Mini Review

Development of new anti-TNF therapy

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We have generated the first TNFR1-selective antagonistic TNF mutant based on structural human TNF variants using our phage display technology. This TNF mutant did not activate TNFR1-mediated responses, although its affinity for TNFR1 was equivalent to human wild-type TNF (wtTNF). The TNF mutant neutralized wtTNF-induced TNFR1-mediated bioactivity without influencing TNFR2-mediated bioactivity. In hepatitis mouse models, the antagonistic TNF mutant significantly blocked liver injury caused by inflammation. These results indicate that antagonistic TNF mutants may be clinically useful for anti-TNF therapy and that phage display libraries of protein ligands can be used to select for receptor subtype-selective antagonists.

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Inflammation is induced by physiological and chemical stimulation and is known to be mediated by the association of many biological factors. Inflammation-mediating proteins, typified by cytokines and chemokines, act in the host defense system by stimulating lymphocytes, macrophages, and endothelial cells to heal external injuries[1]. When a productive balance of these mediators collapses, inflammatory exacerbation occurs. Long-term over-expression of cytokines causes autoimmune disease[2]. Thus, development of therapeutic techniques to remedy the imbalance of cytokine production is necessary.

Tumor necrosis factor-α (TNF) is a major inflammatory cytokine and has a central role in host defense and inflammation[3]. To exert its biological function, TNF binds to two receptor subtypes, TNFR1 and TNFR2, which form homotrimers by preassembling on the cell surface[4]. Deregulation of TNF production promotes TNF-dependent pathologies and correlates with the severity and progression of inflammatory diseases such as rheumatoid arthritis (RA)[5], inflammatory bowel disease[6], septic shock[7] and hepatitis[8]. TNF blocking agents (monoclonal antibodies or soluble receptors) have shown significant clinical efficacy in certain inflammatory diseases. The major impact of TNF blocking agents on the immunological system, however, raises some concerns about the safety of this approach, especially with regard to severe infections[9], malignancies[10] and immune-mediated diseases[11]. For example, in rheumatoid arthritis and Crohn's disease, studies indicated a higher incidence of tuberculosis reactivation[12] and the induction of demyelination[13].

Although the distinction between the role of TNFR1 and TNFR2 on the immune system remains unclear, TNF secreted from activated immune cells in these diseases predominantly

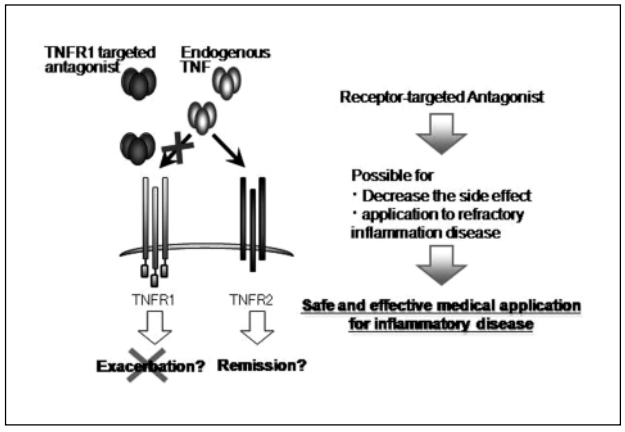

Fig.1 Generation antagonistic protein mutant for receptor targeting

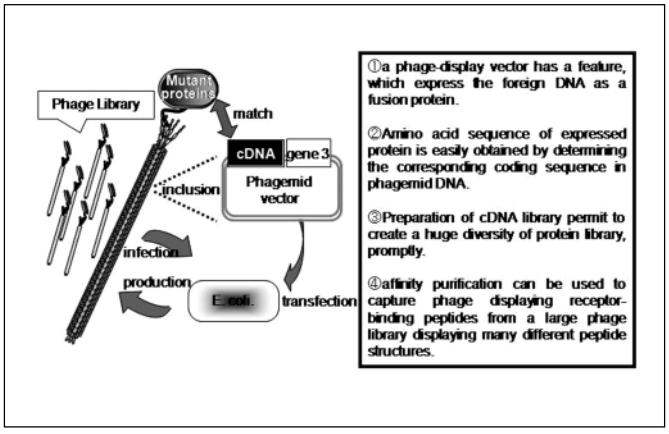

Fig.2 Benefit for engineering protein library using phage display system

activates TNFR1 and accelerates inflammation. In addition, previous studies using animal models of diseases such as arthritis[14] and hepatitis[15] indicated that mainly TNFR1 caused development and exacerbation of inflammation. Moreover, given that in mice lacking the TNFR1 the clinical course of EAE is suppressed both at the pro-inflammatory and the autoimmune phases, the TNFR1 is clearly indicated as an important target for therapy[16]. From this perspective, blocking TNFR1 signal transduction may emerge as a powerful and effective therapy for certain inflammatory diseases (Fig.1).

To develop receptor-selective protein ligands, several studies have described useful mutant proteins created by the substitution of amino acids using a site-directed mutagenesis method, as typified by Kunkel's method[17,18]. It is difficult, however, to obtain an exhaustive and functional panel of protein mutants using this mutagenesis method. Alternatively, the phage display system is a powerful *in vitro* technique that enables polypeptides with desired properties to be selected from a large collection of variants encoded by cDNAs in phagemid vectors (Fig.2). Filamentous phage display of peptide or protein variants has been widely used for rapid selection of protein variants that bind with improved affinity and specificity to target molecules[19]. The key feature of such selection schemes is that the genotype of a particular variant packaged inside a virion particle is linked to the phenotype of a displayed protein or peptide that has been fused to phage coat proteins, i.e., the gene III protein. Phage particles can be selected by binding to an affinity matrix propagated in E. coli and identified by DNA sequencing. These procedures allow phage libraries to be subjected to a selection step, called "affinity panning". Recovered clones are identified by sequencing and re-grown for further rounds of selection.

Using the phage display system, we previously isolated a lysine-deficient TNF mutant from a protein library in which all six lysine residues in the TNF molecule, including the receptor-binding site, were simultaneously replaced with other amino acids[19,20]. This strategy created novel mutant TNFs that exhibited only a slightly different mode of receptor-binding. In the present study, we used the phage display system to isolate novel TNFR1-selective antagonistic TNF mutants that efficiently inhibited a wide variety of TNFR1 mediated effects *in vitro* and *in vivo* without affecting TNFR2-mediated bioactivity.

The selection of amino acids to be altered was based on data from a point mutation study and a TNF structure-function study. Residues (amino acids 89-94) that were shown to contribute to TNFR binding were mapped onto the three-dimensional structure of human TNF. Then, these and other nearby residues were selected for randomization to generate phage libraries (Fig.3). Randomization of each of these residues was performed by PCR with mutated primers in which an NNS codon was incorporated at each randomized position. Each library contained a total of six randomized residues.

To select TNF mutants from phage library that bound strongly to human TNFR1, the mutant TNF phage library was panned against human TNFR1. As a result, we identified ten candidates as TNFR1-selective antagonists and selected the most suitable mutant that possessed the strongest antagonistic activity. To investigate the properties of this antagonistic clone, we examined the binding kinetics and binding specificities of this mutant for TNFR1 and TNFR2 using BIAcore and ELISA techniques, respectively. The antagonistic TNF mutant had an affinity for

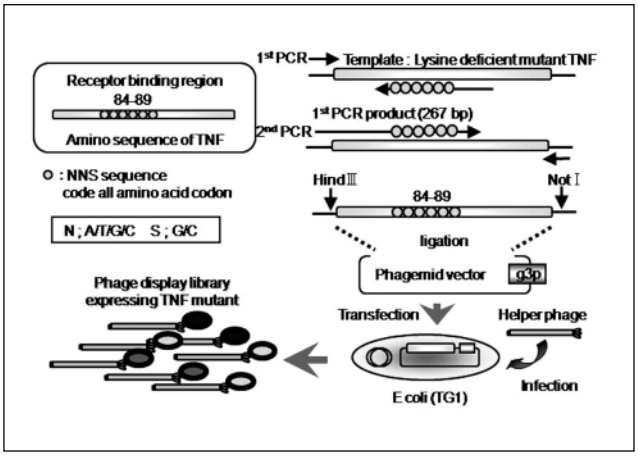

Fig.3 Engineering TNF mutant phage display library

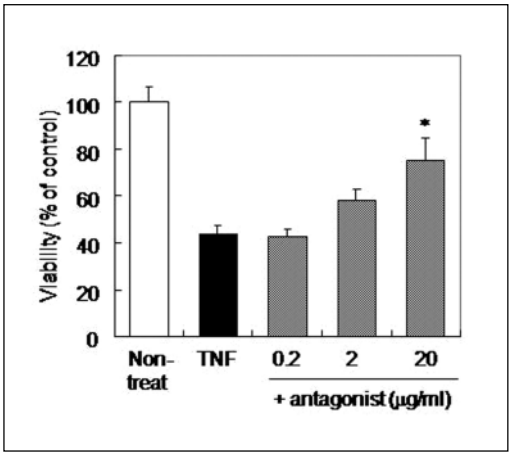

Fig.4 Inhibitory effect of antagonistic mutant TNF on TNF-induced cytotoxicity

Mouse fibrosarcoma L-M cells were treated with wild-type TNF (10 ng/ml). and serial diluted mutant TNF. After 48 hr incubation, ratio of cell death were determined by methylene blue assay.

TNFR1 equivalent to wtTNF, but almost no affinity for TNFR2. We also measured the bioactivity of the TNF mutant via TNFR-mediating response assays. The antagonistic TNF mutant bound to TNFR1 but did not transmit the death signal. To determine the ability of the TNF mutant to compete with wtTNF, we measured TNFR1-selective responses in the presence of both wtTNF and TNF mutant. The antagonistic TNF mutant inhibited wtTNF-induced cytotoxicity (Fig.4), caspase activation, and NF-κB activation through TNFR1 in a dose-dependent manner. These results suggest that the antagonistic TNF mutant is a competitive antagonist, inhibiting TNFR1-mediated pathways.

For the therapy of autoimmune disease, TNF blockades (etarnercept, as p75-IgG Fc fusion protein and lenercept as p55-IgG Fc fusion protein) have been developed. However, differences exist in the mechanisms of action of these agents that might confer risks of infection and immunogenisity. There are some reports that tuberculosis disease is a potential adverse reaction from treatment with etanercept. Moreover, antibody formation against lenercept was a significant problem which resulted in significant reduction of the half-life of the receptor. Thus, much is expected from the development of TNF receptor-selective agents that inhibit disease-causing TNF bioactivity without interfering host defense system against infection and antibody formation. In the present report, we generated a receptor-selective antagonistic TNF mutant through the use of phage display. However, there is a possibility of expressing the new function, which binds to another receptor like as TNF receptor superfamily. Therefore, the reasons of showing agonistic or antagonistic activity should be examined via structural analysis of binding sites. We are now analyzing the crystal structures of the complex formed between the antagonistic TNF mutant and TNFR1 so as to better understand the mechanisms of receptor subtype-selectivity.

While the functions of TNF and its receptors are unclear, their signaling specificities are being examined in many TNF-related studies. In this review, we studied mutant TNF antagonist that bound selectively to TNFR1. The findings from our TNFR1 and TNFR2 study are applicable to the receptors in the TNFR superfamily that do not contain a cytoplasmic death domain. However, we also have produced TNF agonist that binds to TNFR1 and TNFR2. These selective agonists and antagonists are not only therapeutically useful, but also are effective analytical tools for elucidating TNF receptor function. Further functional studies of TNF receptors could uncover interesting receptor biology and may yield additional targets for immunotherapy.

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References

1) Tracey KJ, Cerami A: Tumor necrosis factor: a pleiotropic cytokine and therapeutic target. Annu Rev Med, 45: 491-503, 1994.
2) Cope AP: Regulation of autoimmunity by proinflammatory cytokines. Curr Opin Immunol, 10(6): 669-676, 1998.
3) Ware CF: Network communications: lymphotoxins, LIGHT, and TNF. Annu Rev Immunol, 23: 787-819, 2005.
4) Chan FK, Chun HJ, Zheng L, Siegel RM, Bui KL, Lenardo MJ: A domain in TNF receptors that mediates ligand-independent receptor assembly and signaling. Science, 288 (5475): 2351-2354, 2000.
5) Brennan FM, Feldmann M: Cytokines in autoimmunity. Curr Opin Immunol, 4(6): 754-759, 1992.
6) Van Deventer SJ: Tumour necrosis factor and Crohn's disease. Gut, 40(4): 443-448, 1997.
7) Dinarello CA, Gelfand JA, Wolff SM: Anticytokine strategies in the treatment of the systemic inflammatory response syndrome. Jama, 269(14): 1829-1835, 1993.
8) Bradham CA, Plumpe J, Manns MP, Brenner DA, Trautwein C: Mechanisms of hepatic toxicity. I.TNF-induced liver injury. Am J Physiol, 275(3 Pt 1): G387-G392, 1998.
9) Gardam MA, Keystone EC, Menzies R, Manners S, Skamene E, Long R, Vinh DC: Anti-tumour necrosis factor agents and tuberculosis risk: mechanisms of action and clinical management. Lancet Infect Dis, 3(3): 148-155, 2003.
10) Cohen RB, Dittrich KA: Anti-TNF therapy and malignancy--a critical review. Can J Gastroenterol, 15(6): 376-384, 2001.
11) Shakoor N, Michalska M, Harris CA, Block JA: Drug-induced systemic lupus erythematosus associated with etanercept therapy. Lancet, 359(9306): 579-580, 2002.
12) Keane J, Gershon S, Wise RP, Mirabile-Levens E, Kasznica J, Schwieterman WD, Siegel JN, Braun MM: Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent. N Engl J Med, 345(15): 1098-1104, 2001.
13) Sicotte NL, Voskuhl RR: Onset of multiple sclerosis associated with anti-TNF therapy. Neurology, 57(10): 1885-1888, 2001.
14) Mori L, Iselin S, De Libero G, Lesslauer W: Attenuation of collagen-induced arthritis in 55-kDa TNF receptor type 1 (TNFR1)-IgG1-treated and TNFR1-deficient mice. J Immunol, 157(7): 3178-3182, 1996.
15) Tsuji H, Harada A, Mukaida N, Nakanuma Y, Bluethmann H, Kaneko S, Yamakawa K, Nakamura SI, Kobayashi KI, Matsushima K: Tumor necrosis factor receptor p55 is essential for intrahepatic granuloma formation and hepatocellular apoptosis in a murine model of bacterium-induced fulminant hepatitis. Infect Immun, 65(5): 1892-1898, 1997.
16) Kollias G, Kontoyiannis D: Role of TNF/TNFR in autoimmunity: specific TNF receptor blockade may be advantageous to anti-TNF treatments. Cytokine Growth Factor Rev, 13(4-5): 315-321, 2002.
17) Kunkel TA: Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc Natl Acad Sci USA, 82 (2): 488-492, 1985.
18) Yamagishi J, Kawashima H, Matsuo N, Ohue M, Yamayoshi M, Fukui T, Kotani H, Furuta R, Nakano K, Yamada M: Mutational analysis of structure--activity relationships in human tumor necrosis factor-alpha. Protein Eng, 3(8): 713-719, 1990.
19) McCafferty J, Griffiths AD, Winter G, Chiswell DJ: Phage antibodies: filamentous phage displaying antibody variable domains. Nature, 348(6301): 552-554, 1990.
20) Shibata H, Yoshioka Y, Ikemizu S, Kobayashi K, Yamamoto Y, Mukai Y, Okamoto T, Taniai M, Kawamura M, Abe Y, Nakagawa S, Hayakawa T, Nagata S, Yamagata Y, Mayumi T, Kamada H, Tsutsumi Y: Functionalization of tumor necrosis factor-alpha using phage display technique and PEGylation improves its antitumor therapeutic window. Clin Cancer Res, 10(24): 8293-8300, 2004.