Characterization of a Novel Human Tumor Necrosis Factor- α Mutant with Increased Cytotoxic Activity

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Various novel recombinant human tumor necrosis factor- α (TNF) mutants were prepared using protein engineering techniques, and their cytotoxic activity was compared with that of the intact form of TNF (intact TNF). Mutant 471 (a TNF mutant molecule with the deletion of 7 amino acids at the amino-terminal and the substitution of Pro⁸Ser⁹Asp¹⁰ by ArgLysArg) had a 6-fold higher cytotoxic activity against murine L929 cells. The mutant TNF had an increased ability to bind to TNF receptor on murine L929 fibroblasts cells. A cross-linking study revealed that mutant 471 had an increased ability to form an active trimer. Mutant 471 also showed higher cytotoxic activity against human KYM myosarcoma cells and human MIA PaCa-2 pancreatic carcinoma cells. The possible cachectin activity of the mutant was almost the same as that of intact TNF. These results suggest that mutant 471 might be a more promising candidate as an anticancer agent than intact TNF.

Key words: TNF mutant — Protein engineering — Cytotoxic activity — Receptor binding — Cross-linking

Tumor necrosis factor- α (TNF) was identified by Carswell *et al.*¹⁾ in the serum of mice infected with *Bacillus Calmett-Guerin* and subsequently treated with endotoxin. Since TNF has been found to cause hemorrhagic necrosis of Meth A sarcoma and other transplanted tumors without destroying normal tissues, this substance appears highly promising as an anticancer agent.

The gene for human TNF has now been cloned and expressed in *Escherichia coli*,^{2–6)} making available large quantities of pure TNF. Recent studies using recombinant TNF have revealed that TNF has pleiotropic biological activities.^{7–15)} In clinical trials, TNF was found to have unexpected side effects, which might have been caused by these pleiotropic activities.

Modification of proteins by protein engineering techniques has led to interesting changes in the biological properties of the proteins.^{16–18)} In order to use TNF as an anticancer drug, we are investigating the structure-function relationship of TNF and trying to establish novel TNF mutant molecules having high antitumor activity. We have already synthesized and cloned the gene encoding human TNF.¹⁹⁾ Subsequently, in order to investigate the essential part of TNF for cytotoxic activ-

ity, we prepared mutants which lacked 2, 6, 7, 10 and 12 amino-terminal (N-terminal) amino acids, and compared their cytotoxic activity against L929 cells.²⁰⁾ The mutant which lacked 10 N-terminal amino acids showed a slight reduction in cytotoxicity, and the mutant which lacked 12 N-terminal amino acids completely lacked cytotoxic activity. The others retained the cytotoxic activity. In this group, the mutant which lacked the first 7 amino acids (Val¹-Thr⁷) had the highest cytotoxic activity against L929 cells, 3-fold higher than that of the intact form of TNF (intact TNF). These results suggested that Pro⁸Ser⁹Asp¹⁰ might be an essential structure for the cytotoxic activity. The N-terminal region of TNF contains predominantly amino acids with polar and small hydrophobic side chains. Previously, Soma *et al.*²¹⁾ reported that increase in the basicity of an amino acid at position 10 from the N-terminus broadened the cytotoxic spectrum of TNF. All these findings prompted us to investigate the effect of electric charge changes in the Pro⁸Ser⁹Asp¹⁰ region of TNF on its cytotoxic activity.

In this study, we prepared TNF mutants which had replacements of Pro⁸Ser⁹Asp¹⁰ with basic or acidic amino acids, and compared their cytotoxic activity against L929 cells. One of them, mutant 471 (a TNF- α mutant molecule with the deletion of 7 N-terminal amino acids and the substitution of Pro⁸Ser⁹Asp¹⁰ by ArgLysArg), had an increased cytotoxic activity. Binding ability to TNF receptor, cachectin activity and other characteristics of mutant 471 are also presented.

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MATERIALS AND METHODS

Bacterial strain and cell lines The host used in the present study was *E. coli* C600 r^-m^- (ATCC 33525).

L929 (mouse fibroblasts), RPMI (human colon carcinoma) and HeLa (human uterine cervix cancer) cells were grown in RPMI1640 medium at 37°C under 5% CO₂ in air. McCoy's 5a medium was used to grow ME180 (human cervical carcinoma) and G402 (human renal leiomyoblastoma) cells. PANC-1 (human pancreatic carcinoma), KYM (human myosarcoma), HEL (human embryonic lung, normal diploid fibroblast) and MIA PaCa-2 (human pancreatic carcinoma) cells were cultured in Dulbecco's modified Eagle's medium, DM-160 medium, and Eagle's minimal essential medium, respectively. Each medium was supplemented with 10% fetal bovine serum (FBS).

Chemical synthesis of DNA and cloning techniques Oligonucleotides were synthesized using a Model 380A DNA synthesizer (Applied Biosystems, Foster City, CA). Synthesized oligonucleotides were purified by polyacrylamide gel electrophoresis. The cloning techniques used were as described in the manual by Maniatis *et al.*²²⁾

Purification of TNFs produced by *E. coli* *E. coli* with each expression plasmid was inoculated into M9 medium containing 1% Casamino acids, 1% glucose and 30 mg/ml ampicillin. The cells were cultured at 37°C until the optical density at 600 nm reached 0.7; then, after adding 50 mg/ml of indoleacrylic acid, the culture was continued overnight at 37°C. After the end of this incubation period, the cells were pelleted by centrifugation and disrupted by sonication. Then the cell debris was removed by centrifuging to form lysates. The lysates were applied to an Affi-Gel 10 affinity column (Bio-Rad, Richmond, CA) coupled with an anti-human TNF monoclonal antibody.²³⁾ Mutants and intact TNF were eluted from column with 100 mM citrate buffer (pH 3.0). The purity of TNFs was more than 99% as evaluated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE).²⁴⁾

Cytotoxicity assays Cytotoxic activity was determined by the method of Ruff and Gifford,²⁵⁾ with a minor modification,²⁰⁾ using L929 cells. One unit (U) was defined as the quantity of TNF required to kill 50% of 4×10^4 L929 cells in the presence of actinomycin D (1.0 mg/ml) for 18 h. Cytotoxic activity was assayed against 8 cell lines (KYM, G402, MIA PaCa-2, PANC-1, ME180, HeLa, RPMI, and HEL). Target cells were cultured on a 96-well microplate (1×10^4 cells/0.2 ml/well) containing various concentrations of TNFs for 24 h, and the number of surviving cells in each well was measured by the dye uptake method.²⁶⁾

TNF receptor binding assay Intact TNF was labeled with ¹²⁵I using Enzymobeads (Bio-Rad) at a specific

radioactivity of 422 MBq/mg. Cytotoxic activity against L929 cells of intact TNF after ¹²⁵I-labeling was more than 95% of the initial value (data not shown). The binding of mutant TNF to TNF receptor on L929 cells was then measured by a competitive binding assay using ¹²⁵I-labeled intact TNF according to the method of Yoshie *et al.*²⁷⁾ In brief, ¹²⁵I-labeled intact TNF and various concentrations of unlabeled TNFs were incubated with L929 cells (2×10^5 cells) for 2 h at 4°C. The cells were washed three times with ice-cold medium and solubilized in 0.1% sodium dodecylsulfate solution, then the radioactivity taken up by the cells was determined using a gamma-counter.

Lipoprotein lipase assay Murine 3T3-L1 preadipocytes (ATCC CL173) were grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS. Lipoprotein lipase (LPL) activity was measured as described by Kawakami *et al.*²⁸⁾

Cross-linking of TNFs Intact TNF or mutant 471 was cross-linked at 30°C by incubation with 10 mM dimethyl suberimidate (DMS; Sigma Chemical Co., St. Louis, MO). The reaction was stopped after 30 min by addition of ammonium acetate, and the mixture was subjected to SDS-PAGE. After the electrophoresis, the proteins in the gel were stained with Coomassie Brilliant Blue R-250 (Bio-Rad). The stained gel was subjected to densitometric analysis using a chromatoscanner (Model CS-930, Shimadzu, Kyoto), and the proportion of monomers, dimers and trimers was estimated.

Other methods The N-terminal amino acid sequence was determined with a Model 470A amino acid sequencer (Applied Biosystems). Gel filtration was carried out on a G3000 SW column (TSK-GEL, Tosoh, Tokyo) equilibrated with 20 mM sodium phosphate buffer (pH 7.4) containing 0.5 M NaCl. Isoelectric point was determined by isoelectric focusing (IEF) using a Multiphor II (LKB, Bromma, Sweden). Polyacrylamide gel electrophoresis under nondenaturing conditions (native PAGE) was carried out according to the method of Davis.²⁹⁾

RESULTS

Construction of expression plasmids encoding TNF mutants Expression plasmids containing various TNF mutant genes were derived from the plasmid coding for human intact TNF (pTNF401A)¹⁹⁾ by cassette mutagenesis. In brief, pTNF401A was digested with the restriction enzymes *Cla*I, *Hind*III and *Hap*II to obtain a large DNA fragment and a DNA fragment containing the TNF gene (Fig. 1). The DNA fragments corresponding to the modified part of TNF mutant genes were chemically synthesized. The synthetic oligonucleotides were phosphorylated, annealed and ligated with two DNA fragments previously obtained to construct pTNF

604, 609, 467, 475, 603, 473, 608 and 471. All the mutants commonly have the deletion of 7 N-terminal amino acids. Mutants 604, 609, 467, 475, 603, 473, 608 and 471 have the substitutions of Pro⁸Ser⁹Asp¹⁰ by ProAspAsp, AspAspAsp, ProSerArg, ArgSerArg, ProArgArg, ProLysArg, ArgArgArg, and ArgLysArg, respectively. The mutant numbers refer to those of plasmids constructed above.

From SDS-PAGE and immunoblotting analysis of the lysates of *E. coli* carrying the plasmids encoding TNF mutants, it was demonstrated that all the TNF mutants were produced in soluble forms. Densitometric studies of SDS-PAGE indicated that the quantity of TNF mutants produced by *E. coli* accounted for as much as 20% of the total soluble protein constituents of the *E. coli* cells (data not shown).

Fig. 1. Construction of expression plasmids (A) and DNA sequences of synthetic oligonucleotides used (B). P_{trp}, tryptophan operon promoter region.

B

Mutant no.	Synthetic DNA
604	5'-CGATAATGCCGGACGACAAGCCTGTAGCCCATGTTGTAGCAAACCCCTCAAGCTGAGGGGCAGCTCCAGTGGCTGAACCGC-3' 3'-TATTACGGCCTGCTGTTTCGGACATCGGGTACAACATCGTTTGGGAGTTCGACTCCCCGTCGAGGTCACCGACTTGGCGGC-5'
609	5'-CGATAATGGATGACGATAAGCCTGTAGCCCATGTTGTAGCAAACCCCTCAAGCTGAGGGGCAGCTCCAGTGGCTGAACCGC-3' 3'-TATTACCTACTGCTATTTCGGACATCGGGTACAACATCGTTTGGGAGTTCGACTCCCCGTCGAGGTCACCGACTTGGCGGC-5'
467	5'-CGATAATGCCCTCGAGGAAGCCTGTAGCCCATGTTGTAGCAAACCCCTCAAGCTGAGGGGCAGCTCCAGTGGCTGAACCGC-3' 3'-TATTACGGGAGCTCCTTCGGACATCGGGTACAACATCGTTTGGGAGTTCGACTCCCCGTCGAGGTCACCGACTTGGCGGC-5'
475	5'-CGATAATGCGCTCGAGGAAGCCTGTAGCCCATGTTGTAGCAAACCCCTCAAGCTGAGGGGCAGCTCCAGTGGCTGAACCGC-3' 3'-TATTACGGGAGCTCCTTCGGACATCGGGTACAACATCGTTTGGGAGTTCGACTCCCCGTCGAGGTCACCGACTTGGCGGC-5'
603	5'-CGATAATGCCGGCGCGGAAGCCTGTAGCCCATGTTGTAGCAAACCCCTCAAGCTGAGGGGCAGCTCCAGTGGCTGAACCGC-3' 3'-TATTACGGCGCGCCTTCGGACATCGGGTACAACATCGTTTGGGAGTTCGACTCCCCGTCGAGGTCACCGACTTGGCGGC-5'
473	5'-CGATAATGCCGAAGCGCAAGCCTGTAGCCCATGTTGTAGCAAACCCCTCAAGCTGAGGGGCAGCTCCAGTGGCTGAACCGC-3' 3'-TATTACGGCTTCGCGTTTCGGACATCGGGTACAACATCGTTTGGGAGTTCGACTCCCCGTCGAGGTCACCGACTTGGCGGC-5'
608	5'-CGATAATGCCCGCGCGGAAGCCTGTAGCCCATGTTGTAGCAAACCCCTCAAGCTGAGGGGCAGCTCCAGTGGCTGAACCGC-3' 3'-TATTACGGCGCGCCTTCGGACATCGGGTACAACATCGTTTGGGAGTTCGACTCCCCGTCGAGGTCACCGACTTGGCGGC-5'
471	5'-CGATAATGCGTAAGCGCAAGCCTGTAGCCCATGTTGTAGCAAACCCCTCAAGCTGAGGGGCAGCTCCAGTGGCTGAACCGC-3' 3'-TATTACGCATTCGCGTTTCGGACATCGGGTACAACATCGTTTGGGAGTTCGACTCCCCGTCGAGGTCACCGACTTGGCGGC-5'

Cytotoxic activities of TNF mutants In the initial experiments, we compared the cytotoxic activities of TNFs against L929 cells. As shown in Table I, cytotoxic activity of mutants with the substitutions of Pro⁸Ser⁹Asp¹⁰ by basic amino acids (mutants 467, 475, 603, 473, 608 and 471) was higher than that of mutants with substitutions by acidic amino acids (mutants 604 and 609). In the former group, mutant 471 had the highest cytotoxic activity, 6-fold higher than that of intact TNF.

TNF receptor binding of mutant 471 The binding of mutant 471 to TNF receptor on L929 cells is shown in Fig. 2, together with that of intact TNF. The amount of unlabeled intact TNF required to inhibit 50% of the binding of ¹²⁵I-labeled intact TNF to TNF receptor was 2-fold as much as that of unlabeled mutant 471. This result indicates that mutant 471 has an increased ability to bind to TNF receptor on L929 cells in comparison with intact TNF.

Cytotoxic activity of mutant 471 against human cell lines We compared the cytotoxic activity of mutant 471 and intact TNF using 7 human tumor cell lines (KYM, G402, MIA PaCa-2, ME180, PANC-1, HeLa, and RPMI). Dose-response curves for the cytotoxic action of TNFs against 7 cell lines are shown in Fig. 3. Mutant 471 showed higher cytotoxic activity against KYM myosarcoma cells and MIA PaCa-2 pancreatic carcinoma cells than intact TNF, although the profiles on the other 5 cell lines were similar. Mutant 471 was not cytotoxic to HEL cells, a human lung fibroblast-like normal cell line, as well as intact TNF.

Effect of mutant 471 on LPL activity TNF has been shown to be identical with cachectin, which is presumed to be responsible for cachexia in cancer patients.^{30,31)} So we compared the cachectin activities between mutant 471 and intact TNF. *In vitro* cachectin assay was performed by monitoring the suppression of LPL activity by TNFs.

Table I. Comparison of Relative Cytotoxic Activity of TNF Mutants

Mutant no.	N-Terminal amino acid sequence					Relative activity
	1	7	8	9	10	
Intact	Val-	Thr	Pro	Ser	Asp	1.0 ^{a)}
604			Pro	Asp	Asp	1.1
609			Asp	Asp	Asp	0.5
467			Pro	Ser	Arg	0.8
475			Arg	Ser	Arg	3.5
603			Pro	Arg	Arg	3.9
473			Pro	Lys	Arg	3.3
608			Arg	Arg	Arg	2.4
471			Arg	Lys	Arg	6.0

a) 1.4×10^7 U/mg.

As shown in Fig. 4, the suppression of LPL activity by mutant 471 was similar to that by intact TNF.

Cross-linking of mutant 471 We investigated the structure of mutant 471 and intact TNF in aqueous solution by SDS-PAGE analysis of cross-linked TNFs (Fig. 5). The content of mutant 471 trimer was 4-fold higher than that of intact TNF trimer. This result indicates that mutant 471 has an increased ability to form the trimer in comparison with intact TNF. Cross-linked TNFs migrated as a doublet at the monomer and dimer region. This result may possibly be explained by intramolecular cross-linking.

Physicochemical properties of mutant 471 The characteristics of mutant 471 are summarized in Table II. N-Terminal amino acid sequence analysis of mutant 471 indicated the presence of an N-terminal methionine residue originating from the initiation codon. The amino acid sequence in the N-terminal region was consistent with that predicted from the DNA sequence of pTNF 471. As shown in Fig. 5, the molecular mass of mutant 471 was approximately 17 kilodaltons (kDa) based on SDS-PAGE. On the other hand, the molecular mass, when estimated by gel filtration, was 35–40 kDa (Fig. 6). The isoelectric point was determined to be 8.1, as shown in Fig. 7. Intact TNF had a multiplicity of at least 4 to 5 bands, but mutant 471 showed essentially a single band in IEF (Fig. 7) and native PAGE (Fig. 8).

Fig. 2. Competition of TNFs with ¹²⁵I-labeled intact TNF. L929 cells were incubated with 37 kBq of ¹²⁵I-labeled intact TNF and unlabeled intact TNF (○) or mutant 471 (●) at the indicated molar ratio. After incubation at 4°C for 2 h, the cells were washed, solubilized, and counted in a gamma-counter. Values are the mean of duplicate determinations.

Fig. 3. Dose-response curves of human cell lines to the cytotoxic action of TNFs. Cells were seeded into 96-well microplates at 1.0×10^4 cells/well, and then various concentrations of intact TNF ($\circ$) or mutant 471 ($\bullet$) were added to the wells. After incubation for 24 h, the number of surviving cells was measured by dye uptake methods.²⁶⁾ Values shown are the means of triplicate determinations.

DISCUSSION

We have previously reported that the Pro⁸Ser⁹Asp¹⁰ region might be one of the essential parts of TNF for cytotoxic activity.²⁰⁾ In the present study, we examined the effect of electric charge changes in Pro⁸Ser⁹Asp¹⁰ on cytotoxic activity. Various novel TNF mutants in which Pro⁸Ser⁹Asp¹⁰ had been replaced with amino acids having positive or negative electric charge, and with deletion of

the 7 N-terminal amino acids, were prepared, and their cytotoxic activities were compared with that of intact TNF. The substitution of Pro⁸Ser⁹Asp¹⁰ by amino acids with positive charge greatly increased the cytotoxicity. Mutant 471, which has the deletion of 7 N-terminal amino acids and the substitution of Pro⁸Ser⁹Asp¹⁰ by ArgLysArg, had a 6-fold higher cytotoxic activity than intact TNF against L929 cells. Mutant 471 also showed an increased binding activity to TNF receptors on L929

Fig. 4. Effect of TNFs on LPL activity. The indicated concentrations of intact TNF (○) or mutant 471 (●) were added to 3T3-L1 cultures and the cells were incubated at 37°C for 18 h. After incubation, the LPL activity released into the medium by heparin addition was assayed.²⁸⁾ The data represent the mean $\pm$ SD (n=4).

Fig. 5. Cross-linking of TNFs with DMS. Intact TNF (lanes 3 and 4) or mutant 471 (lanes 1 and 2) was incubated with 10 mM DMS (lanes 1 and 3) or without DMS (lanes 2 and 4) for 30 min prior to quenching by ammonium acetate. SDS-PAGE (10–20% gradient gel) of nonreduced samples containing monomers (a), dimers (b) and trimers (c) are shown. Molecular mass markers are indicated on the right.

Table II. Physicochemical Properties of Mutant 471

N-Terminal amino acid sequence	Met-Arg-Lys-Arg-Lys-Pro-Val-Ala
Isoelectric point	8.1
Molecular mass	17kDa by SDS-PAGE 35–40 kDa by gel filtration

Fig. 6. Gel filtration chromatography of mutant 471. Mutant 471 (100 µg) was injected onto a TSK-Gel G3000 SW column equilibrated with 20 mM sodium phosphate buffer (pH 7.4) containing 0.5 M NaCl. Gel filtration was carried out at a flow rate of 0.2 ml/min. The absorbance at 280 nm was monitored, and the chromatogram was then plotted.

cells, so the greater direct cytotoxicity of mutant 471 was probably due to its higher receptor-binding ability. But the 2-fold difference in binding affinity is not in agreement with the 6-fold difference in cytotoxicity. This disagreement might be accounted for by the difference in experimental conditions or in effects after internalization. Furthermore, mutant 471 showed higher cytotoxic activity against human KYM myosarcoma cells and MIA PaCa-2 pancreatic carcinoma cells, though its cytotoxicity against the other human tumor cell lines tested was similar to that of intact TNF. The difference in the response of tumor cells between mutant 471 and intact TNF might be accounted for by a difference in the species of related receptor molecules or in the pathways of signal transduction. The possible cachectin activity of mutant 471 was almost the same as that of intact TNF. From this result, it seems unlikely that mutant 471 would show less side-effects *in vivo* than intact TNF. But, from the clinical point of view, it is important that the cachectin activity was not increased in parallel with the acquisition of higher cytotoxic activity by mutant 471. It is also noteworthy that the cytotoxicity of mutant 471 on human normal HEL cells was similar to that of intact TNF. Differential responses of cells to TNFs (rep-

Fig. 7. IEF analysis of TNFs. Mutant 471 (lane 1) or intact TNF (lane 2) was analyzed by IEF on an Ampholine PAG plate, pH 3.5–9.5 (Pharmacia) using the LKB Multiphor II electrophoresis system, and the gel was stained with Coomassie Brilliant Blue R-250. Ten micrograms of mutant 471 or 15 μ g of intact TNF was put on each lane. Isoelectric point markers are indicated on the right.

resented by LPL inhibition in adipocytes and cytotoxicity to human tumor cell lines) suggest that it might be possible to design a new TNF with increased cytotoxic activity and without cachectin activity.

The molecular mass of mutant 471 was 17 kDa by SDS-PAGE, but 35–40 kDa by gel filtration, indicating, as in the case of intact TNF,²⁰⁾ that mutant 471 takes an oligomeric form in aqueous solution. In this study, 20 mM phosphate buffer (pH 7.4) containing 0.5 M NaCl was adopted as the equilibration buffer for gel filtration. A buffer containing 0.1 M NaCl was typically used for gel filtration of intact TNF.³²⁾ However, mutant 471 was not eluted from the column when 20 mM sodium phosphate (pH 7.4), 0.1 M NaCl was used (data not shown). These results indicate that mutant 471 may exist as an oligomeric form different from that of intact TNF. It cannot be ruled out that mutant 471 has an increased affinity with the gel filtration column via interaction between the positive charge in the Arg⁸Lys⁹Arg¹⁰ region of mutant 471 and the negative charge of silanol residues

Fig. 8. Native-PAGE analysis of TNFs. Intact TNF (lane 1) or mutant 471 (lane 2) was analyzed by native-PAGE on 4–15% gradient gel, and the gel was stained with Coomassie Brilliant Blue R-250. Fifteen micrograms of intact TNF or 10 μ g of mutant 471 was put on each lane.

on the resin used for gel filtration. The isoelectric point of mutant 471 was much more basic than that of intact TNF. Thus, we could purify mutant 471 to homogeneity from the lysate of *E. coli* carrying pTNF471 by CM-Sepharose CL-6B (Pharmacia, Uppsala, Sweden) chromatography (data not shown). Furthermore, the electrophoretic mobility of mutant 471 was lower than that of intact TNF in native-PAGE. These results may be also due to the increased positive charge in the mutant 471 molecule. Klostergaard *et al.*³³⁾ have demonstrated a strong correlation between the extent of acetylation of TNF and the loss of biological activity. Acetylation of amino groups of TNF probably causes loss of positive charge, and this may account for the lower biological activity of acetylated TNF. Our results partially support their observations. Alzani *et al.*³⁴⁾ have reported that suramin, a polysulfonated naphthylurea, causes dissociation of biologically active TNF trimers into inactive monomers. Suramin is rich in negative electric charge. It is tempting to hypothesize that the negative charge of suramin neutralizes the positive charge of TNF that is important for bioactivity, thereby leading to the inhibition of TNF activity.

The N-terminal amino acid sequence of intact TNF was heterogeneous due to the presence/absence of the N-terminal methionine residue originating from the initiation codon and partial deletion of Val¹Arg².¹⁹⁾ The pro-

portion of intact molecules without N-terminal methionine was more than 80%. N-Terminal amino acid sequence analysis of mutant 471 indicated the presence of the N-terminal methionine residue. However, we do not believe that the presence/absence of N-terminal methionine can explain the difference in activity between mutant 471 and intact TNF. Carlino *et al.*³⁵⁾ have demonstrated that a TNF mutant which lacks the 10 N-terminal amino acids and contains N-terminal methionine shows a lower binding ability to TNF receptor and a lower cytotoxic activity than intact TNF. Intact TNF formed about 4–5 bands in native-PAGE and IEF. This diversity of intact TNF may be due not only to variations in the N-terminal amino acid sequence but possibly also to a factor such as variation in oligomer formation. On the other hand, mutant 471 is more uniform than intact TNF. Several authors have demonstrated that human TNF assumes a compact trimeric structure in aqueous solution and that a trimer is the active form.^{36,37)} A cross-linking study showed that mutant 471 had an increased ability to form

a trimer in comparison with intact TNF. These results suggest that the mutant 471 molecule forms a uniform trimer favorable for binding to TNF receptors.

In conclusion, the present results suggest that mutant 471 might be a superior anticancer agent because of its greater cytotoxic effect as compared with intact TNF, without any increase in the cachectin activity. A next step towards examining the availability of mutant 471 as an anticancer drug will be detailed analysis of its receptor-binding activity with various human cells. We are currently investigating its interaction with the human TNF-R55 receptor³⁸⁾ and the human TNF-R75 receptor.³⁹⁾

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