Extracellular Production of Human Tumor Necrosis Factor- α by *Escherichia coli* Using a Chemically-synthesized Gene

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A DNA fragment of approximately 490 base pairs encoding human TNF was chemically synthesized and expressed within *Escherichia coli* cells. Furthermore, extracellular production of human TNF and several N-terminal deletion mutants of TNF was attempted using the excretion vector pEAP8. The TNF mutant with two N-terminal amino acids deleted (N42-TNF) was efficiently excreted into the culture medium by *E. coli* carrying the plasmid pEXTNF3. In this clone, the signal peptide was correctly processed during the excretion. The *E. coli*-excreted N42-TNF had higher antitumor activity than wild-type TNF or N42-TNF produced intracellularly by *E. coli*.

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 transplantable 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 large quantities of pure TNF available for clinical trials in patients with advanced cancer. However, the TNF which is produced within *E. coli* cells has failed to provide satisfactory anticancer effects when administered alone, particularly by systemic administration. This is largely due to the fact that the dose required for the anticancer effect is very close to the toxic level, and therefore adequate quantities cannot be safely administered. On the other hand, before the availability of recombinant TNF, basic experi-

ments had been done using natural TNF produced by stimulation of animals other than man. In those systems, no toxicity of TNF was observed, and a wide gap between the effective and toxic doses was documented.⁷⁾ Thus, TNF produced within *E. coli* cells and natural TNF display widely different activities, and one reason for this may be differences in the tertiary structures of the two substances.^{7,8)}

We have bred a strain of *E. coli* producing extracellularly the Fc region protein of human immunoglobulin G₁ by weak activation of the *kil* gene, which is present in a dormant state in the plasmid pMB9.⁹⁾ This Fc protein, when produced intracellularly by *E. coli*, assumes a monomeric form.^{10,11)} However, extracellularly-excreted Fc protein is dimeric, joined by disulfide bonds at the hinge region,⁹⁾ and resembles natural Fc protein with respect to its tertiary structure and functional aspects.¹²⁾ The above facts suggest that extracellular production may constitute an effective means of obtaining a suitable tertiary structure for

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microbially-produced proteins.

In this study, a DNA fragment of approximately 490 base pairs (bp) encoding human TNF was designed and chemically synthesized, and then this synthetic gene was expressed intracellularly in *E. coli*. Furthermore, with a view to obtaining recombinant TNF with a tertiary structure closer to that of natural TNF, extracellular production of wild-type TNF as well as TNF mutants with various amino terminus (N-terminus) deletions was attempted. One of these, a TNF mutant with a deletion of the two amino acids at the N-terminus (N42-TNF), was efficiently excreted into the culture medium by *E. coli* cells. The biological characteristics of this excreted N42-TNF were also investigated.

Materials and Methods

Bacterial strains and plasmids. The hosts used in these studies were *E. coli* C600r⁻m⁻ (ATCC 33525) and *E. coli* HB101 (ATCC 33694).

The vector used for intracellular expression was pAA41,¹¹⁾ while pEAP8¹³⁾ was used for extracellular expression.

Chemical synthesis of DNA and cloning techniques. Oligonucleotides were synthesized using a Model 380A DNA synthesizer (Applied Biosystems). Synthesized oligonucleotides were purified by polyacrylamide gel electrophoresis (PAGE).

The cloning techniques used were as described in the manual by Maniatis *et al.*¹⁴⁾

Analysis of TNF gene expression. *E. coli* with extracellular expression plasmids was cultivated at 37°C for at least 24 hr in LB medium (0.5% yeast extract, 1% tryptone, and 1% sodium chloride; pH 7.2). Fractionation of the extracellular, periplasmic, and cytoplasmic fractions of the culture material was done by the method of Kato *et al.*¹³⁾ Each fraction was concentrated by the acetone precipitation method.

E. coli with intracellular expression plasmids was inoculated into M9 medium¹⁴⁾ containing 1% casamino acids, 1% glucose, and 30 µg/ml ampicillin. The cells were cultured at 37°C until the optical density at 600 nm reached 0.7; then, after adding 50 µg/ml of indoleacrylic acid, the culture was continued overnight at 37°C. After the end of this incubation period, the bacteria were pelleted by centrifugation, the cells were disrupted by sonication, and the cell debris was removed by centrifuging to form a lysate, which corresponded to the cytoplasmic fraction of

E. coli harboring extracellular expression plasmids.

The sample fractions thus obtained were electrophoresed on a sodium dodecylsulfate-polyacrylamide gel (SDS-PAGE)¹⁵⁾ at a gel concentration of 15% under reducing conditions, and the proteins in the gel were transferred to nitrocellulose filters by the Western blotting method.¹⁶⁾ The TNF proteins on nitrocellulose filters were specifically stained by the indirect method, using anti-human TNF monoclonal antibody¹⁷⁾ and an Immunoblot kit (Bio-Rad). TNF was also detected by the sandwich solid-phase enzyme-linked immunosorbent assay (ELISA) technique, using two types of anti-human TNF monoclonal antibodies and purified human TNF intracellularly produced by *E. coli* as a control.

Purification of TNF produced by *E. coli*. The culture supernatants or lysates of *E. coli* carrying expression plasmids were put on an affinity column coupled with anti-human TNF monoclonal antibody. TNF was eluted from the column with a 3 M sodium thiocyanate solution (pH 7.0).

The amino acid composition of the purified TNF was analyzed with a PICO-TAG amino acid analysis system (Waters), and the amino acid sequence of the N-terminus was identified with a Model 470A amino acid sequencer (Applied Biosystems).

Assessment of antitumor activity. The *in vitro* antitumor activity of each sample containing *E. coli*-produced TNF was assayed by the method of Ruff and Gifford,¹⁸⁾ using murine L929 cells (ATCC CCL-929). One unit (U) was defined as the quantity of TNF required to kill 50% of a sample of 4×10^4 cells in the presence of actinomycin D in 18 hr.

***In vivo* activity** was assayed by the method of Carswell *et al.*¹⁾ In brief, 5×10^5 murine Meth A sarcoma cells¹⁹⁾ were suspended in 50 µl of RPMI1640 medium (Flow Laboratories) and subcutaneously transplanted to the lateral abdomen of BALB/c mice (male; 6–8 weeks of age) (Charles River). Seven to ten days after transplantation, when the tumor had grown to a diameter of 6–8 mm, *E. coli*-produced TNF was injected into the caudal vein, and the degree of hemorrhagic necrosis in the tumors was assessed after 24 hr by the method of Carswell *et al.*¹⁾

Results

Chemical synthesis of TNF gene and its intracellular expression in E. coli

The synthetic gene encoding human TNF was designed by selecting appropriate codons corresponding to the amino acid sequence of human TNF protein,²⁾ and then constructed by chemically synthesizing this sequence of codons. When designing this synthetic TNF

Fig. 1. DNA Sequence of the Synthetic Human TNF- α Gene.

Arrows indicate the positions of the oligonucleotides 1-17 used to assemble the TNF- α gene. The amino acid sequence and restriction endonuclease cleavage sites are shown above and below the DNA sequence, respectively.

gene, the previously reported DNA sequence of human TNF cDNA²⁾ was regarded as the fundamental structure, and suitable restriction enzyme cleavage sites were established to facilitate subsequent cloning procedures and gene modifications. Also, a translation initiation codon (ATG) and tandem stop codon (TGA-TAG) were added upstream to the 5'-end and downstream to the 3'-end, respectively, so that the resulting reading frames coincided. The DNA sequence of the TNF gene designed

in this manner is shown in Fig. 1.

A DNA fragment consisting of approximately 490 bp was chemically synthesized in separate portions of 17 single-stranded oligonucleotides of approximately 40-70 bases each (Fig. 1). After purification, these synthetic oligonucleotides were kinased, annealed, and ligated separately in three parts. These three blocks were combined by cloning into the expression vector pAA41, thereby forming the TNF gene intracellular expression plasmid,

Fig. 2. Construction of an Intracellular Expression Plasmid.

The phosphorylated oligonucleotides were annealed and ligated to prepare pTNF1BR, pTNF2N, and pTNF3. The ClaI-SalI fragment of pTNF1BR, the SalI-PvuII fragment of pTNF2N, and the PvuII-HindIII fragment of pTNF3 were ligated and inserted between the ClaI and HindIII sites of pAA41 to create an intracellular expression plasmid, pTNF401A.

pTNF401A (Fig. 2).

From SDS-PAGE and immunoblotting analysis of the lysate of *E. coli* C600r⁻m⁻ carrying pTNF401A (*E. coli* C600r⁻m⁻ [pTNF401A]), it was demonstrated that TNF was produced in soluble form within the *E. coli* cells. Densitometric studies indicated that the quantity of TNF produced intracellularly by *E. coli* (hereinafter referred to as cytoplasmic TNF) accounted for as much as 20% of the total soluble protein constituents of the *E. coli* cells. Also, investigation of the *in vitro* antitumor activity of *E. coli* lysates containing cytoplasmic TNF yielded positive results (data not shown).

Construction of extracellular expression plasmids and extracellular production of TNF

Homology searches between human TNF and human lymphotoxin²¹ suggest that the N-terminus of TNF is not directly involved in its antitumor activity. In fact, N42-TNF and N47-TNF produced intracellularly by *E. coli* have antitumor activity.^{3,4,20,21} Moreover, mature human TNF protein is encoded by a region extending into both exons-3 and -4 in the genomic TNF gene,²² but the greater part (starting from Ala-18) is encoded by exon-4,

and the functions of this portion are of principal interest. On the other hand, the signal peptide of the penicillinase produced by the alkalophilic *Bacillus* sp. No. 170 strain used in this study would be expected to be cleaved most efficiently when the N-terminus of the mature protein is a serine residue (the N-terminus of mature penicillinase protein is serine²³). In view of the foregoing considerations, the intracellular bacterial expression plasmid, pTNF401A, was used as a starting material, and extracellular expression plasmids encoding fused proteins containing wild-type TNF, N42-TNF, N47-TNF, and N417-TNF downstream to the penicillinase signal peptide were constructed. Figure 3 shows the methods for the construction of these plasmids.

E. coli HB101 strain cells transformed with the extracellular expression plasmids or the vector plasmid pEAP8 were cultivated, and the extracellular, periplasmic, and cytoplasmic fractions were collected. Immunoblotting analysis of each fraction was done (Fig. 4). The extracellular fraction of *E. coli* HB101 [pEXTNF3] showed a band believed to represent mature mutant TNF, while the cytoplasmic fraction showed a band corresponding to the precursor of the mutant TNF.

Fig. 3. Construction of Extracellular Expression Plasmids (A) and DNA Sequences of Synthetic Oligonucleotides Used (B).

The plasmid pTNF401A was digested with *Ava*I (for pEXTNF4 and pEXTNF3) or *Hap*II (for pEXTNF2), and *Hind*III to obtain a TNF-gene-containing fragment. Oligonucleotides were kinased, annealed, and used as linkers. The DNA fragments containing the TNF gene and synthetic oligonucleotides were ligated and cloned into the *Hind*III site of pEAP8 to construct pEXTNF4, pEXTNF3, and pEXTNF2. Moreover, pEAP8 was digested with *Hind*III and filled in with Klenow fragment to obtain a linear pEAP8 fragment with flush ends. The TNF-gene-containing *Ava*I-*Hind*III fragment cleaved from pTNF401A was also filled in with Klenow fragment and then ligated with the linear pEAP8 fragment to construct pEXTNF1. Ex, Ex promoter; kil, *kil* gene; P, penicillinase promoter region; S, penicillinase signal peptide coding region; *P*_{trp}, tryptophan operon promoter region.

In this clone, the excretion efficiency was estimated about 85% of total TNF mutant protein by measuring the density of the appropriate product band on a nitrocellulose filter. The cytoplasmic fraction of *E. coli*

HB101[pEXTNF1] also showed a band of the precursor of the mutant TNF, although no band was detected in the periplasmic and extracellular fractions. The fractions of the other clones showed no remarkable band in

Fig. 4. Immunoblotting Analysis of Expressed TNF.

E. coli HB101[pEXTNF3] and *E. coli* C600r⁻ m⁻ [pTNF401A] were grown and fractionated. Samples were electrophoresed by 15% SDS-PAGE in the presence of 2-mercaptoethanol and assayed immunologically. Lane 1, cytoplasmic fraction of *E. coli* HB101[pEAP8]; lane 2, periplasmic fraction of *E. coli* HB101[pEAP8]; lane 3, extracellular fraction of *E. coli* HB101[pEAP8]; lane 4, cytoplasmic fraction of *E. coli* HB101[pEXTNF4]; lane 5, periplasmic fraction of *E. coli* HB101[pEXTNF4]; lane 6, extracellular fraction of *E. coli* HB101[pEXTNF4]; lane 7, cytoplasmic fraction of *E. coli* HB101[pEXTNF3]; lane 8, periplasmic fraction of *E. coli* HB101[pEXTNF3]; lane 9, extracellular fraction of *E. coli* HB101[pEXTNF3]; lane 10 and 12, prestained molecular weight marker (Bio-Rad); lane 11, cytoplasmic fraction of *E. coli* C600r⁻ m⁻ [pTNF401A]; lane 13, cytoplasmic fraction of *E. coli* HB101[pEXTNF1]; lane 14, periplasmic fraction of *E. coli* HB101[pEXTNF1]; lane 15, extracellular fraction of *E. coli* HB101[pEXTNF1]; lane 16, cytoplasmic fraction of *E. coli* HB101[pEXTNF2]; lane 17, periplasmic fraction of *E. coli* HB101[pEXTNF2]; lane 18, extracellular fraction of *E. coli* HB101[pEXTNF2]. Each sample corresponds to 300 μ l of the original culture.

Table I. DISTRIBUTION OF TNF EXPRESSED IN *E. coli*^a

Plasmid	TNF coded (N-terminal amino acid)	Activity in Each fraction (U/ml)		
		Cytoplasmic (%)	Periplasmic (%)	Extracellular (%)
pEAP8	—	0	0	0
	(—)	(—)	(—)	(—)
pEXTNF4	Wild-type	1,700	2,000	8,310
	(Val)	(14.1)	(16.6)	(69.3)
pEXTNF3	N42	8,710	15.100	166,000
	(Ser)	(4.6)	(8.0)	(87.4)
pEXTNF1	N47	648	31	75
	(Pro)	(85.9)	(4.1)	(10.0)
pEXTNF2	N417	0	0	0
	(Ala)	(—)	(—)	(—)

^a Amount of TNF in each fraction is shown as *in vitro* antitumor activity.

immunoblotting analysis. Table I shows the distribution of *in vitro* antitumor activity in the fractions. *In vitro* antitumor activity was shown by the culture supernatants from those transformed with pEXTNF3, pEXTNF4, and pEXTNF1, demonstrating that excretion of TNF with the various N-terminal deletions did occur in the *E. coli* carrying these plasmids.

The results shown in Fig. 4 and Table I indicate that *E. coli* HB101[pEXTNF3] was the most effective among these various clones. In *E. coli* HB101[pEXTNF3], about 85% of the mutant TNF protein and nearly 90% of the antitumor activity were detected in the extracellular fraction, and according to the ELISA results, 8.6 mg of the mutant TNF per liter of culture was produced extracellularly by this clone. The various properties of the mutant TNF excreted by *E. coli* HB101[pEXTNF3] were investigated in subsequent experiments.

Physicochemical properties of recombinant TNFs

The cytoplasmic TNF was purified from the lysate of *E. coli* C600r⁻m⁻[pTNF401A] by affinity chromatography using anti-human TNF monoclonal antibody. The relative molecular mass (M_r) of the cytoplasmic TNF as indicated by SDS-PAGE was approximately 17 kirodaltons (kDa) (data not shown), which agrees well with the M_r of 17,356 daltons inferred from the primary structure of the sequence of 157 amino acid residues. On the other hand, after gel filtration, a substance was eluted at the position corresponding to an M_r of about 30–35 kDa, suggesting that the cytoplasmic TNF assumes an oligomeric form in solution. Also, investigation of the amino acid sequence in the vicinity of the N-terminus of the cytoplasmic TNF found three species of TNF molecules; that is, one retaining the terminal methionine residue originating from the initiation codon, a second from which this methionine residue had been cleaved off, and a third lacking the two last residues at the N-terminus. The third molecular species is believed to arise from the action of proteases present in *E. coli* cells. The proportion of this species could be reduced by adding protease inhibitors during the purification process, but in any case, it was shown that the N-termini of the cytoplasmic TNF were not uniform.

The mutant TNF was also purified by affinity chromatography from culture supernates of *E. coli* HB101[pEXTNF3]. The M_r of the *E. coli*-excreted mutant TNF as measured by

SDS-PAGE was approximately 17 kDa, as compared with approximately 35–40 kDa by gel filtration (data not shown), indicating, as in the case of cytoplasmic TNF, a strong possibility that this mutant TNF assumes an oligomeric form in solution. The N-terminal amino acid sequence of the mutant TNF excreted by *E. coli* was H₂N-Ser-Ser-Ser-Arg-Thr-Pro-SerAsp-. This showed that the signal peptide was properly cleaved during the process of excretion, and that mature protein in the form of N42-TNF was extracellularly excreted as anticipated. The amino acid composition of the mutant TNF excreted by *E. coli* (hereinafter referred to as excreted N42-TNF) was mostly consistent with that calculated from the DNA sequence (data not shown).

Fig. 5. Native-PAGE Analysis of Cytoplasmic TNF and Excreted N42-TNF.

Native 4–15% polyacrylamide gel electrophoresis was done, and the gel was stained with Coomassie Brilliant Blue R-250. Lane 1, excreted N42-TNF from *E. coli* HB101[pEXTNF3]; lane 2, cytoplasmic TNF from *E. coli* C600r⁻m⁻[pTNF401A]. About 10 μ g of purified TNF was put on each lane.

Figure 5 shows the results of native-PAGE of cytoplasmic TNF and excreted N42-TNF. Cytoplasmic TNF had a multiplicity of at least 4 to 5 bands, but the pattern for excreted N42-TNF consisted of nearly just a single band. Moreover, in isoelectric focusing (IEF), cytoplasmic TNF displayed several bands in the region of pH 5.4–6.5, while the band pattern of excreted N42-TNF was almost entirely concentrated at the position corresponding to pH 5.7 (data not shown). This again demonstrated that cytoplasmic TNF is not uniform in composition, while excreted N42-TNF has a nearly uniform composition, consisting almost entirely of N42-TNF.

Biological activities of recombinant TNFs

The *in vitro* antitumor activities of purified cytoplasmic TNF and excreted N42-TNF are

Table II. SPECIFIC ACTIVITY OF CYTOPLASMIC TNF, CYTOPLASMIC N42-TNF AND EXCRETED N42-TNF

TNF	Specific activity ^a (U/mg)	Ratio
Cytoplasmic TNF	1.4×10^7	1.0
Cytoplasmic N42-TNF	1.8×10^7	1.3
Excreted N42-TNF	3.3×10^7	2.4

^a *In vitro* antitumor activity to murine L929 cells in the presence of actinomycin D in 18 hr. Each assay was done in triplicate, and the average of six independent assays are shown.

Table III. NECROSIS OF METH A SARCOMA CAUSED *in vivo* BY CYTOPLASMIC TNF, CYTOPLASMIC N42-TNF AND EXCRETED N42-TNF

TNF	Dose (μg/ mouse)	Necrotic response ^a				
		—	+	++	+++	++++
		Number of mice				
Saline	—	5	0	0	0	0
Cytoplasmic TNF	10	0	5	2	0	0
Cytoplasmic N42-TNF	10	0	2	1	0	0
Excreted N42-TNF	10	0	1	3	4	0

^a Tumor necrosis was assessed as follows¹¹: (—) no change, (+) slight necrosis, (++) moderate necrosis, (++++) extensive necrosis.

shown in Table II. Excreted N42-TNF had 2.4 times higher specific activity than cytoplasmic TNF. Furthermore, the intracellular expression plasmid pTNF428A encoding N42-TNF was derived from the plasmid pTNF401A using suitable synthetic oligonucleotides as described previously.²¹ Cytoplasmic N42-TNF was purified from the lysate of *E. coli* C600r[−]m[−] [pTNF428A] and its *in vitro* antitumor activity was measured. The specific activity of cytoplasmic N42-TNF was only half as much as that of excreted N42-TNF, although cytoplasmic N42-TNF had 1.3 times higher specific activity than cytoplasmic TNF.

Ten-micrograms intravenous doses of purified excreted N42-TNF, cytoplasmic TNF or cytoplasmic N42-TNF were administered to mice with transplanted Meth A sarcoma. The resulting degrees of hemorrhagic necrosis are shown in Table III. These results demonstrate that excreted N42-TNF has higher antitumor activity than cytoplasmic TNF and cytoplasmic N42-TNF, not only *in vitro* but *in vivo* as well.

Discussion

An approximately 490-bp DNA fragment encoding human TNF was synthesized chemically. The synthesized TNF gene was designed to facilitate cloning into an expression vector and subsequent genetic modification. An intracellular expression plasmid was constructed by introducing this TNF gene into an expression vector. *E. coli* C600r[−]m[−] transformed with pTNF401A produced TNF in the cytoplasm in the form of a soluble protein, and this TNF reached 20% of the total soluble protein in the cell.

Also, several varieties of plasmids for extracellular expression of the TNF gene with N-terminal deletions were constructed by introducing synthetic TNF genes into an extracellular expression vector. *E. coli* HB101 with a plasmid pEXTNF2 encoding N417-TNF had no detectable antitumor activity. The gene product also could not be detected by immunoblotting analysis in this clone;

therefore, detailed discussion in this case is difficult. Antitumor activity was detected in the culture supernatant of *E. coli* HB101 transformed with extracellular expression plasmids other than pEXTNF2. The productivity was low for *E. coli* HB101 with a plasmid pEXTNF4 encoding wild-type TNF, and the excretion efficiency of this clone could not be estimated due to its low productivity. A thick band corresponding to the precursor form of mutant TNF was detected in the cytoplasmic fraction of *E. coli* HB101 with a plasmid pEXTNF1 encoding N47-TNF, but not in the periplasmic and extracellular fractions, suggesting the low excretion efficiency of this clone. On the other hand, *E. coli* HB101 transformed with plasmids pEXTNF3 encoding N42-TNF was found to produce large quantities of precursor mutant TNF, and about 85% of the mutant TNF protein produced was excreted. About 90% of the antitumor activity was detected in the extracellular fraction in this clone. The amino acid sequence of N42-TNF commences with an N-terminal serine residue, as does the mature penicillinase protein, suggesting that the signal peptide is more readily cleaved in this clone. Furthermore, we constructed the extracellular expression plasmid pEXTNF9 encoding N44-TNF, which had a serine residue at the N-terminus. Also in *E. coli* HB101[pEXTNF9], about 75% of the antitumor activity was detected in the extracellular fraction. In the case of *E. coli* HB101[pEXTNF3], more than 95% of the antitumor activity was detected in the periplasmic and extracellular fractions, although 15% of (precursor) mutant TNF was retained in the cytoplasm. It appears to be due to the low specific activity of precursor mutant TNF compared with mature N42-TNF in periplasmic and extracellular fractions. The other discrepancy between the density of bands in immunoblotting analysis and antitumor activity might be also due to the difference of specific activity of each (precursor) mutant TNF species.

The N-terminus of cytoplasmic TNF was not uniform, and even as regards the amino acid

sequence alone, a mixture of three different molecular species was present. When electrophoresed by native-PAGE and IEF, this cytoplasmic TNF formed about 5–8 bands. This diversity in the composition of the cytoplasmic TNF may be due not only to variations in the N-terminal amino acid sequence but possibly also to factors such as variations in oligomer formation and modification of amino acid side chains. On the other hand, excreted N42-TNF is more uniform than cytoplasmic TNF. The amino acids at the N-termini of a portion of the cytoplasmic TNF are cleaved by proteases, but since excreted N42-TNF lacks the two N-terminal amino acids *ab initio*, this molecule is less susceptible to the effects of proteases. Moreover, the effects of intracellular proteases can also be avoided by inducing extracellular excretion.⁹⁾ Therefore, the uniformity of excreted N42-TNF appears to be synergistically elevated. Regarding the molecular mass calculated from the elution pattern of gel filtration, the results for excreted N42-TNF were in the range of 35–40 kDa, compared with the slightly lower range of 30–35 kDa for cytoplasmic TNF. Recent reports have stated that human TNF assumes a compact trimeric structure in aqueous solutions.^{24,25)} Perhaps the excreted N42-TNF and cytoplasmic TNF molecules are packed in different ways when forming trimers.

Excreted N42-TNF had higher antitumor activity both *in vitro* and *in vivo* than cytoplasmic TNF and cytoplasmic N42-TNF. The higher antitumor activity of excreted N42-TNF cannot be explained solely on the basis of deletion of the two N-terminal amino acids, and appears to be largely due to some optimization of the tertiary structure during the process of excretion. Further studies are under way to define the tertiary structure of excreted N42-TNF by NMR and X-ray crystallography.

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