. Total cellular RNA was extracted from wild-type A549 cells and transfected A549 clones. We obtained four clones with low expression of TNF-R55 mRNA (A549-R55L) and one

clone with high expression of TNF-R55 mRNA (A549-R55H) (Fig. 2A). Although TNF-R55 mRNA was detected in wild-type A549 cells, the expression of TNF-R55 mRNA in *TNF-R55* gene-transfected clones of both A549-R55H and A549-R55L was higher than that of wild-type A549 cells (Fig. 2A). As for the expression of *TNF-R75* gene (Fig. 2B), TNF-R75 mRNA was detected in a *TNF-R75* gene-transfected clone (A549-R75) but not in the wild-type A549 cells. Weak expression of TNF-R75 mRNA detected in U937 cells was used as a control.[21] The expression of *TNF-R75Del* gene was also detected in a *TNF-R75Del* gene-transfected clone (A549-R75Del). The molecular size of TNF-R75Del mRNA was smaller than that of TNF-R75 mRNA.

Protein expression of transfected genes was analyzed by flow cytometry (Fig. 3). As for TNF-R55 on the cell surface, its expression in wild-type A549 cells was detected, but only weakly. However, both high and low expressions of TNF-R55 were obtained in *TNF-R55* gene-transfected clones (A549-R55H and A549-R55L, respectively). On the other hand, TNF-R75 was detected on the cell surface of both A549-R75 and A549-R75Del. In spite of the high expression of TNF-R75 and TNF-R75Del
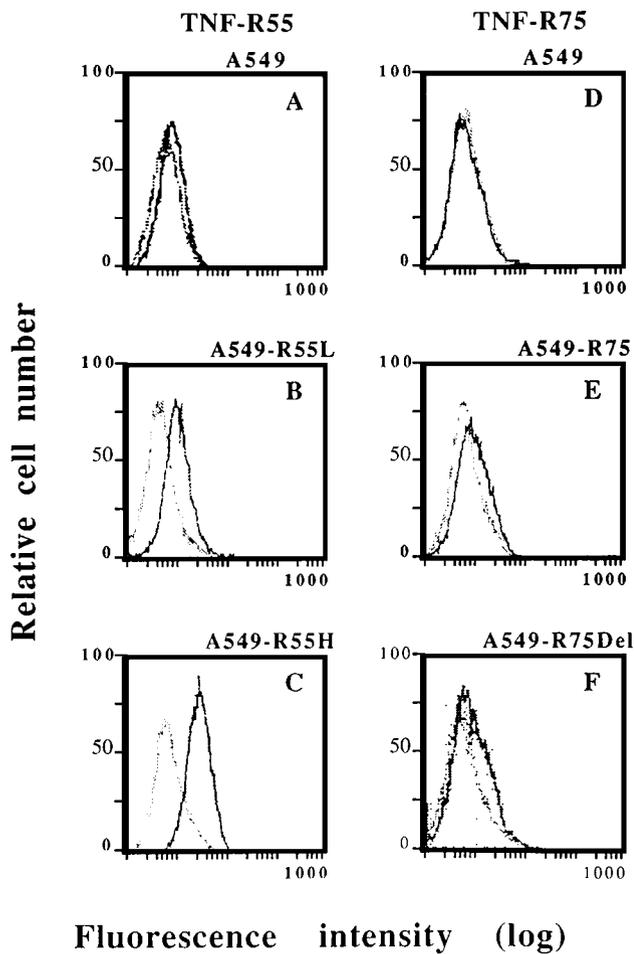

Fig. 3. Flow cytometric analysis of TNF receptor gene-transfected clones. The left column contains histograms from cells stained with anti-human TNF-R55 monoclonal antibody, and the right column contains histograms from cells stained with anti-human TNF-R75 monoclonal antibody. Cells were stained with FITC-conjugated goat anti-mouse IgG or goat anti-rat IgG after treatment with (bold curve) or without (dotted curve) anti-human TNF-R55 or TNF-R75 monoclonal antibody. A and D, wild-type A549; B and C, TNF-R55-transfected A549 clones of A549-55L and A549-55H (A549-55L is one representative clone out of four low-expression clones); E, complete TNF-R75 cDNA-transfected clone; F, C-terminal truncated *TNF-R75* gene-transfected clone. Ten thousand cells were analyzed per sample. Histograms show relative cell number (x-axis) versus log fluorescence intensity (y-axis).
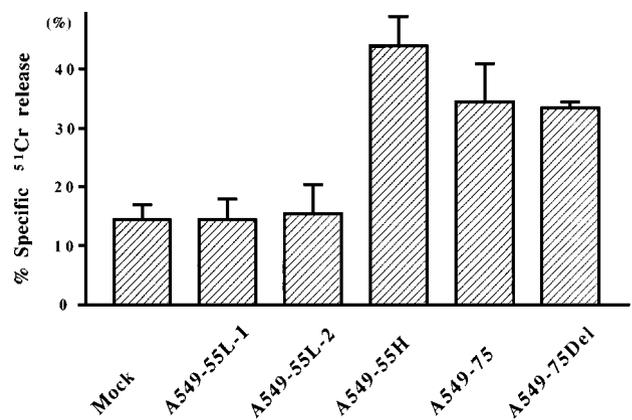

Fig. 4. TNF-α-mediated tumor cell lysis of TNF receptor gene-transfected clones. Ninety-six-well microtiter plates seeded with ^{51}Cr-labeled target cells (5×10^4 cells/well) were treated with or without 1000 U/ml of recombinant human TNF-α for 18 h at 37°C in the presence of cycloheximide (1 μg/ml). Target cells were derived from the following: lane Mock, clone from A549 cell line transfected with the pRC/RSV plasmid without a cDNA insert; lane A549-R55L-1 and A549-R55L-2, two representative clones of *TNF-R55* gene-transfected clones with low expression from the A549 cell line; lane A549-R55H, *TNF-R55* gene-transfected clone with high expression from the A549 cell line; lane A549-R75, a complete *TNF-R75* gene-transfected clone from the A549 cell line; lane A549-R75Del, a C-terminal truncated *TNF-R75* gene-transfected clone from the A549 cell line. All tests were performed in triplicate assays. Results are mean±SEM of three independent experiments.

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mRNA, the expression of the receptor proteins on the cell surface of A549-R75 and A549-R75Del clones was weak. We could not detect TNF-R75 on the cell surface of wild-type A549 cells.

TNF-α-mediated tumor cell lysis of gene-transfected clones We used TNF-α-mediated tumor cell lysis as a biological functional assay for TNF-α. In agreement with previous reports,[7, 20, 21] mock-transfected A549 cells were insensitive to TNF-α. However, wild-type A549 cells became sensitive to TNF-α when TNF-R55 was highly expressed (A549-R55H), but not when it was weakly expressed (A549-R55L) (Fig. 4). On the other hand, the introduction of TNF-R75 (A549-R75) changed the insensitive wild-type A549 cells to cells sensitive to TNF-α-mediated tumor cell lysis. Furthermore, the introduction of a deletion mutant of TNF-R75 (A549-R75Del) also changed the wild-type A549 cells to sensitive cells, and the level of sensitivity to TNF-α-mediated cytotoxicity in the mutant receptor-transfected clone was almost the same as that in the complete *TNF-R75* gene-transfected clone. These results indicate that a high expression of TNF-R55 is necessary for obtaining TNF-α-mediated tumor cell lysis in the absence of TNF-R75. However, transfection of TNF-R75 strongly enhances the sensitivity to TNF-α-mediated cytotoxicity even if its expression is very low, and even without the generation of an intracellular signal through TNF-R75.

DISCUSSION

A human adenocarcinoma cell line, A549, is insensitive to TNF-α-mediated tumor cell lysis, although both expression of TNF-R55 mRNA and TNF-α surface binding to A549 cells are observed.[7, 20, 21] Furthermore, we showed previously that the number of TNF-α binding sites on an A549 cell was half that on TNF-sensitive human lung cancer cells.[21] TNF-R75 mRNA and its cell surface protein were not detected. In this study, we found that TNF-α-mediated tumor cell lysis was observed in the *TNF-R55* gene-transfected clone with high expression, but not in the clone with low expression. This suggests that a certain level of expression of TNF-R55 on the cell surface is required for TNF-α-mediated tumor cell lysis when the cytotoxic responses are triggered by TNF-R55 itself. Thus, one of the TNF-α-resistance mechanisms of A549 cells might be the lack of adequate signal transduction through the cell surface TNF-R55. Previous studies have demonstrated that TNF-R55 is the receptor that signals most of the pleiotropic activities of TNF-α, including cytotoxicity, fibroblast proliferation, and anti-viral activity.[5, 13, 27, 28] The signaling of cytotoxicity is known to be transmitted by the intracellular death domain of TNF-R55, which is conserved with Fas antigen.[14, 29] Signal transduction of TNF-α through TNF-R55 requires the self

association of the death domain induced by TRADD (TNF-R55 associated death domain protein) and the aggregation of TNF receptors, which form a network with TNF-α trimer.[30–33] In this connection, a certain level of TNF-R55 expression on the cell surface may be needed for this network to form.

In contrast to TNF-R55, the role of TNF-R75 in signal transduction of TNF-α is still under investigation.[34, 35] Recently, several reports have provided evidence for the direct signaling of TNF-α-mediated cytotoxicity through TNF-R75.[15, 33, 36, 37] Given the finding that there is a complete absence of homology between the intracellular domains of TNF-R55 and TNF-R75,[10] the mechanism of cytotoxicity through TNF-R75 remains unclear. We have shown in this paper that the expression of TNF-R75 on the cell surface of A549 cells induced a significant enhancement of TNF-α-mediated cytotoxicity, as did the expression of the deletion mutant of TNF-R75. Taking these findings into consideration, we speculate that the contribution of TNF-R75 to the cytotoxic action induced by TNF-α appeared to be in supporting or modulating the ligand interaction with TNF-R55 rather than in cooperating with TNF-R55 in intracellular signaling. Recently, Tartaglia *et al*. proposed a hypothesis for the function of TNF-R75 which they called the ligand passing model.[17] They observed that association and dissociation of TNF-α to TNF-R75 are quite fast, resulting in an increase in local TNF-α concentration in the vicinity of TNF-R55 molecules, and that TNF-R75 increases the rate of association of TNF-α with TNF-R55 by about 10-fold. Thus, according to their ligand passing model, TNF-R75 may play an indirect role in TNF-R55 responses by binding TNF-α and delivering it to the lower affinity TNF-R55. The data obtained in our experimental model using human lung cancer cell line A549 are consistent with the ligand passing model.

In a recent report, a rat/mouse T cell hybridoma (PC60) was transfected either with TNF-R55 or TNF-R75, or with both cDNAs[38]; the TNF-R75 clones showed low or high expression, but the TNF-R55 clones showed low expression only. In contrast, we obtained TNF-R55 clones with both low and high expression, but only low-expression TNF-R75 clones in human lung cancer cell line A549. Although the expressions of TNF-R55 and TNF-R75 are independently regulated,[39, 40] cells of epithelial origin usually express a large excess of TNF-R55, while cells from lymphoid tissue express predominantly TNF-R75 in addition to TNF-R55.[8, 41] The cause of the low translation efficiency of TNF receptors remains unknown. However, the translational control of both TNF receptors may depend on the cell lines or origin of the cells.

In conclusion, we analyzed the mechanism of TNF-α resistance in human lung cancer cells which expressed TNF-R55 on their cell surfaces. We showed that the TNF-

α resistance is due to insufficient cell surface TNF-R55 or to the lack of the contribution of TNF-R75. We previously showed that many primary lung cancer specimens express TNF-R55; however, we could not detect the expression of TNF-R75 protein in any of the lung cancer specimens.[21] Our present study suggests that the transfection of the *TNF-R75* gene rather than the *TNF-R55* gene into human lung cancer cells may effectively induce sensitivity to TNF-α, even if its expression is low.

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