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Development of human tumor necrosis factor-α muteins with improved therapeutic potential

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Tumor necrosis factor-α (TNF-α) exhibits cytotoxicity towards various tumor cells *in vitro* and induces apoptotic necrosis in transplanted tumors *in vivo*. It also shows severe toxicity when used systemically for the treatment of cancer patients, hampering the development of TNF-α as a potential anticancer drug. In order to understand the structure-function relation of TNF-α with respect to receptor binding, we selected four regions on the bottom of the TNF-α trimer that are in close contact with the receptor and carried out mutagenesis studies and computational modeling. From the study, various TNF-α muteins with a high therapeutic index were identified. These results will provide a structural basis for the design of highly potent TNF-α for therapeutic purposes. By conjugating TNF-α muteins with a high therapeutic index to a fusion partner, which targets a marker of angiogenesis, it could be possible to develop TNF-α based anticancer drugs. [BMB reports 2009; 42(5): 260-264]

INTRODUCTION

Apoptosis, or programmed cell death, is a set of ordered events that play an essential role in controlling cell number in many developmental and physiological stages. Components of this signaling network, which include ligands, such as TNF-α, TNF-β (also known as LT-α), LT-β, FasL (Apo-1L or CD95L), TRAIL (Apo-2L) and OPGL, as well as downstream molecules, such as caspases and Bcl-2 family members, are crucial targets for drug development and gene therapy of cancer and other diseases (1,2).

Among various apoptosis-related molecules, TNF-α, which is a cytokine produced primarily by activated monocytes and macrophages, is known to be cytotoxic towards various tumor cell lines *in vitro* and can induce apoptotic necrosis in transplanted tumors in mice (3).

The remarkable ability of TNF-α, especially in combination with interferon-γ or melphalan, to inhibit the growth of malignant tumor cells is so far unmatched. Unfortunately, severe toxic effects, such as hypertension, abnormalities in liver function, leukopenia, chills, and thrombus formation were found when wild-type TNF-α was used systemically for the treatment of cancer patients (4-6). An elegant manner to circumvent this problem is the use of isolated limb and liver perfusion therapy for the treatment of melanoma, soft tissue sarcoma and liver tumors, respectively, although the latter method can lead to a reversible hepatotoxicity. The isolated limb perfusion (ILP) therapy of TNF-α along with melphalan, showed a high complete remission rate in patients with melanoma or sarcoma (7,8).

Based on the results of preclinical and clinical trials, it has been estimated that the therapeutic index of TNF-α must increase by about 10-fold before it can be employed as a potential anticancer drug that can be used systemically (9).

One way to increase the therapeutic index is the targeted delivery of TNF-α to the tumor sites. A fusion protein (L19mTNFalpha) composed of mouse TNF-α and a high-affinity antibody fragment (L19scFv) to the extra domain B (ED-B) of fibronectin, a marker of angiogenesis, was reported to selectively target tumor neovasculature in tumor-bearing mice and to show a greater anticancer therapeutic activity than mouse TNF-α (10). Murine TNF fused with a CNGRC peptide (NGR-TNF), which is an aminopeptidase N (CD13) ligand that targets activated blood vessels in tumors, was 12∼15 times more efficient than murine TNF in decreasing the tumor burden in lymphoma and melanoma animal models, whereas its toxicity was similar (9). Therefore, targeting markers that are selectively expressed or upregulated in angiogenic tumor vessels appears to be a promising choice for developing TNF conjugates with improved activity (11).

Another way to increase the therapeutic index is to produce TNF-α muteins that have a higher anti-tumor activity and reduced side effects, for example, by way of removing several amino acids at the amino-terminus and/or inducing random mutagenesis (12-14).

TNF-α exists as a homotrimer and each subunit consists of two antiparallel β-pleated sheets with a jelly roll topology. Various mutation studies have shown that regions corresponding to the lower part of each cleft between subunits are important for receptor binding (14,15). Previously, our laboratory has designed a novel TNF-α mutein that contains substitutions

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at positions 52 and 56 on the bottom region of the TNF-α trimer. This mutein showed a high cytotoxic activity towards various human tumor cells and exhibited anticancer activities against transplanted human tumors in nude mice (16-18).

The diverse activities of TNF are mediated by binding to each of the two receptors, TNF-R55 (55 kDa) and TNF-R75 (75 kDa), on the cell surface. It is known that the TNF and TNF-R55 complex signals various activities, such as cytotoxicity, manganese superoxide dismutase induction, fibroblast proliferation, apoptosis and NF-κB induction (19-22). On the other hand, the TNF and TNF-R75 complex is known to transduce signals for the proliferation of primary thymocytes and T cells (23).

In order to understand the structure-function relation of TNF-α with respect to receptor binding, we have selected four regions on the bottom of the TNF-α trimer that are in close contact with the receptor (Table 1) and carried out mutagenesis study and computational modeling.

RESULTS AND DISCUSSION

The expression of TNF-α muteins in *E. coli* was induced by the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to cultures grown to an OD_{600} of 0.5. The IPTG induction was preceded by heat shock at 42°C for 30 min to produce bacterial GroEL/ES chaperones (24). After 5 h of induction, cells were harvested by centrifugation and resuspended in 10 mM phosphate buffer (pH 7.6). Cells were lysed by ultrasonication and TNF-α muteins were purified from the supernatant using Mono-Q anion-exchange chromatography. The purity was more than 95% as measured by SDS-PAGE and analytical reverse-phase HPLC. The TNF-α muteins prepared in this study are summarized in Table 1. This method, which utilized bacterial GroEL and GroES chaperones, was very effective in producing soluble TNF-α muteins, which otherwise form inclusion bodies. The soluble expression followed by a single-step ion-exchange chromatography dramatically simplified the downstream processes, which deals with the purification of many different mutant proteins.

The cytotoxic activities of purified TNF-α muteins were measured on TNF-sensitive murine fibrosarcoma L929 cells and the results are summarized in Table 2. As shown previously by our laboratory (17), the most dramatic increase in cytotoxic activity arise from the deletion of 7 N-terminal amino acid residues in region 1 and mutations in region 2 (mutein R2-3). Further mutations either in regions 1 and/or 4 in addition to the mutations in region 2 resulted in the decrease of cytotoxic activities. This could be due to the conformational changes induced by introducing additional bulky side chains. Another interesting phenomenon was found when mutations in region 3 were carried out (R3-1 ~ 3). Introduction of charged residues increased the cytotoxic activities 8 ~ 9-fold regardless of the type and number of charges.

The systemic toxicities of wild-type TNF-α and selected muteins were measured using the lethality test, and summarized in Table 3. Compared to the wild-type, R4-1, R4-2, R4-4, R1-2 and R3-1 showed less systemic toxicities. Interestingly, all these mutations have positive charges in either region 1 or 3.

The ratio of the drug dose that produces the desired effects

Table 1. Human TNF-α muteins and their mutated amino acid residues

TNF-α derivatives	Region-1	Region-2	Region-3	Region-4
Wild type	VRSSSRTPSD	SEGLY	AVSYQ	AL
R1-1	PSD	–	–	–
R1-2	RRRTPSD	–	–	–
R2-1	PSD	SEGLF	–	–
R2-2	PSD	IEGLY	–	–
R2-3	PSD	IEGLF	–	–
R3-1	PSD	–	AVKYQ	–
R3-2	PSD	–	AVSEQ	–
R3-3	PSD	–	AVEYE	–
R4-1	RKR	–	–	AF
R4-2	RKR	IEGLF	–	AF
R4-3	PSD	IEGLF	–	DF
R4-4	RKR	IEGLF	–	DF

Table 2. *In vitro* cytotoxic activities of wild-type TNF-α and its muteins on murine fibrosarcoma L929 cell line

TNF-α derivatives	No. of A.A.	Specific activity (units/mg)	Relative specificity
Wild-type	157	6.24×10^6	1.00
R1-1	150	7.49×10^6	1.20
R1-2	154	6.36×10^7	10.2
R2-1	150	1.72×10^7	2.75
R2-2	150	1.86×10^7	2.98
R2-3	150	1.85×10^8	29.7
R3-1	150	5.30×10^7	8.50
R3-2	150	5.80×10^7	9.30
R3-3	150	5.24×10^7	8.40
R4-1	150	5.24×10^7	8.40
R4-2	150	2.50×10^7	4.00
R4-3	150	7.99×10^7	12.8
R4-4	150	6.86×10^6	1.10

Table 3. Acute lethal toxicities of wild-type TNF-α and selected muteins in D-galactosamine-sensitized ICR mice

TNF-α derivatives	LD_{50} (μg/kg)	Relative toxicity
Wild-type	12	1
R1-2	42	0.29
R2-3	3	4
R3-1	21	0.57
R3-2	8	1.5
R3-3	3	4
R4-1	85	0.14
R4-2	30	0.4
R4-3	5	2.4
R4-4	21	0.57

to the dose that causes an undesired effect is referred to as the therapeutic index and indicates the selectivity of the drug and consequently its usability. The relative therapeutic index was calculated as the value of relative specificity divided by relative toxicity. The relative therapeutic index of selected muteins in comparison to the wild-type TNF-α increased as follows; R1-2 (35-fold), R2-3 (7.4-fold), R3-1 (15-fold), R3-2 (6.2-fold), R3-3 (2.1-fold), R4-1 (60-fold), R4-2 (10-fold), R4-3 (5.3-fold) and R4-4 (1.9-fold). Among all the muteins tested, R1-2, R3-1 and R4-1 had significantly high therapeutic indices, more than 10 times higher than that of the wild-type, where R4-1 was nearly 60 times higher than that of the wild-type.

To determine whether the cytotoxic activity or the systemic toxcicity was correlated with the binding energies of TNF-α muteins to the TNF receptor, we calculated the contact energies between the various human TNF-α muteins and the TNF-R55 (55kDa) receptor. Unfortunately, we could not carry out the energy calculations for the TNF-α muteins and TNF-R75 (75kDa) receptor, because the crystal structure of TNF-R75 bound to any of the TNF family members is still unknown.

The calculated contact energy shows good correlation with relative activity (specificity) (Fig. 1A) and relative toxicity (Fig. 1B). Values of relative activity and toxicity reflected the potency of muteins relative to wild-type TNF-α. It is difficult to calculate atomic-detailed interaction energies between proteins in close contact, because the typical potential energy function gives very high energy values in the case of atomic crashes and finding the exact location of side chain atoms is almost impossible with limited computational resources especially without x-ray data. In contrast, residue contact potential, such as the Miyazawa-Jernigan (MJ) potential (29), does not require any atomic details of side chain atoms and only considers neighbor residues within a cutoff distance. Therefore, the contact energy does not depend on side chain atoms and unrealistic high energy values due to atomic crashes of side chains can be avoided. This type of computational mutagenesis can be used to predict the activity of muteins prior to any wet-lab experiments, which ultimately saves time and cost.

TNF-α is an important mediator in inflammation, immune responses and infection-related phenomena and these activities contribute to the severe toxicity seen when TNF-α is used as an anticancer agent. The first step in the mechanism of action is the specific binding of the ligand to its receptor and dissection of the molecular mechanism involved in this interaction is an important step towards the development of strong agonistic and antagonistic TNF-α analogs. First, strong antagonistic TNF analogs can be used to dampen the potentially lethal or debilitating effects of the overproduction of the cytokine (as in septic shock or rheumatoid arthritis). Secondly, a TNF mutein that has retained its cytotoxic activity in tumor cells without inducing deleterious systemic toxicity could be used as a potent anticancer agent.

In the present study, we generated TNF-α muteins by mutating selected regions on the bottom of the human TNF-α trimer. The cytotoxic activities of the TNF-α muteins and the acute lethal toxicities of selected muteins indicate that cytotoxicity, and systemic toxicity presented by acute lethal toxicity, were not directly correlated, and thus, the therapeutic index of TNF-α can be dramatically improved by mutations. These experimental results were different from the contact energy calculations between TNF-α muteins and the TNF-R55 receptor, where contact energies were relatively well correlated with both cytotoxicities and lethal toxicities. These discrepancies may be resolved when the crystal structure of TNF and TNF-R75 become available and contact energy calculations of the TNF-α muteins and the TNF-R75 receptor are carried out.

As shown previously (9, 10), targeting markers that are selectively expressed or upregulated in angiogenic tumor vessels appears to be a promising choice for developing TNF conjugates with improved activity (11). By conjugating TNF-α muteins with a high therapeutic index (as shown in this report) to a fusion partner, which targets a marker of angiogenesis, it could be possible to develop TNF-α based anticancer drugs. In this regard, our findings that the cytotoxic activity of TNF-α

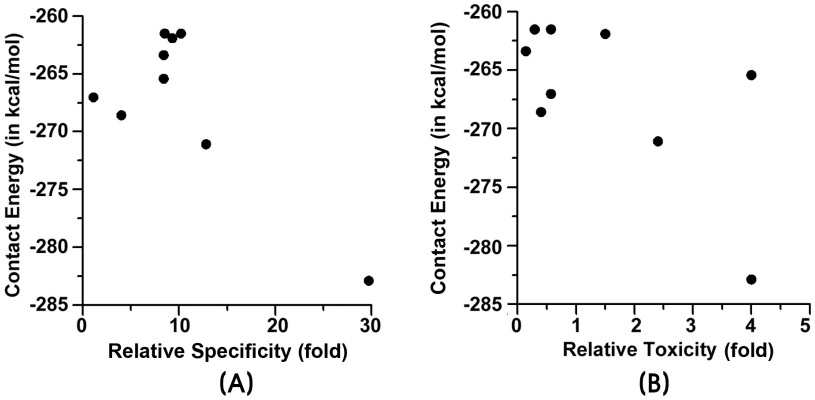

Fig. 1. Correlations between relative specificity and contact energy (A), and between relative toxicity and contact energy (B).

can be significantly increased by mutations in the bottom of the TNF-α trimer will provide a structural basis for the design of highly potent TNF-α for therapeutic purposes.

MATERIALS AND METHODS

Preparation of recombinant human TNF-α muteins

The human TNF-α gene was prepared through polymerase chain reaction (PCR) using Lambda gt11 U937 human monocyte cDNA library (Clontech, Palo Alto, CA, USA) as template DNA. The gene was cloned into plasmid pT7-7 containing a T7 promoter system and the resulting plasmid, pT7-TNF, was introduced into *E. coli* strain BL21 (DE3), which carried the plasmid pOF39 containing the bacterial GroEL and GroES genes (24). The gene encoding the human TNF-α mutein was prepared by site-directed mutagenesis using the plasmid pT7-TNF as a template and cloned into plasmid pT7-7 by the same procedure described above.

Cytotoxicity assay

The cytotoxic activities of TNF-α and its muteins were measured on actinomycin-D treated murine fibrosarcoma L929 cells (ATCC CCL-929) as previously described (25), with some modifications. Briefly, L929 cells were seeded at 1×10^4 cells/well into 96-well microtiter plate in Dulbecco's modified medium containing 2% fetal calf serum. Eighteen hours later, medium containing 2 μg/ml actinomycin-D was added to the cells together with various concentrations of TNFs. The cells were incubated for an additional 18 hr at 37°C. Cell viability was determined by measuring the cellular metabolic activity with a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay (26).

Acute lethal toxicity

Eight weeks-old female ICR mice with an average body weight of 33 g were used for the lethality tests. Several doses of wild-type TNF-α or muteins in 500 μl of saline containing 30 mg of D-galactosamine were administered intraperitoneally (i.p.). Lethality was checked 24 hr after the administration of wild-type TNF-α or muteins, and lethal toxicity was expressed as 50% lethal dose (LD_{50}).

Computational Mutagenesis

Contact energies between various human TNF-α muteins and the TNF-R55 (55kDa) receptor were investigated using computational methods in order to find any correlations between contact energy and relative specificity. The crystal structure of TNF-α (PDBID: 1TNF) (27) was taken from the Protein Data Bank (www.rcsb.org). The monomer structure of TNF-α was converted into the trimer structure by a symmetry operation. Since the crystal structure of the TNF-α:TNF-R55 complex is unknown, the crystal structure of the human TNF-β:TNF-R55 complex (PDBID:1TNR) (28) was used to generated a plausible structure of the TNF-α:TNF-R55 complex. The generated TNF-α:TNF-R55 structure was minimized using the CVFF Force Field and Steepest Minimization method, which was implemented using the Insight II software package (Accelrys Software Inc., San Diego, CA, USA). The various TNF-α muteins:TNF-R55 complexes were generated by manual point mutation using the Insight II software package and the energy minimization method was used to remove any steric hindrances. Using the minimized structures of various TNF-α muteins:TNF-R55 complexes, contact energies were calculated using the MJ potential (29), in order to determine correlations with relative specificity and relative toxicity. The contact energy was calculated by summing the contact energy of the MJ potential between all Cα pairs within the cutoff distance of 6.5Å.

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REFERENCES

1. Los, M., Burek, C. J., Stroh, C., Benedyk, K., Hug, H. and Mackiewicz, A. (2003) Anticancer drugs tomorrow: apoptotic pathways as targets for drug design. *Drug Discov. Today* **8**, 67-77.
2. Gonzalez-Guerrico, A. M., Meshki, J., Xiao, L., Benavides, F., Conti, C. J. and Kazanietz, M. G. (2005) Molecular mechanisms of protein kinase c-induced apoptosis in prostate cancer cells. *J. Biochem. Mol. Biol.* **38**, 639-645.
3. Fiers, W. (1991) Tumor necrosis factor. Characterization at the molecular, cellular and *in vivo* level. *FEBS Lett.* **285**, 199-212.
4. Blick, M., Sherwin, S. A., Rosenblum, M. and Gutterman, J. (1987) Phase I study of recombinant tumor necrosis factor in cancer patients. *Cancer Res.* **47**, 2986-2989.
5. Creaven, P. J., Plager, J. E., Dupere, S., Huben, R. P., Takita, H., Mittelman, A. and Proefrock, A. (1987) Phase I clinical trial of recombinant human tumor necrosis factor. *Cancer Chemother. Pharmacol.* **20**, 137-144.
6. Kimura, K., Taguchi, T., Urushizaki, I., Ohno, R., Abe, O., Furue, H., Hattori, T., Ichihashi, H., Inoguchi, K. and Majima, H. (1987) Phase I study of recombinant human tumor necrosis factor. *Cancer Chemother. Pharmacol.* **20**, 223-229.
7. Lejeune, F. J., Kroon, B. B., Di Filippo, F., Hoekstra, H. J., Santinam, M., Lienard, D. and Eggermont, A. M. (2001) Isolated limb perfusion: the European experience. *Surg. Oncol. Clin. N. Am.* **10**, 821-832.
8. Hundsberger, H., Verin, A., Wiesner, C., Pflüger, M., Dulebo, A., Schütt, W., Lasters, I., Männel, D. N., Wendel, A. and Lucas, R. (2008) TNF: a moonlighting protein at the interface between cancer and infection. *Front Biosci.* **13**, 5374-5386.
9. Curnis, F., Sacchi, A., Borgna, L., Magni, F., Gasparri, A.

and Corti, A. (2000) Enhancement of tumor necrosis factor alpha antitumor immunotherapeutic properties by targeted delivery to aminopeptidase N (CD13). *Nat. Biotechnol.* **18**, 1185-1190.
10. Borsi, L., Balza, E., Carnemolla, B., Sassi, F., Castellani, P., Bernt, A., Kosmehl, H., Biro, A., Siri, A., Orecchia, P., Grassi, J., Neri, D. and Zardi, L. (2003) Selective targeted delivery of TNF-α to tumor blood vessels. *Blood* **102**, 4384-4392.
11. Corti, A. (2004) Strategies for improving the anti-neoplastic activity of TNF by tumor targeting. *Methods Mol. Med.* **98**, 247-264.
12. Zhang, X. M., Weber, I. and Chen, M. J. (1992) Site-directed mutational analysis of human tumor necrosis factor-alpha receptor binding site and structure-functional relationship. *J. Biol. Chem.* **267**, 24069-24075.
13. Van Ostade, X., Tavernier, J., Prangé, T. and Fiers, W. (1991) Localization of the active site of human tumour necrosis factor (hTNF) by mutational analysis. *EMBO J.* **10**, 827-836.
14. Van Ostade, X., Tavernier, J. and Fiers, W. (1994) Structure-activity studies of human tumor necrosis factors. *Protein Eng.* **7**, 5-22.
15. Shin, H. C. and Cho, K. H. (2005) Mutational analysis of human tumor necrosis factor-α. *Biotechnol. Lett.* **27**, 107-112.
16. Seong, J., Maross, C. G., Hunter, N. R., Shin, H. C. and Milas, L. (1997) Potentiation of antitumor efficacy of paclitaxel by recombinant tumor necrosis factor-alpha. *Anticancer Drugs* **8**, 80-87.
17. Shin, N. K., Lee, I., Chang, S. G. and Shin, H. C. (1998) A novel tumor necrosis factor-α mutant with significantly enhanced cytotoxicity and receptor binding affinity. *Biochem. Mol. Biol. Int.* **44**, 1075-1082.
18. Cha, S. S., Kim, J. S., Cho, H. S., Shin, N. K., Jeong, W., Shin, H. C., Kim, Y. J., Hahn, J. H. and Oh, B. H. (1998) High resolution crystal structure of a human TNF-α mutant with low systemic toxicity. *J. Biol. Chem.* **273**, 2153-2160.
19. Engelmann, H., Holtmann, H., Brakebusch, C., Avni, Y. S., Sarov, I., Nophar, Y., Hadas, E., Leitner, O. and Wallach, D. (1990) Antibodies to a soluble form of a tumor necrosis factor (TNF) receptor have TNF-like activity. *J. Biol. Chem.* **265**, 14497-14504.
20. Espevik, T., Brockhaus, M., Loetscher, H., Nonstad, U. and Schalaby, M. R. (1990) Characterization of binding and biological effects of monoclonal antibodies against a human tumor necrosis factor receptor. *J. Exp. Med.* **171**, 415-426.
21. Schalaby, M. R., Sundan, A., Loetscher, H., Brockhaus, M., Lesslauer, W. and Espevik, T. (1990) Binding and regulation of cellular functions by monoclonal antibodies against human tumor necrosis factor receptors. *J. Exp. Med.* **172**, 1517-1520.
22. Suganuma, M., Kuzuhara, T., Yamaguchi, K. and Fujiki, H. (2006) Carcinogenic Role of Tumor Necrosis Factor-α Inducing Protein of *Helicobacter pylori* in Human Stomach. *J. Biochem. Mol. Biol.* **39**, 1-8.
23. Tartaglia, L. A. and Goeddel, D. V. (1992) Two TNF receptors. *Immunol. Today* **13**, 151-153.
24. Jeong, W, Shin, N. K. and Shin, H. C. (1997) Bacterial chaperones increase the production of soluble human TNF-alpha in Escherichia coli. *Biotechnol. Lett.* **19**, 579-582.
25. Creasey, A. A., Doyle, L. V., Reynolds, M. T., Jung, T., Lin, L. S. and Vitt, C. R. (1987) Biological effects of recombinant human tumor necrosis factor and its novel muteins on tumor and normal cell lines. *Cancer Res.* **47**, 145-149.
26. Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **65**, 55-63.
27. Eck, M. J. and Sprang, S. R. (1989) The structure of tumor necrosis factor-alpha at 2.6 A resolution. Implications for receptor binding. *J. Biol. Chem.* **264**, 17595-17605.
28. Banner, D. W., D`Arcy, A., Janes, W., Gentz, R., Schoenfeld, H. J., Broger, C., Loetscher, H. and Lesslauer, W. (1993) Crystal structure of the soluble human 55 kd TNF receptor-human TNF beta complex: implications for TNF receptor activation. *Cell* **73**, 431-445.
29. Miyazawa, S. and Jernigan, R. L. (1996) Residue-residue potentials with a favorable contact pair term and an unfavorable high packing density term, for simulation and threading. *J. Mol. Biol.* **256**, 623-644.