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Creation of an improved mutant TNF with TNFR1-selectivity and antagonistic activity by phage display technology

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Tumor necrosis factor-α (TNF), which binds two types of TNF receptors (TNFR1 and TNFR2), regulates the onset and exacerbation of autoimmune diseases such as rheumatoid arthritis and Crohn's disease. In particular, TNFR1-mediated signals are predominantly related to the induction of inflammatory responses. We have previously generated a TNFR1-selective antagonistic TNF-mutant (mutTNF) and shown that mutTNF efficiently inhibits TNFR1-mediated bioactivity *in vitro* and attenuates inflammatory conditions *in vivo*. In this study, we aimed to improve the TNFR1-selectivity of mutTNF. This was achieved by constructing a phage library displaying mutTNF-based variants, in which the amino acid residues at the predicted receptor binding sites were substituted to other amino acids. From this mutant TNF library, 20 candidate TNFR1-selective antagonists were isolated. Like mutTNF, all 20 candidates were found to have an inhibitory effect on TNFR1-mediated bioactivity. However, one of the mutants, N7, displayed significantly more than 40-fold greater TNFR1-selectivty than mutTNF. Therefore, N7 could be a promising anti-autoimmune agent that does not interfere with TNFR2-mediated signaling pathways.

1. Introduction

The severity and progression of inflammatory diseases, such as rheumatoid arthritis, Crohn's disease and ulcerative colitis, can be correlated with the serum level of tumor necrosis factor-α (TNF). Thus, TNF blockades such as anti-TNF antibodies and soluble TNFRs, which neutralize the activity of TNF, have been used to treat various autoimmune diseases in clinical practice. However, TNF blockades inhibit both TNFR1 and TNFR2 signaling. Thus, treatment with these drugs can lead to an increased risk of infection (Gomez-Reino et al. 2003; Lubel et al. 2007) and lymphoma development (Brown et al. 2002). TNF has been reported to induce inflammatory response predominantly through TNFR1 (Mori et al. 1996), whereas activation of the immune response is initiated *via* TNFR2 (Kim et al. 2006; Kim and Teh 2001; Grell et al. 1998). Therefore, blocking TNFR1-signaling, but not TNFR2-signaling, is a promising strategy for the safe and effective treatment of inflammatory diseases, which overcomes the risk of infection associated with the use of non-specific TNF blockades (Kollias and Kontoyiannis 2002).

In our previous studies, we used the phage display technique (Imai et al. 2008; Nagano et al. 2009; Nomura et al. 2007) to generate a TNFR1-selective antagonistic mutant TNF (mutTNF) that blocks TNFR1-mediated signals but not those of TNFR2 (Shibata et al. 2008b). Moreover, mutTNF showed superior therapeutic effects using an inflammatory disease mouse model (Shibata et al. 2008a). Thus, a drug for autoimmune diseases that selectively targets TNFR1 is anticipated to display higher efficacy and safety compared to existing treatments. In this study, we have attempted to isolate TNFR1-selective antagonists with higher TNFR1-selectivity than previous mutTNF by constructing a modified phage library displaying mutTNF-based variants.

2. Investigations, results and discussion

Here, we attempted to improve the TNFR1-selectivity of mutTNF using a phage display technique. Firstly, we constructed a phage library of TNF mutant using mutTNF as template. We designed a randomized library of mutTNF to replace the six amino acid residues (aa 29, 31, 32, 145–147) in the predicted receptor binding site. As a result of the 2-step PCR, we confirmed that the mutTNF mutant library consisted of 4×10^7 independent recombinant clones (*data not shown*). To enrich for TNFR1-selective antagonists, the phage library was subjected to two rounds of panning against TNFR1 on a Biacore biosensor chip. After the second panning, supernatants of single clone of *E. coli* TG1 including phagemid were randomly collected and subjected to screening by bioassay and ELISA to evaluate their bioactivity and affinity against each TNF receptor, respectively (*data not shown*). Consequently, twenty candidates of TNFR1-selective mutants with antagonistic activity were isolated (Table).

Next, we determined the detailed biological properties of each candidate. Positive clones were engineered for expression in

Table: Amino acid sequences and biological properties of TNFR1-selective antagonist candidates

TNF	Amino acid sequence						Relative affinity (% K_d)[a)]			Bioactivity via TNFR1	
	29	31	32	145	146	147	TNFR1	TNFR2	TNFR1[b)]/TNCR2	Agonistic[c)] activity	Antagonisti[d)] activity
mutTNF	L	R	R	A	E	S	100.0	100.0	1.0	–	+
N1	S	–	W	R	–	–	550.0	21.6	25.5	+	–
N2	S	–	W	–	–	–	200.0	N.D.	N.D.	+	–
N3	S	–	W	R	D	–	550.0	44.8	12.3	–	±
N4	S	–	W	–	D	–	183.3	19.1	9.6	±	–
N5	S	–	W	–	S	E	275.0	25.8	10.7	±	–
N6	A	D	T	–	–	–	200.0	21.6	9.3	±	–
N7	S	N	D	D	A	–	104.7	2.5	41.9	–	+
N8	R	I	A	D	–	–	169.2	26.7	6.3	+	–
N9	H	H	–	–	N	G	169.2	33.0	5.1	+	–
N10	T	N	N	–	–	–	314.3	28.6	11.0	±	–
N11	T	N	N	S	–	–	275.0	18.3	15.0	±	–
N12	F	S	T	–	–	–	440.0	58.0	7.6	+	–
N13	F	S	T	–	S	E	440.0	73.9	6.0	+	–
N14	R	W	Y	T	N	T	314.3	19.2	16.4	+	–
N15	F	K	T	N	A	T	275.0	24.1	11.4	±	–
N16	M	L	T	N	S	T	367.0	7.7	47.7	+	–
N17	Y	L	A	T	H	T	137.5	1.6	86.0	±	–
N18	Y	L	A	T	H	–	110.0	4.7	23.4	±	–
N19	V	Q	Y	N	N	–	367.0	N.D.	N.D.	±	–
N20	F	S	T	P	Q	R	244.4	N.D.	N.D.	±	–

Conserved residues compared with mutTNF are indicated by an em dash (–) The affinity values are shown as relative values (% mutTNF). N.D.: not detected

[a)] Affinity for immobilized TNFR1 and TNFR2 was assessed by SPR using BIAcore3000. The dissociation constant (K_d) of TNF mutants were calculated from their sensorgrams by BIAEVALUATION 4.0 software

[b)] TNFR1-selectivity was defined as relative affinity [TNFE1]/ relative affinity [TNFR2] for mutTNF

[c)] TNFR1-mediated agonistic activity was measured, using a HEp-2 cell cytotoxicity assay. The intensity in agonistic activity was evaluated as the following. Cell viability at 10^4 ng/ml each mutant. 0–25% (of non treatment); (+), 25–50%; (±), 50–100%; (–)

[d)] TNFR1-mediated antagonistic activity of mutant TNFs on wtTNF induced cytotoxicity in HEp-2 cells was measured. The intensity in antagonistic activity was evaluated as the following. Cell viability at 10^5 ng/ml each mutant in present of 5 ng/ml wtTNF. 0–25% (of non treatment); (–), 25–50%; (±), 50–100%; (+)

E. coli BL21λDE3 and each recombinant protein was purified as described previously (Yamamoto 2003). As anticipated, gel electrophoresis confirmed the mutant TNF proteins to have a molecular weight of 17 kDa. Moreover, gel filtration chromatography established that each mutant forms a homotrimeric complex in solution, as is the case for wild-type TNF (wtTNF) (*data not shown*). To analyze the binding properties of these TNFR1-selective TNF candidates, their dissociation constants (K_d) for TNFR1 and TNFR2 were measured using a surface plasmon resonance (SPR) analyzer. Our previous SPR analysis showed that although mutTNF has an almost identical affinity to TNFR1 as to wtTNF, it displays more than 17,000-fold greater selectivity for TNFR1. As shown in the Table, all the candidates exhibited higher affinity for TNFR1 than mutTNF. Furthermore, clones N1, N7, N16, N17 and N18 showed more than 20-fold higher TNFR1-binding selectivity compared to mutTNF. To examine the bioactivity of all candidates *via* TNFR1, we subsequently performed a cytotoxicity assay using

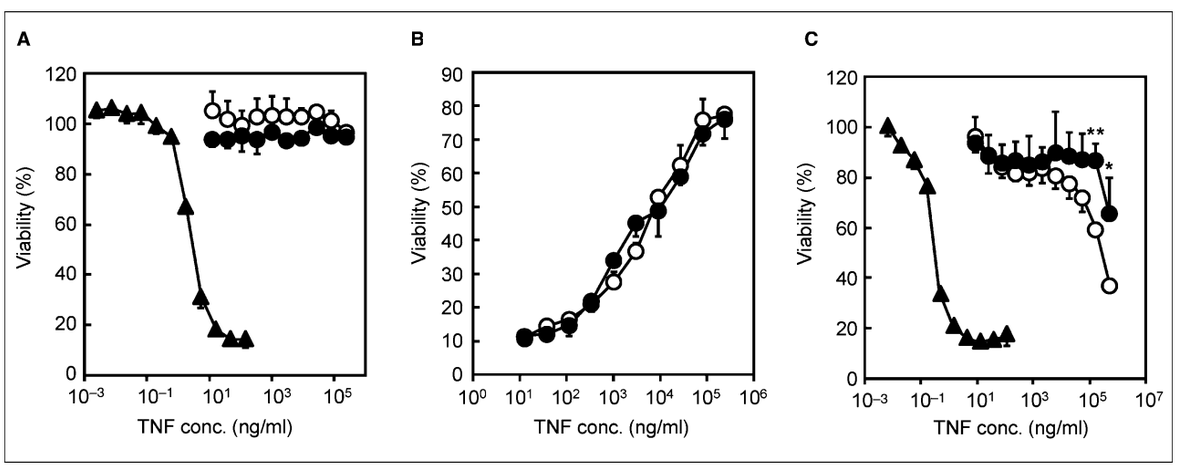

Fig.: Bioactivities and antagonistic activities of N7. (A) To determine the TNFR1-mediated bioactivities, several dilutions of wtTNF (closed triangle), mutTNF (open circle) and N7 (closed circle) were added to L-M cells and incubated for 4 h at 37 °C. (B) Indicated dilutions of mutTNF (open circle) and N7 (closed circle) and constant of wtTNF (5 ng/ml) were mixed and added to L-M cells and incubated for 4 h at 37 °C. TNFR1-mediated antagonistic activity was assessed as described in the Experimental section. (C) To determine the TNFR2-mediated bioactivities, diluted wtTNF (closed triangle), mutTNF (open circle) and N7 (closed circle) were added to hTNFR2/mFas-preadipocyte cells and incubated for 48 h at 37 °C. After incubation, cell viability was measured using the methylene blue assay. Data represent the mean ± S.D. and were analyzed by Student's t-test (*$p < 0{,}05$, **$p < 0{,}01$ vs mutTNF)

HEp-2 cells (Table). As anticipated, mutTNF was unable to activate TNFR1. Likewise clones N3 and N7 do not activate TNFR1 signaling, even when tested at high concentrations. The TNFR1-mediated antagonistic assay demonstrated that N7 showed the highest activity of all the TNFR1-selective antagonist candidates. The Figure show details of bioactivities and antagonistic activities of N7. The TNFR1-mediated agonistic activity using L-M cells showed that wtTNF displays TNFR1-mediated agonistic activity in a dose-dependent manner. In contrast, N7, in addition to mutTNF, barely displays any agonistic activity (Fig. A). Moreover, N7 had an almost identical antagonistic activity for TNFR1-mediated bioactivity to that of mutTNF (Fig. B). Next, TNFR2-mediated activities of these TNFR1-selective antagonists were measured using hTNFR2/mFas-preadipocyte cells. The bioactivity of mutTNF and N7 *via* TNFR2 was much lower than that of wtTNF. Remarkably, TNFR2-mediated agonistic activity of N7 was lower than that of mutTNF, in agreement with the reduced affinity for TNFR2 (Fig. C).

In conclusion, we have succeeded in creating a TNFR1-selective antagonist with improved TNFR1-selectivity over that of mutTNF. This was achieved by constructing a library of mutTNF variants using a phage display technique. While TNFR1 is believed to be important for immunological responses (Rothe et al. 1993), TNFR2 is thought to be important for antiviral resistance and is effective for controlling mycobacterial infection by affecting membrane-bound TNF stimulation (Saunders et al. 2005; Olleros et al. 2002). Therefore, use of N7 might reduce the risk of side effects, such as infections, when applying TNF blockade as a therapy for autoimmune disease. We are currently evaluating the therapeutic effect of N7 using a mouse autoimmune disease model.

3. Experimental

3.1. Cell culture

HEp-2 cells (a human fibroblast cell line) were provided by Cell Resource Center for Biomedical Research (Tohoku University, Sendai) and were maintained in RPMI 1640 medium supplemented with 10% FBS and 1% antibiotics cocktail (penicillin 10,000 units/ml, streptomycin 10 mg/ml, and amphotericin B 25 μg/ml). L-M cells (a mouse fibroblast cell line) were provided by Mochida Pharmaceutical Co. Ltd. (Tokyo, Japan) and were maintained in minimum Eagle's medium supplemented with 1% FBS and 1% antibiotics cocktail. hTNFR2/mFas-preadipocyte cells were established previously in our laboratory (Abe et al. 2008) and were maintained in Dulbecco's modified Eagle's medium supplemented with Blasticidin S HCl, 10% FBS, 1 mM sodium pyruvate, 5×10^{-5} M 2-mercaptoethanol, and 1% antibiotic cocktail.

3.2. Construction of a novel gene library displaying mutTNF variants

The pCANTAB phagemid vector encoding mutTNF was used as template for PCR. The mutTNF was created in previous study and showed TNFR1-selective antagonistic activity (Shibata et al. 2008b). The six amino acid residues at the receptor binding site (amino acid residues; 29, 31, 32 and 145–147) of mutTNF were replaced with other amino acids using a 2-step PCR procedure as described previously (Mukai et al. 2009).

3.3. Selection of TNFR1-selective antagonist candidates from a mutTNF mutated phage library

Human TNFR1 Fc chimera (R&D systems, Minneapolis, MN) was immobilized onto a CM3 sensor chip as described previously. Briefly, the phage display library (1×10^{11} CFU/100 μl) was injected over the sensor chip at a flow rate of 3 μl/min. After binding, the sensor chip was washed using the rinse command until the association phase was reached. Elution was carried out using 4 μl of 10 mM glycine-HCl. The eluted phage pool was neutralized with 1 M Tris-HCl (pH 6.9) and then used to infect *E. coli* TG1 in order to amplify the phage. The panning steps were repeated twice. Subsequently, single clones were isolated and supernatant from each clone was collected and used to determine the cytotoxicity in the HEp-2 cytotoxic assay and the affinity for TNFR1 by ELISA, respectively (Shibata et al. 2008b). We screened clones having almost no cytotoxicity but significant affinity for TNFR1. The phagemids purified from single clones were sequenced using the Big Dye Terminator v3.1 kit (Applied Biosystems, Foster City, CA). Sequencing reactions were analyzed on an ABI PRISM 3100 (Applied Biosystems).

3.4. Surface plasmon resonance assay (BIAcore® assay)

The binding kinetics of the proteins were analyzed by the surface plasmon resonance technique by BIAcore® (GE Healthcare, Amersham, UK). Each TNF receptor was immobilized onto a CM5 sensor chip, which resulted in an increase of 3,000–3,500 resonance units. During the association phase, all clones serially diluted in running buffer (HBS-EP) were allowed to pass over TNFR1 and TNFR2 at a flow rate of 20 μl/min. Kinetic parameters for each candidate were calculated from the respective sensorgram using BIAevaluation 4.0 software.

3.5. Cytotoxicity assay

In order to measure TNFR1-mediated cytotoxicity, HEp-2 or L-M cells were cultured in 96-well plates in the presence of TNF mutants and serially diluted wtTNF (Peprotech, Rocky Hill, NJ) with 100 μg/ml cycloheximide for 18 h at 4×10^4 cells/well or for 48 h at 1×104 cells/well. Cytotoxicity was then assessed using the methylene blue assay as described previously (Mukai et al. 2009; Shibata et al. 2004). For the TNFR1-mediated antagonistic assay, cells were cultured in the presence of 5 ng/ml human wtTNF and a serial dilution of the mutTNF. For the TNFR2-mediated cytotoxic assay, hTNFR2/mFas-preadipocyte cells were cultured in 96-well plates in the presence of TNF mutants and serially diluted wtTNF (1×10^4 cells/well) (Abe et al. 2008). After incubation for 48 h, cell survival was determined using the methylene blue assay.

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References

Abe Y, Yoshikawa T, Kamada H, Shibata H, Nomura T, Minowa K, Kayamuro H, Katayama K, Miyoshi H, Mukai Y, Yoshioka Y, Nakagawa S, Tsunoda S, Tsutsumi Y (2008) Simple and highly sensitive assay system for TNFR2-mediated soluble- and transmembrane-TNF activity. J Immunol Methods 335: 71–78.

Aggarwal BB (2003) Signalling pathways of the TNF superfamily: a double-edged sword. Nat Rev Immunol 3: 745–756.

Brown SL, Greene MH, Gershon SK, Edwards ET, Braun MM (2002) Tumor necrosis factor antagonist therapy and lymphoma development: twenty-six cases reported to the Food and Drug Administration. Arthritis Rheum 46: 3151–3158.

Feldmann M (2002) Development of anti-TNF therapy for rheumatoid arthritis. Nat Rev Immunol 2: 364–371.

Goldbach-Mansky R, Lipsky PE (2003) New concepts in the treatment of rheumatoid arthritis. Annu Rev Med 54: 197–216.

Gomez-Reino JJ, Carmona L, Valverde VR, Mola EM, Montero MD (2003) Treatment of rheumatoid arthritis with tumor necrosis factor inhibitors may predispose to significant increase in tuberculosis risk: a multicenter active-surveillance report. Arthritis Rheum 48: 2122–2127.

Grell M, Becke FM, Wajant H, Mannel DN, Scheurich P (1998) TNF receptor type 2 mediates thymocyte proliferation independently of TNF receptor type 1. Eur J Immunol 28: 257–263.

Imai S, Mukai Y, Takeda T, Abe Y, Nagano K, Kamada H, Nakagawa S, Tsunoda S, Tsutsumi Y (2008) Effect of protein properties on display efficiency using the M13 phage display system. Pharmazie 63: 760–764.

Kim EY, Priatel JJ, Teh SJ, Teh HS (2006) TNF receptor type 2 (p75) functions as a costimulator for antigen-driven T cell responses *in vivo*. J Immunol 176: 1026–1035.

Kim EY, Teh HS (2001) TNF type 2 receptor (p75) lowers the threshold of T cell activation. J Immunol 167: 6812–6820.

Kollias G, Kontoyiannis D (2002) Role of TNF/TNFR in autoimmunity: specific TNF receptor blockade may be advantageous to anti-TNF treatments. Cytokine Growth Factor Rev 13: 315–321.

Lubel JS, Testro AG, Angus PW (2007) Hepatitis B virus reactivation following immunosuppressive therapy: guidelines for prevention and management. Intern Med J 37: 705–712.

Mori L, Iselin S, De Libero G, Lesslauer W (1996) Attenuation of collagen-induced arthritis in 55-kDa TNF receptor type 1 (TNFR1)-IgG1-treated and TNFR1-deficient mice. J Immunol 157: 3178–3182.

Mukai Y, Shibata H, Nakamura T, Yoshioka Y, Abe Y, Nomura T, Taniai M, Ohta T, Ikemizu S, Nakagawa S, Tsunoda S, Kamada H, Yamagata Y, Tsutsumi Y (2009) Structure-function relationship of tumor necrosis factor (TNF) and its receptor interaction based on 3D structural analysis of a fully active TNFR1-selective TNF mutant. J Mol Biol 385: 1221–1229.

Nagano K, Imai S, Mukai Y, Nakagawa S, Abe Y, Kamada H, Tsunoda S, Tsutsumi Y (2009) Rapid isolation of intrabody candidates by using an optimized non-immune phage antibody library. Pharmazie 64: 238–241.

Nomura T, Kawamura M, Shibata H, Abe Y, Ohkawa A, Mukai Y, Sugita T, Imai S, Nagano K, Okamoto T, Tsutsumi Y, Kamada H, Nakagawa S, Tsunoda S (2007) Creation of a novel cell penetrating peptide, using a random 18mer peptides library. Pharmazie 62: 569–573.

Olleros ML, Guler R, Corazza N, Vesin D, Eugster HP, Marchal G, Chavarot P, Mueller C, Garcia I (2002) Transmembrane TNF induces an efficient cell-mediated immunity and resistance to Mycobacterium bovis bacillus Calmette-Guerin infection in the absence of secreted TNF and lymphotoxin-alpha. J Immunol 168: 3394–3401.

Rothe J, Lesslauer W, Lotscher H, Lang Y, Koebel P, Kontgen F, Althage A, Zinkernagel R, Steinmetz M, Bluethmann H (1993) Mice lacking the tumour necrosis factor receptor 1 are resistant to TNF-mediated toxicity but highly susceptible to infection by Listeria monocytogenes. Nature 364: 798–802.

Saunders BM, Tran S, Ruuls S, Sedgwick JD, Briscoe H, Britton WJ (2005) Transmembrane TNF is sufficient to initiate cell migration and granuloma formation and provide acute, but not long-term, control of Mycobacterium tuberculosis infection. J Immunol 174: 4852–4859.

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 (2004) Functionalization of tumor necrosis factor-alpha using phage display technique and PEGylation improves its antitumor therapeutic window. Clin Cancer Res 10: 8293–8300.

Shibata H, Yoshioka Y, Ohkawa A, Abe Y, Nomura T, Mukai Y, Nakagawa S, Taniai M, Ohta T, Mayumi T, Kamada H, Tsunoda S, Tsutsumi Y (2008a) The therapeutic effect of TNFR1-selective antagonistic mutant TNF-alpha in murine hepatitis models. Cytokine 44: 229–233.

Shibata H, Yoshioka Y, Ohkawa A, Minowa K, Mukai Y, Abe Y, Taniai M, Nomura T, Kayamuro H, Nabeshi H, Sugita T, Imai S, Nagano K, Yoshikawa T, Fujita T, Nakagawa S, Yamamoto A, Ohta T, Hayakawa T, Mayumi T, Vandenabeele P, Aggarwal BB, Nakamura T, Yamagata Y, Tsunoda S, Kamada H, Tsutsumi Y (2008b) Creation and X-ray structure analysis of the tumor necrosis factor receptor-1-selective mutant of a tumor necrosis factor-alpha antagonist. J Biol Chem 283: 998–1007.

Yamamoto Y, Tsutsumi Y, Yoshioka Y, Nishibata T, Kobayashi K, Okamoto T, Mukai Y, Shimizu T, Nakagawa S, Nagata S, Mayumi T (2003) Site-specific PEGylation of a lysine-deficient TNF-alpha with full bioactivity. Nat Biotechnol 21: 546–552.