Note

The Properties of a Tumor Necrosis Factor Mutant with the Deletion of Alanine-14

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Tumor necrosis factor (TNF) is a pleiotropic cytokine produced mainly by activated macrophages. TNF is secreted as an unglycosylated 157-amino-acid polypeptide and is thought to be trimeric in its active form.⁽¹⁾ Lymphotoxin (LT), a product of activated T lymphocytes, is a cytokine related to TNF. Both TNF and LT have similar biological properties in many respects,⁽²⁾ and are recognized by the same cellular receptors.⁽³⁾ The amino acid sequences of the two cytokines show 28% homology,⁽⁴⁾ suggesting the structural and functional importance of the conserved residues. In this paper, we describe the properties of TNF mutant with the deletion of Ala¹⁴, one of the residues conserved between TNF and LT.

pTNF471⁽⁵⁾ is a plasmid encoding mutant 471. Mutant 471 (a TNF molecule with the deletion of 7 amino acids at the amino-terminal and the substitution of Pro⁸Ser⁹Asp¹⁰ by ArgLysArg) has a higher antitumor activity and lower lethal toxicity against normal mice in comparison with intact TNF. Figure 1 shows the construction of the expression plasmid pTNF483 encoding mutant 483, which is mutant 471 with the deletion of Ala¹⁴.

The plasmid pTNF483 was introduced into *Escherichia coli* C600r^{−m} (ATCC 33525) by the procedure of Norgard *et al.*⁽⁶⁾ Culture of this transformant was done as described previously.⁽⁷⁾ Cells were sonicated and centrifuged to obtain soluble and insoluble fractions, and the

distribution of the product was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).⁽⁸⁾ *In vitro* antitumor activity was measured by the dye uptake method, using mouse L929 cells (ATCC CCL-929) as the target cells.⁽⁹⁾

Mutant 471 protein (molecular weight 16,700) was produced by *E. coli* C600r^{−m} harboring pTNF471 (*E. coli* [pTNF471]) as a soluble and active form in the cytoplasm, with a yield of more than 20% of the total cellular protein.⁽⁵⁾ We could detect neither mutant 483 protein nor antitumor activity in the soluble fraction of *E. coli* [pTNF483]. On the other hand, SDS-PAGE of the insoluble fraction of *E. coli* [pTNF483] showed a thick band at 17 kDa (Fig. 2). Immunoblotting analysis using an anti-TNF monoclonal antibody⁽¹⁰⁾ confirmed that this band corresponded to the TNF-related protein, mutant 483 (data not shown). The yield of mutant 483 was estimated to be 20–25% of the total cellular protein by densitometric analysis of SDS-PAGE.

To examine the localization of mutant 483, electron microscopy was done. One or two high-contrast portions per cell were observed by transmission electron microscopy (TEM) and the finding suggested that mutant 483 formed insoluble inclusion bodies in the cytoplasm (Fig. 3A). High resolution scanning electron microscopy (SEM) showed a band-like swelling encircling the bacterial cell (Fig. 3B). From the SEM view of a section of *E. coli* [pTNF483], it was suggested that the band-like swelling corresponded to the position where the inclusion bodies were formed (data not shown). An *E. coli* [pTNF483] cell suspension was centrifuged at low speed (500–1,000 × *g*) after sonication to sediment inclusion bodies selectively (Fig. 3C). Inclusion bodies thus obtained contained more than 95% mutant 483 protein (Fig. 2, lane 4).

We tried to solubilize the inclusion bodies by adding 7 M urea and to refold the mutant 483 protein by dialysis under several sets of conditions (factors examined: pH, salt concentration, and thiol concentration). However, after mutant 483 dissolved in 7 M urea solution, it easily re-aggregated during the refolding process. Antitumor activity could not be determined in the supernatant of a suspension of mutant 483 aggregates.

Mutant 483, in which Ala¹⁴ was deleted from mutant 471, was produced as insoluble inclusion bodies within *E. coli* cells and could not be converted to a soluble and active form. On the other hand, mutant 471 was produced in a soluble form at a yield as high as that of mutant 483. Therefore, we conclude that the formation of inclusion bodies by mutant 483 was due to an intrinsic property of this protein. These suggested that the Ala¹⁴ and its neighboring residues, which are conserved in the primary structure of TNF and LT, were involved in the maintenance

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Fig. 1. Construction of pTNF483 (A) and DNA Sequences of Synthetic Oligonucleotides Used (B).

The plasmid pTNF471 was digested with *Cla*I and *Hind*III. The small *Cla*I–*Hind*III fragment of pTNF471 was then digested with *Hap*II to obtain a *Hap*II–*Hind*III fragment containing the TNF gene. Synthetic oligonucleotides containing the proposed mutation were phosphorylated, annealed, and ligated with the large *Cla*I–*Hind*III fragment of pTNF471 and the *Hap*II–*Hind*III fragment of the TNF gene to yield pTNF483.

of the correct conformation of TNF. Our finding is in agreement with the report of Yamamoto *et al.*¹²⁾ that His¹⁵ assumes an important role in the antitumor activity of TNF. Eck and Sprang¹³⁾ have proposed, on the basis of the three-dimensional structure of TNF obtained by X-ray crystallography, that Lys¹¹ forms an ion pair with Leu¹⁵⁷ of the adjacent subunit in the TNF trimer. Thus, the deletion of Ala¹⁴ or the substitution of His¹⁵ may destabilize the tertiary or quaternary structure of active TNF molecules.

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Fig. 3. TEM View of an Ultrathin Section of *E. coli* [pTNF483] (A), SEM View of *E. coli* [pTNF483] (B), and SEM View of Inclusion Bodies (C).

Cells were fixed with 2% glutaraldehyde and 2% osmium tetroxide, dehydrated by ethanol-series, sectioned after embedding in Quetol 812, and observed with a Hitachi H-800 transmission electron microscope at 75 kV. SEM views were taken using a Hitachi S-900 high resolution scanning electron microscope at 1.5 kV, as described previously.¹¹⁾ Arrows indicate the positions of inclusion bodies. Bars are 1 μ m.

Fig. 2. SDS-PAGE Analysis of *E. coli* [pTNF483] and Inclusion Bodies.

SDS-PAGE was done on 15% gel (lanes 1-3) and 10-20% gradient gel (lanes 4, 5) in the presence of 2-mercaptoethanol, and stained with Coomassie Brilliant Blue R-250. Lanes 1, 5, prestained molecular weight marker (Bio-Rad); lane 2, insoluble fraction of *E. coli* [pTNF483]; lane 3, soluble fraction of *E. coli* [pTNF483]; lane 4, inclusion bodies. Each lane corresponds to 100 μ l of the original culture.

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