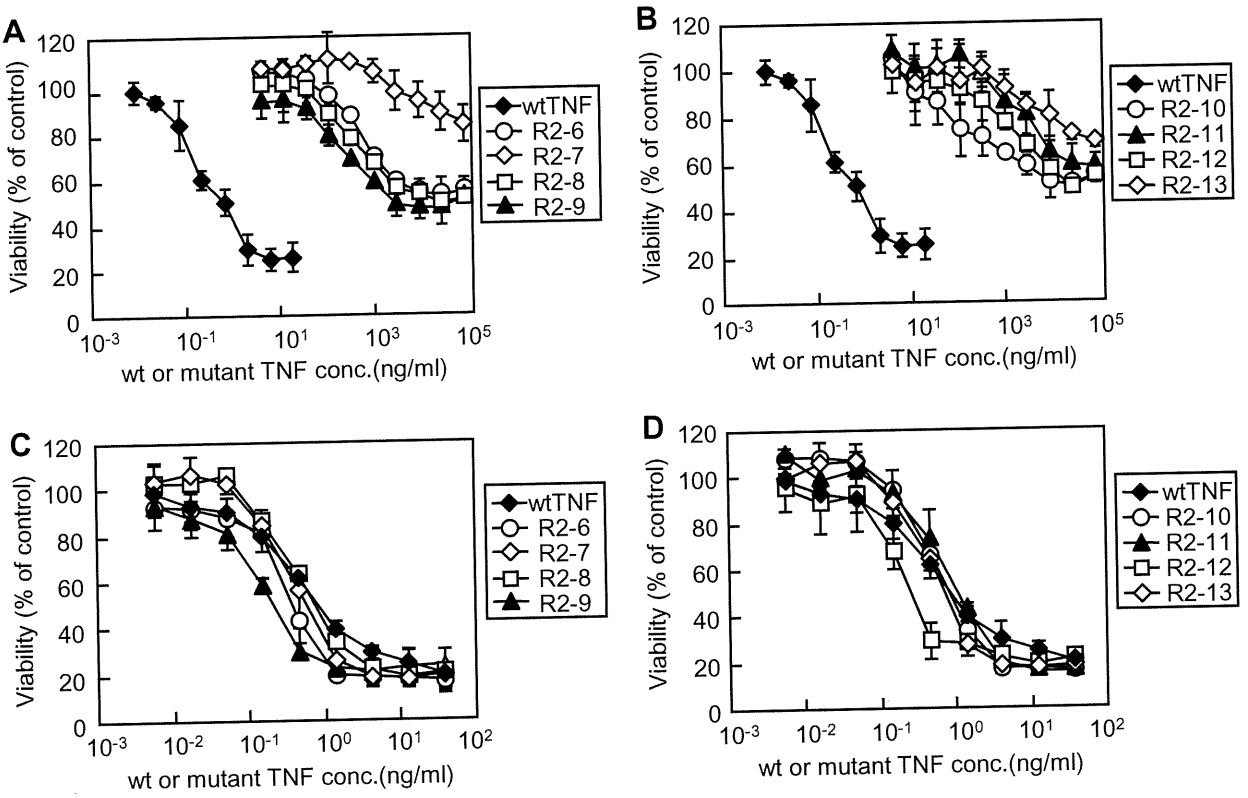

Fig. 4. In vitro bioactivity assay of TNF mutants via TNFR1 or TNFR2. The bioactivity of mutant TNFs via TNFR1 or TNFR2 were measured by cytotoxicity assay against HEp-2 cells (A and B) or hTNFR2/mFas-PA (C and D), respectively. Each point represents the mean ± S.D. of triplicate measurements.

3.2. Selection of TNFR2-selective TNF mutants by one-step competitive panning

To concentrate TNFR2-selective mutant TNFs, the TNF structural mutant displaying phage library was subjected to two rounds of conventional panning or competitive panning against TNFR2 using the BIAcore biosensor. After the second round of panning, *Escherichia coli* (TG1) supernatants of 54 randomly picked clones from each panning procedure were further screened by capture ELISA to analyze their binding specificities for each TNFR (Fig. 3). Consequently, we obtained numerous clones with high-affinity for TNFR2 under all panning conditions. Binding avidities of these clones for TNFR1 tended to decrease depending on the concentration of TNFR1-Fc used for premixing. However, binding avidity of a TNFR2-selective clone, which binds only to TNFR2 (Fig. 3, black bar), tended to increase depending on the concentration of TNFR1-Fc used for premixing. Almost all clones obtained from the conventional and competitive panning with 0.1 pmol of TNFR1-Fc (Fig. 3A and B, respectively) bound to TNFR1, and the panning efficiency for isolating the TNFR2-selective TNF mutants was <2%. In contrast, clones obtained from the subtracted panning with 1 or 10 pmol of TNFR1-Fc (Fig. 3C and D, respectively) contained many TNFR2-selective TNF mutants (>20%). From these panned clones, we eventually identified eight candidate agonists that selectively and strongly bound to the TNFR2. Amino acid sequences of these eight candidate TNFR2-selective TNF mutants are shown in Table 1. TNFR2-slective mutants were mutated near residue 145 and

Table 3
In vitro bioactivities of TNF mutants via TNFR1 or TNFR2.

	TNFR1[a]		TNFR2[b]		TNFR2/TNFR1[e]
	EC50[c] (ng/ml)	Relative Activity[d] (%)	EC50[c] (ng/ml)	Relative activity[d] (%)	
wtTNF	0.6	100	0.56	100	1.0
R2–6	8.1×10^3	7.3×10^{-3}	0.39	144	2.0×10^4
R2–7	$>1.0 \times 10^5$	$<6.0 \times 10^{-4}$	0.51	110	1.8×10^5
R2–8	4.6×10^3	1.2×10^{-2}	0.67	84	7.0×10^3
R2–9	2.1×10^3	2.8×10^{-2}	0.21	267	9.5×10^3
R2–10	1.1×10^4	5.4×10^{-3}	0.72	78	1.4×10^4
R2–11	6.7×10^4	8.9×10^{-4}	0.95	59	6.6×10^4
R2–12	2.6×10^4	2.2×10^{-3}	0.23	243	1.1×10^5
R2–13	$>1.0 \times 10^5$	$<6.0 \times 10^{-4}$	0.63	89	1.5×10^5

[a] Bioactivities of the wtTNF and TNF mutants via TNFR1 were measured by determining the TNF-induced cytotoxicity in HEp-2 cells.
[b] Bioactivities of the wtTNF and TNF mutants via TNFR2 were measured by determining the TNF-induced cytotoxicity in hTNFR2/mFas-PA.
[c] Experimental data were analyzed by a logistic regression model to calculate the mean effective concentration (EC50).
[d] Relative activities were calculated from the EC50 (wtTNF)/EC50 (TNF mutants).
[e] Selectivity for TNFR2 was calculated from the ratio of the relative activity (via TNFR2)/relative activity (via TNFR1).

conserved near residue 30. These findings indicate that the amino acid residues near position 30 are an essential for TNFR2 binding.

3.3. Binding kinetics of TNFR2-selective TNF mutants

To investigate the properties of eight TNFR2-selective TNF mutants in detail, we prepared recombinant protein using the previously described methods [30,31]. TNF mutants expressed as an inclusion body in *E. coli* (BL21λDE3) were denatured and refolded. Then, active TNF mutants were purified by ion–exchange and gel–filtration chromatography. TNF mutant purity was greater than 90% in sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and all mutants were confirmed to form homotrimers in the same manner as the wtTNF by gel-filtration analysis (data not shown). To analyze the binding properties of these TNFR2-selective TNF mutants, we determined their binding dissociation constants (kinetic on- and off-rates) for TNFR1 and TNFR2, respectively, in detail using the surface plasmon resonance technique (Table 2). Our analysis showed that all eight mutant TNFs bound to the TNFR2 with high affinity; in contrast, they bound to the TNFR1 with greatly reduced affinity (typically between 1 and 7% of the wtTNF affinity). The dissociation constants (K_D) of these mutants for TNFR2 were between 2.1–3.6 $\times$ 10^{-10} M, and their relative affinities for TNFR2 were between 169 and 291% of that of the wtTNF. Thus, using the competitive panning technique we successfully obtained a large repertoire of TNFR2-selective TNF mutants with different binding parameters (on- and off-rates and dissociation constants).

3.4. Bioactivities of TNFR2-selective TNF mutants

To examine the bioactivity of these TNF mutants via TNFR1, we subsequently performed a cytotoxicity assay using HEp-2 cells (Fig. 4A and B). All TNF mutants (R2-6 ~ R2-13) showed almost no cytotoxicity, and the bioactivity was much lower than that of the wtTNF. Next, we evaluated the TNFR2-mediated activity of TNF mutants using the hTNFR2/mFas-PA, which were previously constructed in our laboratory [26]. The TNFR2-mediated bioactivities of these 8 mutant TNF proteins were at least same or higher than that of the wtTNF (Fig. 4C and D). As a negative control, we determined TNF cytotoxicity in parental TNFR1$^{-/-}$R2$^{-/-}$ preadipocytes and observed no wtTNF- or mutant TNF-mediated cell death (data not shown). Results of the cytotoxicity assay are summarized in Table 3. R2-7, the most highly TNFR2-selective mutant, exhibited 1.8 $\times$ 10^5 fold higher TNFR2-selectivity than that for the wild-type TNF.

4. Discussion

Recently, it was revealed that the two TNFRs worked together by crosstalk signaling, which suggested that the TNF-mediated signaling in the presence of both TNF receptors actually correlates with their physiological functions [32–34]. To understand the mechanism as well as to analyze the structure–function relationship of the TNFRs, several attempts were made in the past to create TNFR-specific mutant TNFs by conventional site-directed mutagenesis methods (such as Kunkel's method) [35–37]. However, these attempts were not very successful in yielding a desired TNF mutant having high receptor specificity and full bioactivity. For example, the TNFR2-binding affinity of the double mutant D143N-A145R was about 5–10 fold less than the wtTNF [38]. To overcome these problems, we applied phage-display technique and optimized panning method using the BIAcore biosensor (Fig. 1). Using an adequate amount of selective competitive inhibitor (>1 pmol TNFR1-Fc), this one-step competitive panning is ten times more efficient for screening TNFR2-selective TNF mutants, suggesting the competitive panning technology described here is a simple and effective screening method for fine-tuning TNF receptor-selectivity (Fig. 3). As a result of screening, we obtained successfully obtained TNFR2-selective TNF mutants with full bioactivity via TNFR2 (Table 3). Because of its high TNFR2-selectivity and full bioactivity, the TNF mutant R2-7 would help in elucidating the functional role of TNFR2.

One advantage of our phage-display-based technique is that it can be used to obtain the sequence information of many mutants [39,40]. It was previously shown by site-specific mutagenesis technique that mutations at positions 29, 31 and 32 (L29S, R31E and R32W) remarkably reduced the TNF's affinity for binding to TNFR2 [35,37,38]. For most of the TNFR2-selective TNF mutants, amino acids at positions 29, 31 and 32 were indeed identical (except for the R2-7 mutant which contained a conserved L to V substitution at position 29) to those of the wtTNF (Table 1), which is consistent with the previously reported idea that these three amino acids play critical roles in maintaining the binding between the TNF and TNFR2. The amino acid sequence at positions 145, 146 and 147 of the TNFR2-selective TNF mutants were, however, very different from those of the wtTNF. For example, the amino acid residue at position 145 of the TNF mutants R2-7, R2-12 and R2-13 contained an Asp residue in place of the Ala residue, and all of them showed high TNFR2 selectivity. Structural analysis and mutagenesis studies suggested that the loop containing the residues 145–147 is involved in the receptor binding [41–43]. Since Asp is a comparatively large residue, we speculated that this substitution could lead to a steric hindrance disrupting the interaction between the TNFR1 and TNFR2-selective mutants, which may be why they are less TNFR1-selective. However, why this replacement would increase the selectivity for TNFR2 is unclear at this moment. Currently, we are working on determining the structure of the TNF/TNFR2 complex by X-ray crystallography [44] so that structure–activity relationship studies could be initiated in the near future. Additionally, this structural information, in combination with bioinformatics technology, will be useful for designing TNFR-selective inhibitors (peptide mimics and chemical compounds).

5. Conclusions

In this study, we optimized our phage display-based screening using a unique competitive panning technique, which is ten times more efficient for screening TNFR2-selective TNF mutants compared to the conventional panning method. As a result of screening, we have succeeded in isolating several TNFR2-specific TNF mutants with high TNFR2 affinity and full bioactivity via TNFR2. Further analysis of the relationship between the structure and bioactivity of the TNF mutants would offer highly valuable and useful information regarding the TNF/TNFR biology. In conclusion, our fine-tuned competitive panning system is a simple and effective technology for isolating receptor-selective mutant proteins.

Acknowledgment

This study was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and from the Japan Society for the Promotion of Science (JSPS). This study was also supported in part by Health Labour Sciences Research Grants from the Ministry of Health, Labor and Welfare of Japan, and by Health Sciences Research Grants for Research on Publicly Essential Drugs and Medical Devices from the Japan Health Sciences Foundation.

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