Construction and Production of Concatameric Human TNF Receptor-Immunoglobulin Fusion Proteins

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Abstract Tumor necrosis factor-α (TNF-α) and lymphotoxin-α (LT-α, TNF-β) can initiate and perpetuate human diseases such as multiple sclerosis (MS), rheumatoid arthritis (RA), and insulin-dependent diabetes mellitus (IDDM). TNFs can be blocked by the use of soluble TNF receptors. However, since monomeric soluble receptors generally exhibit low affinity or function as agonists, the use of monomeric soluble receptors has been limited in the case of cytokines such as TNF-α, TNF-β, interleukin (IL)-1, IL-4, IL-6, and IL-13, which have adapted to a multicomponent receptor system. For these reasons, very high-affinity inhibitors were created for the purpose of a TNFs antagonist to bind the TNFR and trigger cellular signal by using the multistep polymerase chain reaction method. First, recombinant simple TNFR-Ig fusion proteins were constructed from the cDNA sequences encoding the extracellular domain of the human p55 TNFR (CD120a) and the human p75 TNFR (CD120b), which were linked to hinge and constant regions of human IgG$_1$ heavy chain, respectively using complementary primers (CP) encoding the complementary sequences. Then, concatameric TNFR-Ig fusion proteins were constructed using recombinant PCR and a complementary primer base of recombinant simple TNFR-Ig fusion proteins. For high level expression of recombinant fusion proteins, Chinese hamster ovary (CHO) cells were used with a retroviral expression system. The transfected cells produced the simple concatameric TNFR-Ig fusion proteins capable of binding TNF and inactivating it. These soluble versions of simple concantameric TNFR-Ig fusion proteins gave rise to multiple forms such as simple dimers and concatameric homodimers. Simple TNFR-Ig fusion proteins were shown to have much more reduced TNF inhibitory activity than concatameric TNFR-Ig fusion proteins. Concatameric TNFR-Ig fusion proteins showed higher affinity than simple TNFR-Ig fusion proteins in a receptor inhibitor binding assay (RIBA). Additionally, concatameric TNFR-Ig fusion proteins were shown to have a progressive effect as a TNF inhibitor compared to the simple TNFR-Ig fusion proteins and conventional TNFR-Fc in cytotoxicity assays, and showed the same results for collagen induced arthritis (CIA) in mice *in vivo*.

Key words: TNF, TNFR1, TNFR2, TNFR-Ig, concatameric TNFR-Ig, arthritis

Cytokines and growth factors have an impact on many critical biological processes, with diverse effects ranging from acute regulation of gene expression and cell proliferation to promotion of chronic inflammation. Inhibiting cytokine activity can trigger clinical benefits; for example, blocking tumor necrosis factor-α (TNF-α) is efficacious in patients suffering from rheumatoid arthritis (RA) and inflammatory bowel disease [7, 23, 30]. One approved TNF-α blocker is etanercept (Enbrel), in which the ectodomain of the TNF-α receptor (p75 TNFR) is fused to the constant region (Fc) of human IgG1 [10]. This TNFR-Fc fusion protein dimerizes by means of interchain disulfides between the Fc domains; dimerization increases the affinity for TNF-α, and the Fc domain slows *in vivo* clearance [10, 21]. TNF-α and lymphotoxin-α (LT-α, TNF-β) are pleiotropic cytokines involved in diverse biological processes including immunological, inflammatory, and cytotoxic effects. The biological response to TNF is mediated through two forms of cell surface receptors such as p55R and p75R. Both receptors exist in a soluble form (p55-sR or TBP I, and p75-sR or TBP II) generated by the proteolytic cleavage of the extracellular region of the molecule [1]. The soluble forms may act by binding, therefore they neutralize circulating TNF [1]. The cloning of the 55-kd [5, 15, 25] and 75-kd [5, 14, 27] TNF receptors has opened the way for further studies of TNF effects and signal transduction. Moreover, it appears that truncated receptor molecules, lacking the transmembrane or cytoplasmic domains, are

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capable of interacting with TNF, and have been isolated from urine [9, 26] and serum [25] as TNF inhibitors. The current researchers speculated that derivatives of such molecules might be useful as an antagonist of TNF *in vivo*, high affinity ligands to be applied to a more sensitive assay for TNF, and reagents to be used in defining the molecular interaction between TNF and its receptor. Unfortunately, truncated forms of the TNF receptor are highly unstable *in vivo*; therefore, they are poor substitutes for antibodies as a means of antagonizing TNF action in living animals. The naturally occurring TNF receptor fragments are univalent and therefore have an avidity that is effectively far lower than that of a bivalent ligand. The soluble receptor fragments are active, but produced at low levels, therefore they are difficult to purify.

To circumvent these problems, proteins were engineered so that the extracellular domain of the human TNF receptor, which normally interacts with the TNF molecules, was covalently linked to the hinge region and constant regions of the human IgG, heavy chain. Furthermore, various concatameric TNFR-Ig fusion proteins were engineered in such a way that they contain the extracellular domain of the TNF receptor 1 and TNFR receptor 2, respectively.

Materials and Methods

Materials

Human recombinant TNF-α and TNF-β (LT-α) were purchased from R&D Systems, Inc. (Minneapolis, MN, U.S.A.), and had an activity of ED$_{50}$ (this effect is typically 0.02–0.05 ng/ml, respectively) measured in a cytotoxicity assay using the TNF susceptible murine L-929 cell line in the presence of the metabolic inhibitor actinomycin D (R&D Systems, Inc. Minneapolis, MN, U.S.A.) [18]. The

retroviral expression system was purchased from BD Biosciences Clontech Inc. (Palo Alto, CA, U.S.A.).

Retroviral Expression Vector

Retroviral expression vector pLPCX containing concatameric TNFR-Ig recombinant DNA was transfected into a packaging cell line such as GPG-293 cell line (supplied by N.I.H., U.S.A.). Once in the cell, RNA from the vector was packaged into infectious, replication-incompetent retroviral particles. Retroviral expression vector pLPCX ($P_{CMV\ IE}$, Amp^r, Puro^r, Col E1 ori, and 5'- 3' LTR) did not contain the structural genes (*gag*, *pol*, and *env*) necessary for particle formation and replication. Subsequent introduction of pLPCX, containing ψ^+, transcription, and processing elements, and the gene of interest produced a high-titer, replication-incompetent infectious virus. These retroviral particles could infect target cells such as Chinese hamster ovary (CHO) cells and transmit the gene of interest (which was cloned between the viral LTR sequences), but could not replicate within these cells since the cells lacked the viral structural genes [3, 8, 17, 19, 20, 22].

Construction of TNFR-Ig

The cDNA encoding the extracellular domain (ED) of the human TNFR1, TNFR2 (amino acids 211, 257, respectively) were cloned from total RNA prepared in human activated T-cells using a reverse transcriptase/PCR protocol [13]. The cDNA encoding the Fc region (hinge-CH2-CH3) of a human IgG, heavy chain (amino acid 234) was cloned from total RNA prepared in human activated B-cells using a RT/PCR protocol [13]. The PCR protocol employed in this study was 40 cycles of denaturation (94°C, 1 min), annealing (55°C, 1 min), and extension (72°C, 2 min) on a Biometra® (GreenMate Biotech Co.). Each reaction mixture contained 50 mM KCl, 10 mM Tris-Cl pH 8.0,

Table 1. Primer sequence for TNFR-Ig fusion proteins[a].

Forms	Primers	Sequence (5'-3')	Size (bp)
Simple TNFR-Ig (TNFR1, 2-Ig)	TR1-F	5' CCGGAATTCCGGTCTGGCATGGGCCTCTCCACC 3'	633
	TR1-R	5' CACAAGATTTGGGCTCTGCTGTGGTGCCTGAGTCCTC 3'	
	TR2-F	5' CCGGAATTCCGGGCACCCATGGCGCCCGTCGCC 3'	771
	TR2-R	5' CACAAGATTTGGGCTCTGCGTCGCCAGTGCTCCCTTC 3'	
	IgTR1-F	5' GAGGACTCAGGCACCACAGCAGAGCCCAAATCTTGTG 3'	133
	IgTR2-F	5' GAAGGGAGCACTGGCGACGCAGAGCCCAAATCTTGTG 3'	1473
	Ig-R	5' GCTCTAGAGCTCATTTACCCGGAGACAGGGAGAG 3'	
Human IgG, heavy chain	IgG$_1$-F	5' ATCTGCAGAGCCCAAATCTTGTGAC 3'	702
	IgG$_1$-R	5' TTCTCGAGTCATTTACCCGGAGACAGGGA-3'	
Concatameric TNFR-Ig (TNFR1, 2-TNFR1, 2-Ig)	TR1-F-F	5' CGCGGATCCGGGAACATTTCACTGGTCCCTCACCTAG 3'	1887
	TR1-F-R	5' CGCGGATCCGTCCTCAGTGCCCTTAACATTCTCAATCTG 3'	2022
	TR2-F-F	5' CGCGGATCCAACGCAACTACACCCTACGCCCCGGAG 3'	2028
	TR2-F-R	5' CGCGGATCCGCTCCCTTCAGCTGGGGGGCTG 3'	2163

[a]PCR was performed in a total volume of 50 µl. One microliter of the sample DNA solution was used in each reaction. Number of cycles was 40. Cycling was preceded by an initial denaturation step (94°C, 3 min). Before cooling, an elongation step was performed (72°C, 7 min).

1.5 mM MgCl$_2$, 200 μM of each dNTP (Takara, Co), 20 pmol of each primer, and 1.25 units of Taq polymerase (Promega). After the reaction, one-tenth (10 μl) of the reaction mixture was electrophoresed through 1% agarose (iNtRON biotechnology) gel containing 1 μg/ml ethidium bromide, and the gel was photographed. The human TNFR-ED and human IgG$_1$ heavy chain cDNA were separately amplified by PCR using TR1-F, TR2-F (corresponding to the 5' end of the TNFR1, 2-ED moiety), TR1-R, TR2-R (corresponding to the 3' TNFR1, 2-ED moiety and corresponding to the 5' end of the IgG$_1$ moiety), IgTR1-F, IgTR2-F (corresponding to the 3' end of the TNFR1, 2-ED moiety and corresponding to the 5' end of the IgG$_1$ moiety), and IgR (corresponding to the 3' end of the IgG$_1$ moiety) primers in Table 1. The PCR products were obtained after the first synthetic PCR reaction and they carried the complementary sequence site on the 3' end of TNFR1, 2-ED, and 5' end of the IgG$_1$ (Fig. 1A). The first PCR products were isolated and analyzed by agarose gel electrophoresis. The correct DNAs were mixed and used for a second round of PCR amplification using TR1-F, TR2-F, and IgR (corresponding to the 5' end of TNFR1, 2-ED moiety, and corresponding to the 3' end of the IgG$_1$ moiety) primers in Table 1. The amplified fragment was purified, digested with EcoRI and XbaI (encoded in the 5' end of TR1-F, TR2-F, and 5' end of IgR primers), and cloned into the cloning vector, pBluescript II KS (+), (STRATAGENE, Inc. La Jolla, CA, U.S.A.) derived from pBR322. Concatameric TNFR-Ig fusion proteins were obtained after the simple TNFR1, 2-ED-Ig ligation. Concatameric TNFR-Ig DNAs were obtained by PCR amplification using TR1-F-F, TR2-F-F (corresponding to the 5' end of the simple TNFR1, 2-Ig moiety), TR1-F-R, TR2-F-R (corresponding to the 3' end of the TNFR1, 2-ED moiety), and IgR (corresponding to the 3' end of the IgG$_1$ moiety) primers in Table 1. The four pairs of complementary primers carried the BamHI site (Fig. 1b). Concatameric TNFR-Ig DNAs containing the EcoRI and BamHI (encoding in the 5' end of TNFR1, 2-ED, and 3' end of TNFR-1, 2-ED) sites or the BamHI and XbaI (encoding in the 5' end of TNFR-1, 2-ED-Ig, and 3' end of IgG$_1$) sites were purified. They were then digested with EcoRI and BamHI or BamHI and XbaI, and cloned into the pBluescript II KS (+) vector. The entire sequence was verified by dideoxynucleotide sequencing [25] on both strands. For retroviral expression, the simple concatameric recombinant DNAs were cloned into the EcoRI and NotI sites of the pLPCX retroviral expression vector, respectively.

Expression and Purification of TNFR-Ig

GPG-293 cells (a line that is a derivative of the 293, CRL-1573, human embryonic kidney, HEK 293-based packaging cell line that stably expresses the viral gag, env, and pol genes) were grown in Dulbecco's modified Eagle's medium (DMEM, GibcoBRL, Gaithersburg, MD, U.S.A.) with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and 4.5 g/L glucose supplemented with 0.25 mg/ml G-418, 0.2 mg/ml tetracycline, 0.1 mg/ml puromycin, 100 units/ml penicillin G sodium, 100 μl/ml streptomycin, and 10% fetal bovine serum (FBS, GibcoBRL, Gaithersburg, MD, U.S.A.). Cells were incubated at 37°C with 5% CO$_2$. To produce a high-titer virus for a short while, a retroviral expression vector was transfected into a GPG-293-based packaging cell line by the CaCl$_2$-phosphate precipitation method [4]. On day 1, a confluent 15-cm plate of GPG-293 was split into six-well plates and grown to ~80%, and recombinant DNAs (30 μg) were washed with ice-cold 70% ethanol to remove residual salt. On day 2, for 2 h, media was aspirated off in the six-well plates, and 2 ml of fresh media was added without Ab solution (Neo, Tet, Puro). Recombinant DNAs were resuspended in 185 μl water, and 62.5 μl of 2 M CaCl$_2$ was added. Then, 250 μl of 2× HBS (280 mM NaCl, 10 mM KCl, 1.5 mM Na$_2$HPO$_4$, 12 mM dextrose, and 50 mM HEPES free acid, pH adjusted to 7.05 with NaOH) was added by bubbling using a Pasteur pipette. This solution was incubated for 30 min and dribbled onto the cells. The aspirate was incubated for 12-16 h and new complete media (DMEM+10%FBS without Ab) was added. After 2-4 days, the medium was collected from packaging cells and then filtered through a 0.45-μm cellulose acetate or polysulfonic (low protein binding) filter. A nitrocellulose filter was not used because it binds proteins in the retroviral membrane and destroys the virus. The CHO-K1 cell line was purchased from ATCC (ATCC CCL-61, Cricetulus griseus, hamster-ovarian, Chinese), then it was injected with retroviral particles containing simple concatameric TNFR-Ig fusion protein genes. CHO-K1 cells were grown in DMEM medium with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and 4.5 g/L glucose supplemented with 100 units/ml penicillin G sodium, 100 μl/ml streptomycin, and 10% fetal bovine serum. Stably transfected CHO-K1 cell lines were maintained in a culture of DMEM with 10% FBS, 4 mM L-glutamine supplemented with 4 mg/ml puromycin, 100 units/ml penicillin G sodium, and 100 μl/ml streptomycin, using standard sterile techniques. For purification of the recombinant TNFR-Ig fusion proteins, stably transfected CHO-K1 cells were maintained in serum-free medium, CHO-S-SFMII (GibcoBRL, Gaithersburg, MD, U.S.A.). Cells were incubated at 37°C with 5% CO$_2$. After 1-2 weeks, CHO-K1 cells were removed by filtration and the conditioned medium was used for affinity chromatography. Concatameric TNFR-Ig fusion proteins were produced in serum-free media and assessed by an enzyme linked immunosorbant assay (ELISA, R&D System, Minneapolis, MN, U.S.A.). All the medium components, except for the DMEM and FBS, were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Concatameric TNFR-Ig fusion
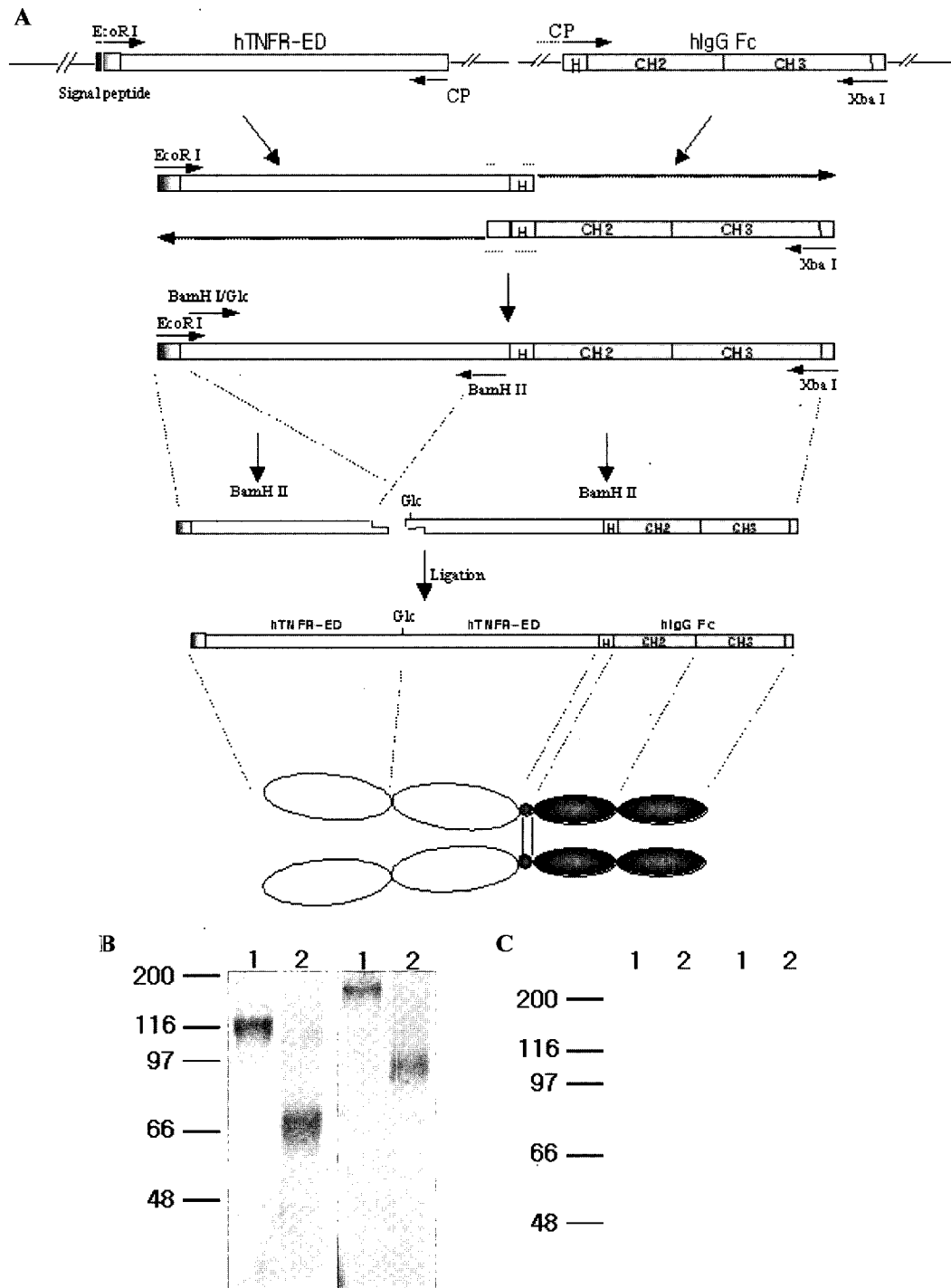
Fig. 1. (A) Schematic structure of the human TNFR-1, TNFR-2, human IgG₁ heavy chain, TNFR-Ig, and TNFR-TNFR-Ig. The extracellular domain (ED), and the IgG₁ heavy-chain hinge region (H) and constant region (domain C_H2 and C_H3) are indicated. TNFR-Ig was constructed by gene fusion of the extracellular domain of TNFR with the hinge region and C_H2 and C_H3 domains of the IgG₁ heavy chain. cDNAs encoding the human TNFR-1 or TNFR-2, and hinge and constant region of human IgG₁ heavy chain were separately amplified by PCR using complementary primers (CP) that add a unique restriction enzyme site. (B-C) Subunit structure and functional domains of TNFR-Ig. Chinese hamster ovary (CHO) cells were transfected with retroviral particles directing expression of TNFR-Ig. The proteins were recovered from serum-free culture supernatants and purified by affinity chromatography on *S. aureus* protein A. SDS/polyacrylamide gel electrophoresis was carried out under nonreducing (lane 1) or reducing (lane 2) conditions. The proteins were stained with Coomassie blue (B *Left*, TNFR1-TNFR1-Ig and B *Right*, TNFR2-TNFR2-Ig) or electroblotted onto nitrocellulose membrane and incubated with antibodies of human IgG₁ heavy chain region (C *Left*, TNFR1-TNFR1-Ig and C *Right*, TNFR2-TNFR2-Ig C). Blots were developed with horseradish peroxidase-conjugated second antibodies (C).

proteins were purified to an apparent homogeneity by passing serum-free medium from infected CHO-K1 cell cultures over the HiTrap™ protein A HP columns (Amersham Bioscience). HiTrap™ Protein A HP (1.6×2.5 cm, 5 ml) is a prepacked, ready to use column for preparative affinity chromatography. HiTrap Protein A HP is designed for the isolation and purification of monoclonal and polyclonal IgG from serum, cell culture supernatants, and ascites. The purified fractions of concatameric TNFR-Ig fusion proteins were used for ELISA, and concentrated with a Centricon 30 concentrator (Amicon) at 2,000 rpm for 30 min at 4°C.

Receptor-Inhibitor Binding Assays

The receptor-inhibitor binding assay (RIBA) was based on the binding of TNF-α or TNF-β to TNFR-Ig fusion proteins (recombinant CHO-K1 derived), which were previously adsorbed onto the microtiter plate. When a sample of TNFR-Ig fusion protein (10 µg/ml) was added, it competed with the TNFR1-Fc (350 ng/ml) or TNFR2-Fc (350 ng/ml) used for the standard (R&D Systems, Inc. Minneapolis, MN, U.S.A.) that was coated to the plate for binding to TNF-α (20 ng/ml). Reactions with recombinant human TNF-α were performed in a phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) for 1 h at 37°C. The amount of bound TNF-α was inversely proportional to the added TNFR-Ig fusion proteins and quantified by a polyclonal antibody (Ab) anti-TNF-α, followed by an HRP-conjugated second Ab. The optical density was measured at 492 nm with a multiscan spectrophotometer (Bio-RAD Model-550 Japan). For TNF-α, concatameric TNFR-Ig fusion proteins and TNFR1, 2-Fc were serially diluted twice in a microtiter plate to obtain a seven-point dilution binding curve [30].

TNF Cytotoxicity Assays

Murine L929 cells derived from strain L cell (3×10⁴ cells/ well) were seeded into 96-well microtiter plates in a high glucose DMEM medium (200 µl) supplemented with 10% fetal bovine serum, 4 mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin (GibcoBRL, Gaithersburg, MD, U.S.A.) and incubated for 24 h at 37°C in a 5% CO₂ incubator. Then, actinomycin (3 µg/ml) was added to the medium. Purified concatameric TNFR-Ig fusion proteins (10 µg/ml) were added to the wells and serially diluted. After that, TNF-α or TNF-β was added to the wells and finally concentrated to 1 ng/ml as 100% cytotoxicity. The plates were incubated for an additional 16 h, and the viable cells were stained with 20% methanol containing 0.5% crystal violet (Wako Pure Chemical Industries, Japan). The dye was eluted with 0.1 M sodium citrate, and 0.1 M citric acid in 50% ethanol, and absorbance was measured at 540 nm using a spectrophotometer (Bio-RAD Model-550, Japan). Reported values are the means of triplicate determinations [18].

CIA Model

Male DBA/1 (H-2ᵠ) mice, 7-8 weeks of age, were purchased from Chondrex, Inc. (Redmond, WA, U.S.A.) and maintained in a pathogen-free animal facility. Bovine type II collagen (Chondrex, Inc.) was dissolved in 0.05 M acetic acid at a concentration of 2 mg/ml by stirring overnight at 4°C, and emulsified in an equal volume of incomplete Freund's adjuvant (IFA, Calbiochem, La Jolla, CA, U.S.A.). DBA/1 mice were immunized intradermally on the first day with type II collagen (CII) emulsified in incomplete Freund's adjuvant at a 2:1 ratio and boosted on day 21 with CII (emulsified in incomplete Freund's adjuvant). The mice were immunized intradermally at the base of the tail with 100 µg of collagen. Arthritis developed 3-4 weeks after the first immunization, and the incidence of arthritis has shown around 80-100%. In addition, the most severe (a score of 5-8) incidence of arthritis was developed at 5-7 weeks. For disease prevention studies, groups of DBA/1 mice (3 mg per mouse) received an intraperitoneal (IP) injection of purified TNFR-Ig fusion proteins [12] and PBS was used as the control. Mice were treated 3 times per week for a total of 8 injections. The arthritis severity score was measured as described in Wooley et al. [32]. Mice were monitored every second day using an established macroscopic scoring system on a 0-4 scale: 0, normal; 1, detectable arthritis with erythema; 2, considerable swelling and redness; 3, severe swelling and redness from joint to digit; 4, maximal swelling and deformity with ankylosis. The macroscopic score was expressed as a cumulative value for all 4 paws with a maximum possible score of 16.

RESULTS AND DISCUSSION

Construction of Concatameric TNFR-Ig and Expression in CHO Cells

Separate constructs encoding the extracellular domains of TNFR-1 or TNFR-2 fused to the Fc portion of human IgG₁ (Fig. 1A) were engineered. Fusing complementary DNAs encoding the extracellular portion of human TNFR-1 and TNFR-2 of the simple TNFR-Ig fusion proteins were created as concatameric TNFR-Ig fusion proteins (Fig. 1A). As the Fc directed formation of disulfide-linked dimers, the infection of retroviral expression constructs encoding TNFR-Ig fusion proteins in CHO-K1 cells has led to the secretion of two distinct dimeric products: simple dimers of TNFR1-Ig and TNFR2-Ig, and concatameric homodimers of TNFR1-TNFR1-Ig and TNFR2-TNFR-2-Ig (Fig. 1B-C). To take advantage of the presence of an IgG Fc domain in the TNFR-Ig fusion proteins, protein-A affinity chromatography was used to recover and purify the protein from cell culture supernatants. The IgG₁ portion of these fusion proteins provided an effective means of purification and detection due to the presence of the reagents derived
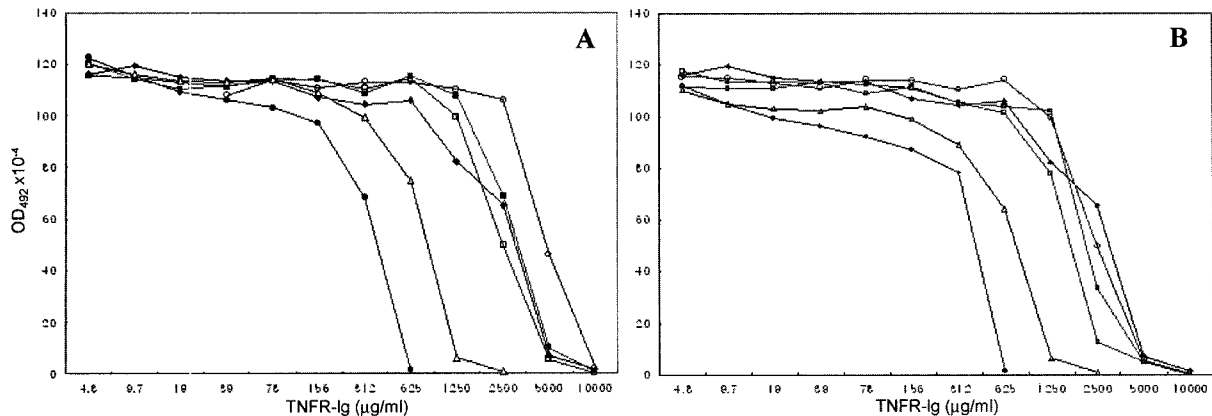
Fig. 2. Competition analysis of TNFR-Ig binding to recombinant human TNF-α. Purified TNFR-Ig was competed in microtiter wells coated with conventional TNFRI-Fc (A), and TNFRII-Fc (B) and incubated with increasing concentration of TNFR-Ig. TNFR1-Ig (□), TNFR2-Ig (■), TNFR1-TNFR1-Ig (●), TNFR2-TNFR2-Ig (▲), TNFRI-Fc (○), and TNFRII-Fc (◆) were incubated with 20 ng/ml TNF-α.

from antibodies against IgG₁ which are readily available. Single-step affinity purification of the TNFR-Ig fusion proteins from serum-free culture media yielded proteins that ranged from ~50 to 90% purity as determinated by SDS-PAGE (Fig. 1B). The subunit structure of TNFR-Ig fusion proteins was examined by SDS/polyacrylamide gel electrophoresis (Fig. 1B-C). Under reducing conditions, a molecular mass of 70– 80 kDa was observed, whereas under nonreducing conditions, it was approximately doubled, indicating that the simple, concatameric TNFR-Ig fusion proteins were disulfide-bonded dimers (Fig. 1B-C). Western blot analyses showed reactivity of TNFR-Ig fusion proteins with antibodies to human IgG Fc (Fig. 1C).

Binding of Concatameric TNFR-Ig to TNF-α

To investigate the binding of TNFR-Ig to TNF-α, saturation and competition binding analyses were carried out by using an assay in which TNFR-Ig fusion proteins were

competitors with conventional TNFR-Fc coated on microtiter wells. The ability of concatameric TNFR-Ig fusion proteins to compete with TNFR1-Fc (R&D) and TNFR2-Fc (R&D) for binding to human TNF-α was measured in three independent experiments. The simple TNFR-Ig fusion proteins, and TNFR 1, 2-Fc, appear to bind to TNF-α with 2- to 8-fold lower affinity than concatameric TNFR-Ig fusion proteins (Fig. 2A-B). These results indicate lower affinity binding of simple TNFR-Ig and TNFR-Fc to TNF-α. Concatameric TNFR-Ig fusion proteins were found to be able to displace the binding of TNF-α from the coated TNFR1-Fc and TNFR2-Fc, confirming the results obtained in the cytotoxicity bioassay (Fig. 3A-B). Notably, the K_d for the binding affinity of TNFR-IgG to TNF-α was significantly lower than values reported for type 1 cell-surface or soluble TNFR (sTNFR) (470– 660 nM) [16, 25, 27]. Thus, TNFR-IgG appears to bind to TNF-α with 6- to 8-fold higher affinity than type 1 cell surface TNFR or
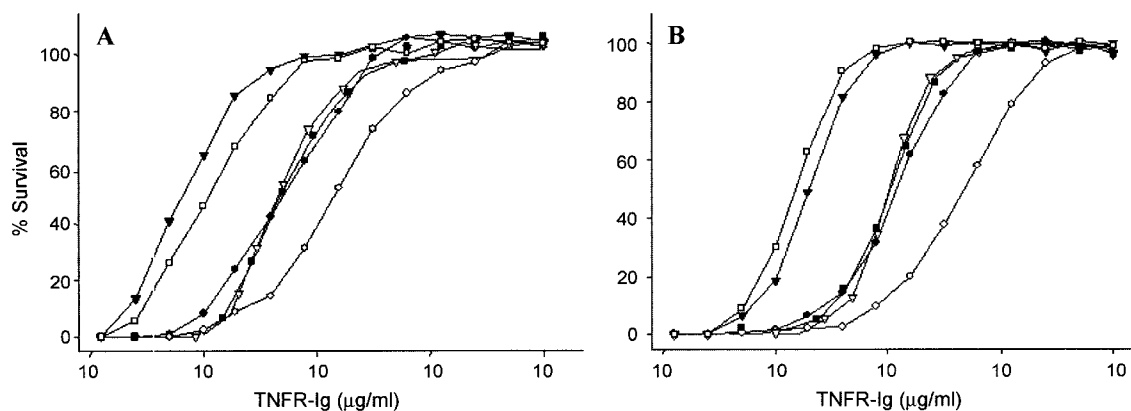

Fig. 3. Inhibition of TNF cytotoxicity by TNFR-Ig in vitro. (A) Effect of TNFR1-TNFR1-Ig (▼), TNFR2-TNFR2-Ig (□), TNFR1-Ig (▽), TNFR2-Ig (●), TNFRI-Fc (■), TNFRII-Fc (○) to human TNF-α on the killing of actinomycin D-treated murine L929 cells induced by TNF-α (1 ng/ml). (B) Effect of TNFR1-TNFR1-Ig (▼), TNFR2-TNFR2-Ig (□), TNFR1-Ig (▽), TNFR2-Ig (●), TNFRI-Fc (■), TNFRII-Fc (○) on cell killing by TNF-β (1 ng/ml).

sTNFR. This higher affinity may be due to a multivalent interaction between TNFR-IgG and TNF-α with a trimeric structure [6, 11, 31]. Two TNFR domains of a TNFR-IgG molecule interact with one TNF-α trimer, which may result in a more stable binding interaction. Therefore, it is concluded that concatameric TNFR-Ig fusion proteins have higher affinity than the simple TNFR-Ig fusion proteins and conventional TNFR-Fc due to increasing structural binding. As a result, concatameric TNFR-Ig fusion proteins showed higher affinity than the simple TNFR-Ig fusion proteins and TNFR-Fc (R&D) in the receptor inhibitor binding assay (RIBA).

Inhibition of Cytotoxicity by Concatameric TNFR-Ig

To examine the ability of TNFR-Ig fusion proteins to antagonize TNF activity in vitro, the effect of TNFR-Ig fusion proteins on the induction of cell lysis was investigated by using TNF-α in actinomycin D-treated murine L929 cells (Fig. 3A). Concatameric TNFR-Ig fusion proteins were able to block the killing of cells completely, with 50% inhibition (IC_{50}) occurring at 9 ng/ml and 15 ng/ml, respectively. For comparison, simple TNFR-Ig fusion proteins were tested and an IC_{50} of 63 ng/ml and 189 ng/ml was found, respectively. Thus, on a molar basis, concatameric TNFR-Ig fusion proteins were 4- to 21-fold more efficient than simple TNFR-Ig fusion proteins in blocking the cytolytic action of TNF-α. The ability of concatameric TNFR-Ig fusion proteins to block the cytolytic activity of TNF-β was also tested (Fig. 3B). Complete inhibition of cell killing was achievable, with an IC_{50} of 20 ng/ml and 25 mg/ml, respectively. For comparison, simple TNFR-Ig fusion proteins were tested and an IC_{50} of 129 ng/ml and 469 ng/ml was found, respectively (Table 2). Thus, on a molar basis, concatameric TNFR-Ig fusion proteins were 6- to 23-fold more efficient than simple TNFR-Ig fusion proteins in blocking the cytolytic action of TNF-β. Thus, concatameric TNFR-Ig fusion proteins were less efficient by a factor of 2 in blocking TNF-β rather than TNF-α, which is consistent with its lower affinity for TNF-β. These results show that concatameric TNFR-Ig fusion proteins act as a full antagonist in vitro against both types of TNF. In fact, soluble TNF-α and

Table 2. IC_{50} Mass ratio: Comparison of efficacy of different TNFR-Ig fusion proteins vs TNF-α and TNF-β.

Soluble TNFR-Ig fusion protein		IC_{50} (ng/ml)[a]	
		TNF-α	TNF-β
Simple TNFR-Ig	TNFR1-Ig	63	129
	TNFR2-Ig	189	469
Concatameric TNFR-Ig	TNFR1-TNFR1-Ig	9	20
	TNFR2-TNFR2-Ig	15	25

The IC_{50} values were calculated by graphic interpolation of the dose-response curve of each experiment.

TNF-β actually promote apoptosis on cells that express the transmembrane forms, TNFR1 or TNFR2 [6, 11, 27, 29]. Thus, the approach of this study combining two receptor extracellular domains transformed an agonist into concatameric TNFR-Ig fusion proteins and validated the TNF trap concept. Concatameric homodimers of TNFR1-TNFR1-Ig and TNFR2-TNFR2-Ig were substantially more potent blockers of TNF action in vitro than the corresponding simple dimers of TNFR1-Ig and TNFR2-Ig (Fig. 3A-B). As a result, concatameric homodimers of TNFR1-TNFR1-Ig and TNFR2-TNFR2-Ig were ~10- and 20-fold more effective than the simple dimers of TNFR1-Ig and TNFR2-Ig in blocking the cytolytic action of TNF-α and TNF-β, respectively, as an antagonist of human TNF activity (Fig. 3A-B). These results show that concatameric TNFR-Ig, a molecule that combines the TNF binding function of the extracellular portion of type 1 TNFR and type 2 TNFR with the dimeric structure of IgG_1, is a potent TNF antagonist. At the molecular level, concatameric TNFR-Ig fusion proteins exhibit significantly higher affinity for TNF-α and TNF-β than simple TNFR-Ig or conventional TNFR-Fc. In addition, a molar binding ratio of 2.5:1 TNF-α trimer to concatameric TNFR-Ig fusion proteins suggests that this higher affinity may be due to an increase in bivalent binding to TNF-α. At the cellular level, concatameric TNFR-Ig fusion proteins completely block the cytolytic action of TNF-α or TNF-β, in murine L929 cells, and are markedly more potent than simple TNFR-Ig or conventional TNFR-Fc in blocking TNF-α. The difference in TNF-α binding affinity between concatameric TNFR-Ig fusion proteins and simple TNFR-Ig probably contributes to the differential efficiency of these forms of TNFR in blocking the TNF-α cytolytic activity. However, the difference between concatameric TNFR-Ig fusion proteins and simple TNFR-Ig in blocking TNF-α (23-fold) is significantly greater than the difference in affinity (2- to 8-fold). Previous work with anti-TNFR antibodies showed that bivalent but not monovalent antibody fragments can activate TNFR [27], indicating that a TNF-α trimer may trigger signal transduction by cross-linking two cell surface TNFR molecules. Also, concatameric TNFR-Ig fusion proteins can simultaneously block four receptor binding sites on two TNF-α trimers, thus rendering TNF-α unable to dimerize cell surface receptors, and may contribute to the greater efficiency of TNFR-Ig versus simple TNFR-Ig in blocking TNF-α. Therefore, in this study, novel concatameric TNFR-Ig fusion proteins were found to be useful as a therapeutic reagent in curing various forms of TNF-related human diseases, such as MS, RA, and IDDM.

Concatameric TNFR-Ig Blocks TNF Action In Vivo

To investigate the ability of concatameric TNFR-Ig fusion proteins as a TNF antagonist in vivo, a model for collagen-induced arthritis (CIA) in mice was used (Fig. 4). First, in
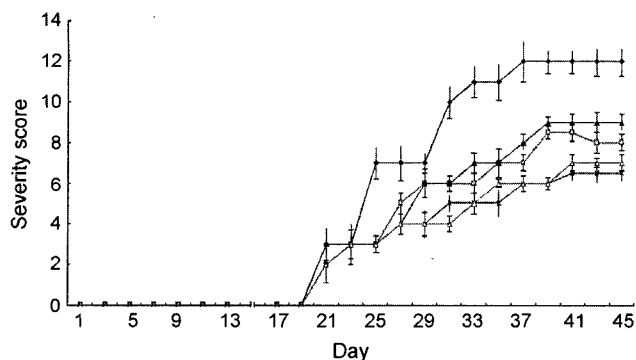
Fig. 4. Arthritis severity score (mean cumulative score of all 4 paws ± s.e.m.) of DBA/1 mice after induction of CIA by collagen immunization and treatment with either PBS (◆; n=5), TNFR1-Ig (■; n=5), TNFR2-Ig (▲; n=5), TNFR1-TNFR1-Ig (✕; n=6), TNFR2-TNFR2-Ig (△; n=6) at 3 mg per mouse 3 times per week for a total of 8 injections starting at day 20.

vivo, the activity of human concatameric TNFR-Ig fusion proteins were tested using mice (3 mg per mouse) which received an intraperitoneal injection of purified TNFR-Ig fusion proteins. Concatameric TNFR-Ig fusion proteins accessed and blocked TNF in the inflamed joints of the mouse CIA model in which the injection of bovine collagen induced an autoimmune arthritic response in DBA/1 mice [12]. As a result, concatameric homodimers of TNFR1-TNFR1-Ig and TNFR2-TNFR2-Ig were 10–30% more effective than the simple dimers of TNFR1-Ig and TNFR2-Ig, respectively (Fig. 4). These results show that concatameric TNFR-Ig fusion proteins can prevent or significantly delay CIA in mice when given prior to or shortly after collagen injection. These data also show that concatameric TNFR-Ig fusion proteins have higher affinity than simple TNFR-Ig fusion proteins and conventional TNFR-Fc as a TNF antagonist *in vivo*. Therefore, concatameric TNFR-Ig fusion proteins are expected to block TNF in human diseases such as MA, RA, and IDDM. At the level of the whole organism, concatameric TNFR-Ig fusion proteins can prevent or protect against CIA in mice, depending on the dose and time of injection. This confirms the hypothesis that TNF-α is a key contributor to rheumatoid arthritis, first suggested by the ability of anti-TNF-α antibodies and TNFR-IgG to protect against rheumatoid arthritis [2, 29]. The ability of concatameric TNFR-Ig fusion proteins to provide protection at the dose tested in this study appears limited to about 7 weeks after collagen injection although this remains to be investigated *in vivo*.

So far, the exact cause of RA is not known. However, in the last decade, researchers have determined that people with RA tend to have an excess of protein called TNF-α. TNF-α triggers inflammation as part of the body's normal immune system response. Overproduction of TNF-α can lead to excessive inflammation such as that found in patients with RA. As TNF-α builds up in the joints, it leads

to joint inflammation, which can ultimately result in joint destruction. Because of its role in the progression of RA, blocking the activity of TNF-α has become a key focus of new RA therapies such as human monoclonal antibody therapy (adalimunab, HUMIRA) and TNFR-Fc fusion protein therapy (Etanercept, Enbrel). Finally, concatameric TNFR-Ig fusion proteins are generated by the recombination of the relevant cDNAs. Concatameric TNFR-Ig fusion proteins were far more effective as a TNF inhibitor than simple TNFR-Ig fusion proteins *in vitro* and *in vivo*. Concatameric TNFR-Ig fusion proteins potently block TNF *in vitro* and *in vivo* and represent a substantial advance towards creating a novel therapeutic candidate for TNF-driven diseases.

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