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Next, we examined the TNFR1 selectivity of R1-6 based on its structure. Because the structure of TNFR2 is thought to be similar to that of TNFR1,[18] we generated a model structure of TNFR2 by manual mutation based on the crystal structure of TNFR1. This TNF–TNFR2 simulation is speculative, but this model, together with the information obtained from previous mutation studies, can be used to form hypotheses regarding the important structural features for TNFR1 selectivity. The binding surface of TNFR2 was composed of Asp54, Glu57, and Glu70, which could cause a strongly negatively charged surface of TNFR2 different from that of TNFR1 (Fig. 5c and d). Arg31 of wtTNF was thought to have an important role in TNFR2 binding by strongly interacting with this surface (Fig. 5c). R1-6 had an R31A mutation, however, which could cause the loss of the affinity of R1-6 for TNFR2 (Fig. 5d). In support of this finding, a single point mutation R31E mutant was previously reported to have a dramatic loss of affinity for TNFR2.[12,14] On the other hand, the R32W mutant is also reported to be a mutant with TNFR1 selectivity.[12] From our library, Arg32 of our TNFR1-selective candidates was replaced with hydrophobic or nonionic amino acids (Trp, Tyr, Phe, and Gly), which might indicate the importance of Arg32 for binding to TNFR2 (Table 1). This structural information, in combination with bioinformatics technology, will be useful for designing more advanced TNFR-selective mutants and TNFR-selective inhibitors (peptide mimics and chemical compounds).

In conclusion, the phage display technique is an attractive method for creating functional mutants, as demonstrated here by the production of TNFR-specific mutants. Application of this method to various cytokines and proteins will enhance the construction of useful receptor-selective mutants and accelerate functional analysis of these proteins. As an advanced application, analysis of the "structure (sequence)–function relationship" using the obtained mutants will be a powerful technique for basic life science research and drug discovery.

Materials and Methods

Cell culture

HEp-2 cells (a human fibroblast cell line) were provided by the Cell Resource Center for Biomedical Research (Tohoku University) and maintained with RPMI 1640 containing 10% fetal bovine serum and antibiotics. PC60-hTNFR2 cells (a mouse–rat fusion hybridoma comprised of human TNFR2-transfected PC60 cells) were provided by Dr. Vandenabeele and maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 5×10^{-5} M 2-mercaptoethanol, 3 μg/ml puromycin, and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B).

Library construction

The pCANTAB phagemid vector (GE Healthcare Ltd., UK) encoding mutTNF-Lys(−) was used as template for PCR. This TNF was previously reported to be a fully active lysine-deficient TNF mutant.[22] Mutations were introduced in TNF at six amino acid codons (Library I: amino acid residues 29, 31, 32, and 145–147; Library II: amino acid residues 84–89) using a two-step PCR. Three primers, Oligos A, B, and C, were used for the construction of Library I. The first PCR was performed using Oligos A and B. The PCR conditions were 5 min at 95 °C, 35 cycles of denaturization at 95 °C for 15 s, and annealing/extension at 68 °C for 2 min. This first PCR product and Oligo C were then annealed to the template, and PCR was performed again under the same conditions. For the construction of Library II, Oligos A, D, and E were used. The first PCR was performed using Oligos A and D. The first PCR product and Oligo E were used as primers for the second PCR. The PCR conditions of Library II were the same as those of Library I. After the second PCR, the PCR products were digested with HindIII and NotI, and then ligated to a pY03′ phagemid vector (modified from pCANTAB) for the display of TNF variants on the phage surface as g3p fusion proteins. The primer sequences used in this experiment are listed below. Oligos A and E were designed to prime to the pCANTAB vector sequence: Oligo A: 5′-GATAACAATTTCACACAGGAAACAGCTATGACCATGATTACGCCAAGCTTTGGAGCC-3′; Oligo B: 5′-CGCCATTGGCCAGGAGGGCATTAGCSNNSNNGTTSNNCCACTGGAGCTGCCCCTCAGCTTGAGGG-3′; Oligo C: 5′-CCAGCGGATCCGGATACGGCACCGGCGCACCTGCGGCCGCGGATCCACCACCACCCAGGGCAATGATCCCAAAGTAGACCTGCCCSNNSNNSNNAAAGTCGAGATAGTCGGGCCGATTGA-3′; Oligo D: 5′-CTGGCAGGGGCTGCGGATGGCAGAGAGGAGATTGACGGGSNNSNNSNNSNNSNNSNNGATGCGGCTGATGGTGTGGGTGAGGAGCAC-3′; Oligo E: 5′-TGCGGCACGCGGTTCCAGCGGATC-3′.

Isolation of receptor-selective TNF mutants from the library (affinity panning and screening)

Human TNFR1 Fc (R&D Systems, Inc., Minneapolis, MN) and TNFR2 Fc (R&D Systems, Inc.) were diluted to 50 μg/ml in 10 mM sodium acetate buffer (pH 4.5) and immobilized on a CM3 sensor chip using an amine coupling kit (GE Healthcare Ltd.), which resulted in an increase of 4000–6000 resonance units. The phage library (1×10^{11} colony-forming units/100 μl) was injected at 3 μl/min over the sensor chip. After binding and until the association phase had been reached, the sensor chip was washed using the rinse command and eluted using 20 μl of 10 mM glycine–HCl. The eluted phage was neutralized with 1 M Tris–HCl (pH 6.9). *E. coli* (TG1) was infected with the collected phage for amplification. This panning cycle was performed two more times. After picking up a single clone of transfected *E. coli*, the phagemid vectors were sequenced using a Big Dye Terminator v3.1 kit and ABI PRISM 3100 (Applied Biosystems Ltd., Pleasanton, CA). After the procedure, the binding affinities of the TNF mutants were assessed by ELISA, and their bioactivities through TNFR1 were determined by cytotoxicity assay in human HEp-2 cells.

Expression and purification of TNF mutants

The protocol for the expression and purification of recombinant protein was the same as that described previously.[21,22] Briefly, TNF mutants were produced in the *E. coli* BL21(DE3) strain. The inclusion body of each

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TNF mutant was washed in 2.5% Triton X-100 and solubilized in 6 M guanidine–HCl, 0.1 M Tris–HCl (pH 8.0), and 2 mM ethylenediaminetetraacetic acid. Solubilized protein at 10 mg/ml was reduced with 10 mg/ml dithioerythritol for 4 h at room temperature and refolded by 100-fold dilution in a refolding buffer (100 mM Tris–HCl, 2 mM ethylenediaminetetraacetic acid, 0.5 M arginine, and 551 mg/L oxidized glutathione). After dialysis with 20 mM Tris–HCl (pH 7.4) containing 100 mM urea, active trimeric proteins were purified by ion-exchange chromatography using Q-Sepharose FF (GE Healthcare Ltd.). Size-exclusion chromatography was performed using a Superose 12 column (GE Healthcare Ltd.).

In vitro bioactivity of TNF mutants

HEp-2 cells were used for cytotoxicity assay in the presence of cycloheximide (50 μg/ml). HEp-2 cytotoxicity was dependent on TNFR1 signaling. HEp-2 cells were cultured in 96-well plates in the presence of TNF mutants and serially diluted mouse or human wtTNF (PeproTech EC Ltd., UK) at 4×10^4 cells/well. For neutralization assay, cells were cultured in the presence of a constant concentration of human (20 ng/ml) wtTNF and a serial dilution of TNF mutants. After incubation for 18 h, cell survival was determined by methylene blue assay, as described previously.[21,22] To evaluate the bioactivity of the TNF mutant binding specifically to TNFR2, PC60-hTNFR2 cells were used as an index of granulocyte–macrophage colony-stimulating factor (GM-CSF) production, as described previously.[26] Briefly, PC60-hTNFR2 cells were cultured at 5×10^4 cells/well with interleukin-1β (2 ng/ml) and serially diluted TNF mutant. After 24 h of incubation, the amount of rat GM-CSF produced was quantified by ELISA in accordance with the manufacturer's protocol (R&D Systems, Inc.).

Affinity assessment using SPR

The binding kinetics of wtTNF and TNF mutants were analyzed using the BIAcore 3000 SPR system (GE Healthcare Ltd.). TNFRs were immobilized on a CM5 sensor chip, which resulted in an increase of 3000–5000 resonance units. During the association phase, TNF mutants or wtTNF diluted in HBS-EP running buffer (10 mM HEPES pH7.4, 150 mM NaCl, 3 mM EDTA, 0.005% Tween20, GE Healthcare Ltd.) at 78.4, 26.1, or 8.7 nM were individually passed over the immobilized TNFR at a flow rate of 20 μl/min. During the dissociation phase, HBS-EP buffer was applied to the sensor chip at a flow rate of 20 μl/min. The data were analyzed globally with BIAEVALUATION 3.0 software (GE Healthcare Ltd.) using a 1:1 binding model.

Competitive binding of TNF to TNFR1 and TNFR2 (ELISA)

Goat anti-human IgG (MP Biomedicals, Inc., Solon, OH) was immobilized on Maxisorb 96-well ELISA plates (Nalge Nunc International KK, Japan), and nonspecific binding to the plates was blocked using Block Ace (Dainippon Sumitomo Pharma Co., Ltd., Japan). Human TNFR1-Fc or human TNFR2-Fc (ALEXIS Corporation, Switzerland) was bound to coated antibody. Serially diluted TNF with 50 ng/ml FLAG-tagged wtTNF (wtTNF–FLAG) was added to TNFR1-Fc or TNFR2-Fc in 0.4% Block Ace. wtTNF–FLAG binding was detected by anti-FLAG M2 antibody (Sigma-Aldrich Corporation, St. Louis, MO) and avidin horseradish peroxidase conjugate (Invitrogen Corporation, Carlsbad, CA). The binding affinity of TNF was assessed by competitive wtTNF–FLAG binding to TNFR (IC_{50} value).

X-ray crystallography

Purified R1-6 was concentrated to 10 mg/ml in 20 mM Tris–HCl (pH 7.4). Initial screening using a Hampton Crystal screen 1-2 and Crystal screen Lite kit (Hampton Research Corporation, Aliso Viejo, CA) was performed by vapor diffusion method with hanging drops (1+1 μl) at 20 °C. After optimization of the crystallization conditions, rhombohedral crystals (0.2 mm × 0.2 mm × 0.3 mm) were obtained with reservoir solution containing 0.5 M ammonium sulfate, 1.2 M lithium sulfate, and 0.1 M trisodium citrate (pH 5.6). The crystals were frozen in a cryopreserving solution containing 15% glycerol as cryoprotectant. X-ray diffraction data to 2.5 Å resolution were collected at BL41XU, SPring-8, under flash cooling to 100 K to reduce the effects of radiation damage. Data integration and scaling were performed using HKL2000.[27] Molecular replacement was performed by the MOLREP program in CCP4i[28] using a crystal structure of the wtTNF (1TNF)[25] as search model. Cycles of manual rebuilding using the O program[29] and refinement using the CNS program[30] led to a refined structure. Final refinement (TLS refinement) was performed using the Refmac program in CCP4i.[28] Final model validation was performed using PROCHECK program in CCP4i.[28] The model complexes of TNF–TNFR1 and R1-6–TNFR1 were constructed based on the crystal structure of the LTα–TNFR1 complex[18] using the superimposing program in CCP4i. Structural models of TNFR2 were constructed based on the TNFR1 structure by manual mutation using the O program.[29]

Accession number

Coordinates and structure factors have been deposited in the PDB with accession number 2ZJC.

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