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—Reviews—

タンパク療法の最適化に向けた新規タンパク性アンタゴニストの創製と DDS への展開

阿部康弘

Development of Novel DDS Technologies for Optimized Protein Therapy by Creating Functional Mutant Proteins with Antagonistic Activity

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In the post-genomic era, cytokine or antibody therapy has received attention for advanced drug therapies. Indeed, attempts are being made to develop a wide variety of therapeutic proteins for diseases including cancer, hepatitis and autoimmune conditions. Unfortunately, however, the utilization of bioactive proteins in clinical practice is often limited because of their inherent instability and pleiotropic actions *in vivo*. Our laboratory aims to overcome two major problems, details of which will be addressed in separate sections to follow. (i) Development of a powerful system to rapidly create functional mutant proteins (muteins) with enhanced receptor affinity and receptor specificity using a phage display technique (biological DDS). (ii) Establishment of a novel polymer-conjugation system to dramatically improve *in vivo* stability and selectively of bioactive proteins (polymeric DDS). We are currently attempting to combine both approaches to create a protein-drug innovation system to further promote pharmaco-proteomic-based drug development. In this review, we will describe DDS-based technology for creating functional mutants for advanced medical applications, using tumor necrosis factor-alpha (TNF) as an example.

Key words——phage display system; tumor necrosis factor-alpha; bioconjugation

1. はじめに

近年の疾患プロテオミクスの進展に伴う国内外の研究から，様々な疾患の発症や悪化に関与するタンパク質（創薬ターゲット）や，逆に病態の治癒に係わるタンパク質（医薬品シーズ）が同定され，[1] これらを医薬品開発へ有効活用しようとするタンパク療法の確立が待望されている．しかし，過去の事例からも明らかなように，タンパク質は一般に，体内安定性に極めて乏しいため，臨床応用の際には大量頻回投与を余儀なくされ，往々にして重篤な副作用を招いてしまう．なかでもサイトカインなどは，多彩な細胞上の複数種類のレセプターを介して，多様な *in vivo* 生理活性を示すため，目的とする治療作用のみならず副作用の原因となる他の作用までをも同時に発揮してしまう．[2-4] そのため，タンパク質の臨床応用は著しく制限されており，医薬品化に成功した例は極めて少ない．したがって，疾患プロテオミクス情報などを有効活用したプロテオーム創薬を推進し，有効かつ安全なタンパク療法を確立していくためには，これらタンパク質固有の問題点を克服しうる創薬テクノロジー，すなわちタンパク療法の最適化を目指した Drug Delivery System（DDS）の確立が，依然として必須となっている．本観点から筆者らは，1）レセプター親和性・特異性等が高く医薬価値に優れた機能性人工タンパク質を迅速創製できるタンパク質分子進化戦略（生物学的 DDS）の構築，2) タンパク質の生体内安定性を向上させ，かつ目的治療作用の選択的発現能を付与できる高分子バイオコンジュゲーション法（高分子化学的 DDS）の確立に関する研究を推進している．本稿では，上述した 1)，2）を融合させた DDS 基盤テクノロジーについて，自己免疫疾患治療薬の創薬ターゲットとして注目されている腫瘍壊死因子

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(Tumor necrosis factor-alpha; TNF) に対する分子標的タンパク医薬の創出を1例に概説する.

2. 抗TNF阻害薬の問題点

慢性関節リウマチや多発性硬化症等の自己免疫疾患は，いまだ克服すべき難病の1つとして広く認識されている．そのため，自己免疫疾患を標的とした創薬プロテオミクス研究が盛んに行われており，広範な炎症の惹起・悪化におけるKey moleculeの1つとして，TNFが創薬ターゲットとなっている.[5-7] 一方でTNFは，発がんや種々感染症に対する生体防御活性の中心を担っていることも明らかとなっているが，病態（炎症）の発症・悪化と生体防御活性の発揮とのバランスや2種類の異なるレセプター（TNFR1及びTNFR2）を介した機能の相違等は十分に理解されていない.

現在，慢性関節リウマチに対する特効薬として，TNFに対する中和抗体や可溶型TNFレセプターが臨床に供されるようになり，患者のQOLを格段に向上させる等，切れ味鋭い治療成績を発揮している.[8,9] しかし上述のように，TNFは本来，宿主の生体防御機構に重要な役割を担っているため，これらTNF阻害薬の使用は，結核等の感染症や発がんに対する宿主の抵抗性を減弱させてしまうため，臨床現場における大きな懸念事項となっている.[10,11] また自己免疫疾患の中でも，多発性硬化症では，逆に病態悪化が認められたことから,[12] TNF阻害薬の使用は禁忌となっており，これら問題点を克服し得る新たな抗TNF治療戦略の確立が求められている.

一方で動物モデルを使った検討から，可溶型TNFのTNFR1を介した過剰な活性発現が炎症反応の惹起・悪化に，可溶型／膜結合型TNFのTNFR2を介した活性発現がウイルス感染防御や多発性硬化症の抑制に関与していることが明らかとなりつつある.[13-15] これは，可溶型TNFのTNFR1を介した活性発現を選択的に阻害することができれば，慢性関節リウマチのみならず，既存のTNF阻害薬では適用外であった多発性硬化症等の自己免疫疾患にも安全かつ有効な新規治療戦略を提示できるものと期待される.

以上の観点から，近年，抗体医薬品の進展に伴い，特定分子をターゲットにする治療戦略として，TNFレセプター中和抗体が，炎症性疾患に対する有効な治療薬に成り得るものとして，その作製が試みられてきた．しかし，各TNFレセプター中和抗体がTNFのような作用，すなわちアゴニスト作用を発現する場合があることが報告され,[16] 上記の疾患モデルにおいて効果のある中和抗体の作製については報告されていない．したがって，TNFR1に選択的な抗体とは機能的・性状的に異なるタンパク性アンタゴニストが作製できたなら，上述した副作用を克服できる可能性があるだけでなく，これまでTNF阻害剤を適応できなかった疾患の治療へ適応可能であり，様々な炎症性疾患に対する画期的な治療戦略を確立できるものと期待される．そこで次項では，後述するファージ表面提示法を駆使することで，TNFR1指向性を有したタンパク性アンタゴニスト（機能性人工TNF）の探索・創出を試みた.

3. ファージ表面提示法を用いた生物学的DDS

タンパク療法の最適化に向け，従来から産官学の多くのバイオ研究機関が，特定レセプターへの親和性や選択性に優れた機能性人工タンパク質などを創製するため，Kunkel法といった点突然変異法を用いた構造変異タンパク質（アミノ酸置換体）の作製を精力的に試みている.[17-19] しかし点突然変異法では，まず構造変異タンパク質の立体構造や機能をシミュレーションし，トライ・アンド・エラーで生理活性タンパク質の構成アミノ酸を1つずつ別の特定アミノ酸に改変することにより，個々の構造変異タンパク質を作製せねばならない．そのうえで目的とする機能性人工タンパク質を探索・同定するため，作製した構造変異タンパク質の諸機能を個別に評価する必要がある．そのため従来法では，時間ばかりが消費され，かつ作製し得る構造変異タンパク質の多様性（種類）にも限界があるなど，期待通りの成果は得られていない.

この点，筆者らはファージ表面提示法を独自に改良することにより10^8（1億）種類以上もの多様性を有した構造変異タンパク質（アミノ酸置換体）を一挙にCombinatorial Biosynthesisし，この構造変異体ライブラリの中から，レセプター親和性（選択性／特異性）や体内安定性，生物活性などを向上あるいは任意に制御した「医薬価値に優れた機能性人工タンパク質」を迅速（2週間以内）かつ効率よく同定できる基盤テクノロジーを確立してきた（Fig. 1)．これまでに筆者らは，この独自のテクノロジー

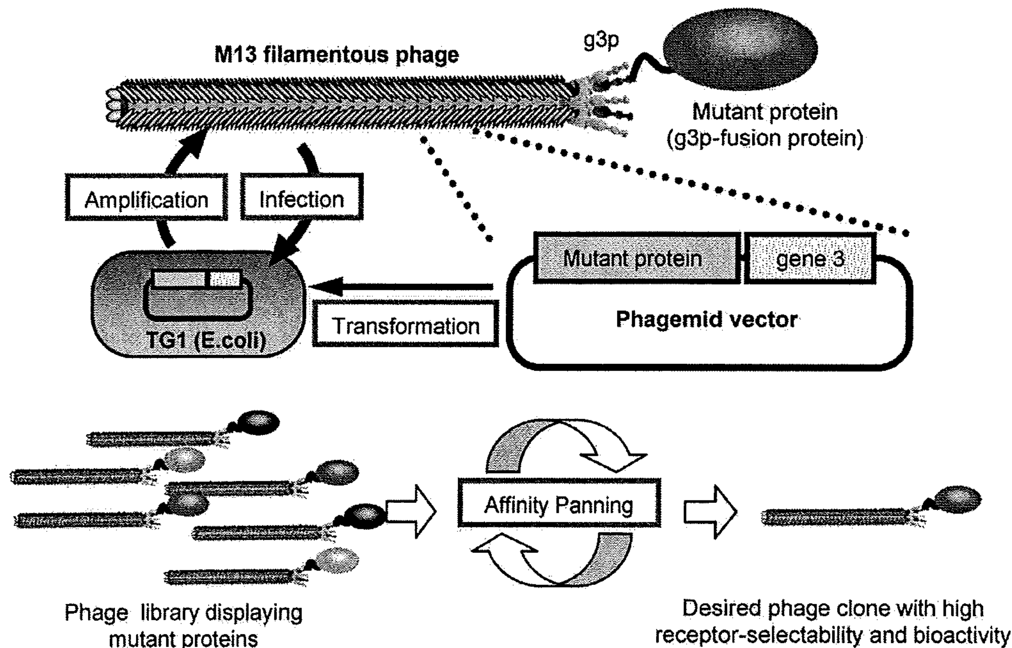

Fig. 1. Creation of Functional Mutant Proteins Using Phage Display

Phage display system has the following main characteristics: 1) proteins can be displayed on the outer shell of the phage where they can interact with their target molecules, such as a receptor or antigen. These protein-displaying phage particles are produced by the integration of a foreign gene into the 5'-terminus of the gene that encodes the outer shell of the phage (*i.e.*, g3p) in phagemid vector or phage genome; 2) the genotype of this phage (the foreign gene inside the phage clone), corresponds with the phenotype (the protein displayed on the phage's surface); 3) phage particles or "libraries", can readily be made, which consist of billions of varieties of protein; 4) a selected phage from the library can be readily amplified by infection of a host bacterial cell. It is therefore possible to screen for, and then isolate, high-affinity binders to target molecules from the phage library.

を駆使し，活性発現や三量体形成に重要な K11 や K65, K90 などを含む全 6 個のリジン残基を一挙に他のアミノ酸へ置換しても，wtTNF と同等さらには 10 倍以上もの生物活性やレセプター親和性を有する機能性リジン欠損 TNF 変異体を創出することに初めて成功している（Table 1).[20] そこで本テクノロジーを駆使することで，TNF のレセプターとの結合領域に位置する計 6 ヵ所のアミノ酸残基を網羅的に他のアミノ酸に置換した構造変異 TNF 発現ファージライブラリを作製し，スクリーニングした結果，「TNFR2 とは結合せず，TNFR1 に対してのみ野生型 TNF と同等の結合親和性を示す TNFR1 指向性アンタゴニスト（TNF-T2)」が初めて創出できた（Fig. 2).[21] これまで，生理活性タンパク質の構造変異体が野生型タンパク質により発現する生物活性に対してアンタゴニスト活性を示すという概念すらなく，この「タンパク性アンタゴニスト」とも言うべき TNFR1 指向性アンタゴニストの創出は，独自に構築した基盤テクノロジーを応用することで初めてなし得たものである．またレセプター指向性を有した TNF-T2 の創出基盤は，生物学的改変により特定のレセプターへのターゲティング能をタンパク質に付与できる点で，分子レベルの DDS であり，いわばタンパク医薬による疾病治療の最適化を目指した「生物学的 DDS」と位置づけられる．

Table 1. Amino Acid Sequences and *in vitro* Bioactivity of Lysine-deficient Mutant TNF (K90R)

	Residue Position						EC_{50}[1) (ng/ml)
	11	65	90	98	112	128	
wtTNF	K	K	K	K	K	K	1.28
K90R	A	S	R	A	L	T	0.12

1) The bioactivities of wild-type TNF (wtTNF) and K90R were measured by cytotoxic assay using HEp-2 cells in the presence of cycloheximide (50 μg/ml). Experimental data were analyzed by a logistic regression model to calculate the mean effective concentration (EC50).

4. 高分子化学的 DDS による部位特異的バイオコンジュゲーション

関節リウマチなどの慢性炎症性疾患を対象とした検討においては，往々にして，血中濃度の維持を目的とした薬物の長期的な投与が必要であるが，

TNF-T2も例外でない．タンパク性薬物の最大の問題点は，その生体内安定性の低さであると考えられる．そのため，慢性の疾患に対しTNF-T2を用いる場合，この問題を克服し，長期投与にも耐え得る戦略が必要となる．主として1980年代以降，DDSを視野においた医薬品開発の分野において，生理活性タンパク質の生体内安定性を改善するために，ポリエチレングリコール（PEG）などの水溶性高分子をタンパク質に結合させた，いわゆる高分子バイオコンジュゲーションが考案されてきた．[22-24] このタンパク質のバイオコンジュゲーションは，分子量増大による腎排泄速度の減少をもたらすだけでなく，バイオコンジュゲーションに用いた修飾高分子によりタンパク質の分子表面が覆われるために，プロテアーゼからの攻撃が立体障害的にブロックされ，結果としてタンパク質の生体内半減期が延長される（Fig. 3）．同様の立体障害効果によって，免疫応答においても抗原性及び免疫原性が低下し，体内クリアランスの減少に直結する．以上に述べた総合的な体内安定化効果により，最終的にタンパク質の生体への投与量・回数を削減することが可能となる．このバイオコンジュゲーションは，数あるDDSの中でもタンパク質の医薬品化に向けた最適DDSと位置づけられてきたが，依然としてその成功例は限られている．この最大の原因は，タンパク質の活性中

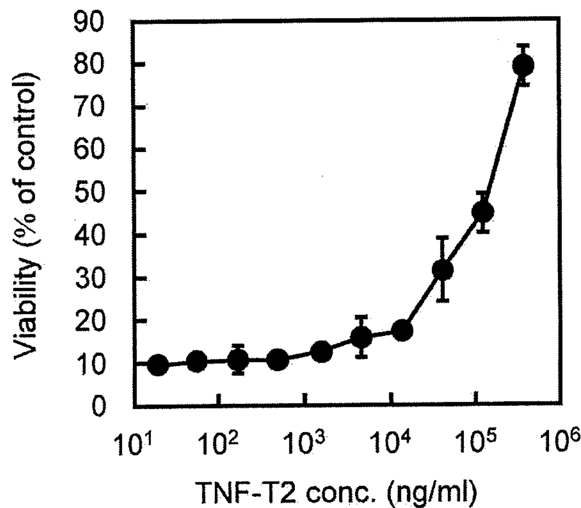

Fig. 2. Antagonistic Activities of the R1antTNF

Serial dilutions of TNF-T2 were mixed with human wtTNF (20 ng/ml) and then applied to HEp-2 cells. After 18 h, the inhibitory effects of TNF-T2 on the cytotoxicity of wtTNF were assessed by using the methylene blue assay. The absorbance of cells without wtTNF was plotted as 100 percent viability. The data represent the mean±SD ($n=3$).

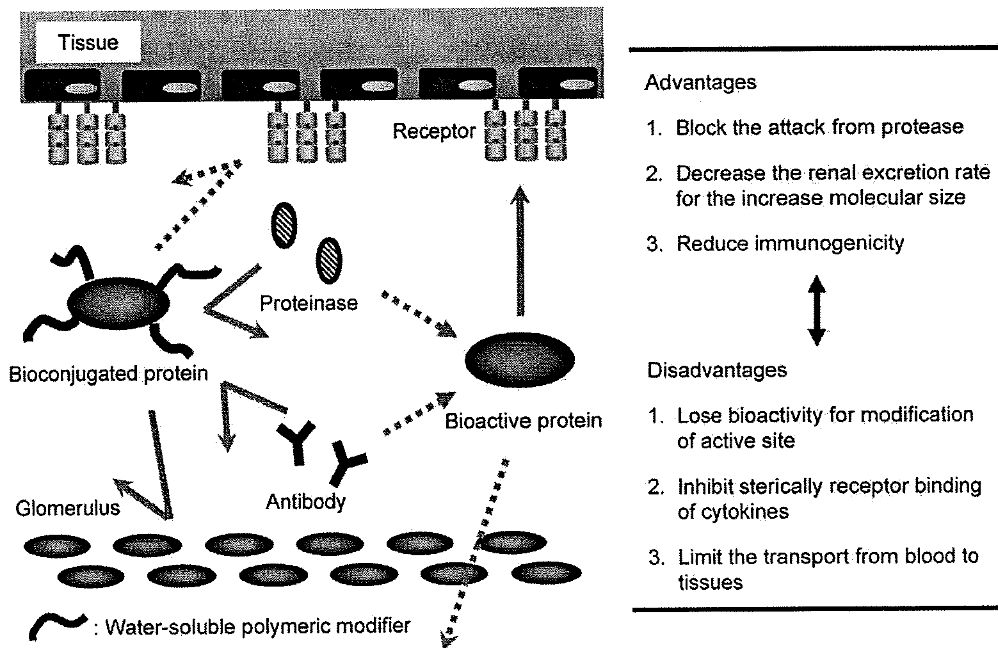

Fig. 3. Characteristics of Bioconjugation

Bioconjugated proteins with water-soluble polymeric modifiers increase their molecular size and steric hindrance, resulting in augmented plasma half-lives and stability.

心に存在するリジン残基へのランダムな水溶性高分子導入による致命的な比活性低下と，バイオコンジュゲート化タンパク質の分子的・機能的不均一性にある．事実，現在C型肝炎の特効薬として市販されているPEG化Interferon-α（PEGASYS）ですら，残存活性10–30％のヘテロ集団であることが報告されている．したがって，タンパク療法の最適化を目指したDDSを推進するためには，部位特異的に効率よく高分子導入でき，高い比活性を有するバイオコンジュゲート体を創製できる方法の確立が待望されている．

この点，筆者らが創出したTNFR1指向性アンタゴニストTNF-T2は，前述したリジン欠損TNF変異体をテンプレートに作製していること，*N*末端アミノ基は活性発現や立体構造形成に無関係であることから，そのアミノ基へのバイオコンジュゲーションを試みた場合，修飾高分子は*N*末端アミノ基にのみ導入されることになり，比活性低下を回避した上で，分子的均一性にも優れたバイオコンジュゲート体を作製可能である．これまでに，*N*末端部位特異的に修飾高分子PEGを導入したPEG化TNF-T2（PEG-T2）が，野生型TNF-T2と比較して，*in vitro*におけるアンタゴニスト活性を低下することなく，血中滞留性が飛躍的に増大していることを見い出している．なお，この革新的な部位特異的バイオコンジュゲーション（高分子化学的DDS）は，前述した生物学的DDSによって機能性リジン欠損タンパク質を創製することで初めて実現可能になるものであり，両テクノロジーの融合で，従来法の諸問題を一挙に克服することに成功したものである．そこで，PEG-T2の新規自己免疫疾患治療薬としての有効性を評価するため，関節リウマチの動物モデルにおける関節炎抑制効果の検討と，既存のTNF阻害薬で致命的問題となっている感染症リスクに及ぼす影響を評価したところ，既存の抗TNF薬とは決定的に異なり，宿主のウイルス感染防御能に全く影響することなく，安全に，関節炎抑制効果を発揮することが判明している（Fig. 4）．すなわち，TNF-T2は内因性のTNFのTNFR2を介した感染防御作用になんら影響しないため，既存の抗TNF阻害薬の致命的問題点であった感染症リスクを回避できることから，安全かつ有効な自己免疫疾患治療薬となり得ることが示された．現在，TNF-T2のさらなる有用性を評価すべく，他の自己免疫疾患モデルに対しての治療実験を進めるとともに，霊長類レベルで医薬品化（関節リウマチ及び多発性硬化症を対象）を目指した研究ステージに移行している．

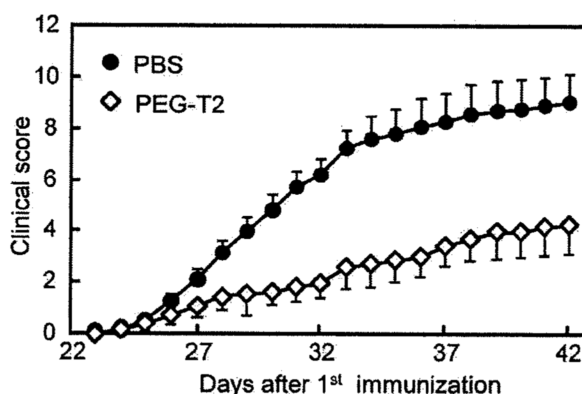

Fig. 4. Clinical Score of Collagen-induced Arthritis (CIA) Mice Treated with PEG-T2

The severity of arthritis in CIA mice ($n=10$) treated with PBS, PEG-T2 (1 μg twice a day) for three weeks from 23 day was assessed every day using an established macroscopic scoring system. Data of severity represent the mean±SEM.

5. おわりに

本稿では，タンパク質そのものに標的指向性を付与する"分子レベルの生物学的DDS"と，機能性人工タンパク質の体内動態制御を可能とする"生体レベルの高分子化学的DDS"の融合開発が，安全かつ有効な次世代型バイオ医薬品の創薬基盤となり得ることを示した．また，プロテオーム創薬は，プロテオミクス及び構造ゲノミクスの進展と，これらの知見を統括したバイオインフォマティクスが駆動力となり，近い将来，上記の「プロテオーム創薬システム」との融合により加速度的に推進されるものと期待される．すなわちこのようなプロテオーム創薬を指向したバイオインフォマティクスの進展は，タンパク質のアミノ酸配列と立体構造，機能との連関を理解可能とするため，近未来的にはタンパク質をコードした塩基配列やタンパク質のアミノ酸配列さえ判明すれば，その立体構造と機能が予測できることになる．これは逆に目的とする機能や立体構造を有した機能性タンパク質の新規デザインを可能とするだけでなく，タンパク質の立体構造やその機能を模倣した低分子化合物の合理的設計をも可能にするものと期待される．このようなバイオインフォマ

ティクスをシステムアップするためには，未知タンパク質の機能解明や立体構造解析に加え，種々のタンパク質について膨大な多様性を有する構造変異体を網羅的に作製し，レセプター・リガンド結合の様式，生物活性等をも含めた機能情報を集積し，立体構造との連関を追求しなければならない．この点筆者らが開発した分子進化戦略は，視点を変えればわずか1週間で1億種類以上もの多様性を有する構造変異体ライブラリを作製し，その機能情報をハイスループットに集積できる基盤技術と言える．本観点から現在，機能性人工TNFを含む様々な構造変異タンパク質の構造–活性相関情報の集積とともに構造情報を基にした低分子阻害剤のドラッグデザインへの研究展開を図ろうとしている．

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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

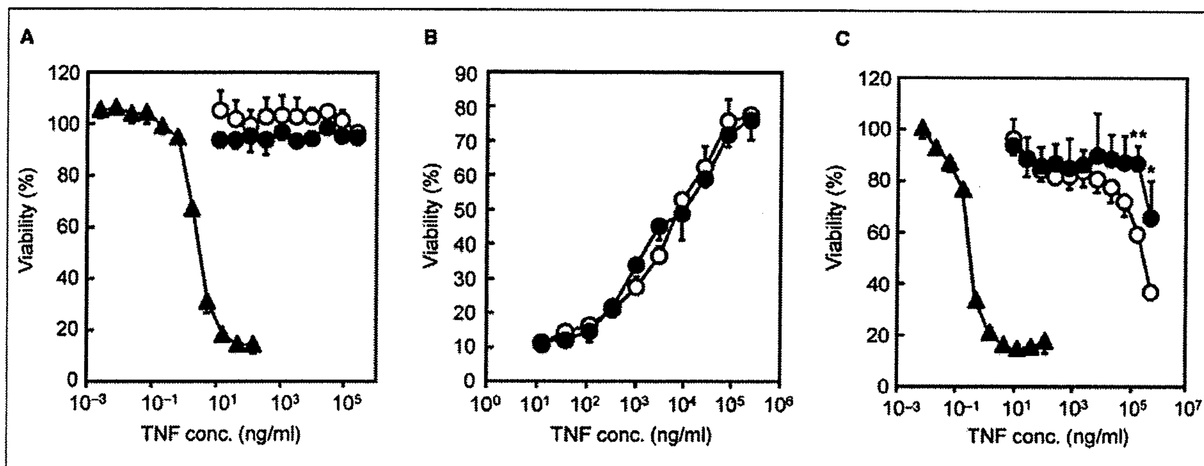

Fig.: Bioactivities and antagonistic activities of N7. (A) To determine the TNFR1-mediated bioactivities, several dilutions of wtTNF (closed triangle), mutTNF (open circle) and N7 (closed circle) were added to L-M cells and incubated for 4 h at 37 °C. (B) Indicated dilutions of mutTNF (open circle) and 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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