ン，増殖因子等）により腫瘍組織へ誘導され，増殖することで，形成されると考えられている．このため，腫瘍組織血管内皮細胞は正常組織血管内皮細胞と構成タンパク質の大部分が類似していると考えられる．事実，われわれが構築した腫瘍組織血管内皮細胞モデルにおいても，2D-DIGE 比較解析の結果，正常組織血管内皮細胞と構成タンパク質の大部分が類似していた．このことからも，パンニングの際，腫瘍組織血管内皮細胞モデルのみを用いてしまうと，正常組織血管内皮細胞と交差性を持つ抗体が選別されてくる可能性が非常に高い．そこで，腫瘍組織血管内皮細胞モデルと抗体ライブラリファージを結合させる前に，正常組織血管内皮細胞と結合させるサブトラクションセルパンニングを行うことで腫瘍組織血管内皮細胞モデル特異抗体の選別・濃縮を図った（Fig. 4）．

まず，抗体ライブラリファージを HUVEC と結合させ，正常組織血管と結合する抗体ファージを除去し，遊離している抗体ファージを Colon26 CM-HUVEC と結合させた．非特異的結合ファージを洗浄後，Colon26 CM-HUVEC 結合抗体ファージを回収し，大腸菌に感染させ，増幅した．このサブトラクションパンニングを計 5 回行った結果，パンニング前のファージ抗体ライブラリ（input ファージ）とパンニングにより回収されたファージ（output ファージ）の比率は，5th パンニング時では，1st パンニング時と比較して，約 10000 倍という高い比率を示したことから，Colon26 CM-HUVEC 特異的に結合する抗体ファージの選別・濃縮に成功したと推測される（Fig. 5）．

さらに，抗体アミノ酸配列を確認するため，各パンニングラウンドのファージ抗体をモノクローン化後，インサート PCR を行い，scFv DNA の増幅が確認されたクローンの scFv アミノ酸シークエンスを解析した．その結果，2 種類の scFv のアミノ酸シークエンス解析に成功した（未発表データ）．獲得した 2 種類の scFv は抗体の抗原認識性を決定する上で非常に重要である 3 ヵ所の相補性決定領域（complementarity-determining regions: CDR1, CDR2, CDR3）を保有し，最も多様性に富むとされている CDR3 領域の中で特に，VH の CDR3 領域においては，アミノ酸の種類，数ともに全く異なっていた．

3-4. 獲得抗体の特性評価　獲得抗体の *in vitro* における抗体特性評価として，ELISA, western blotting による抗原結合性評価，及び抗原分子量の探索を行った（Fig. 6）．

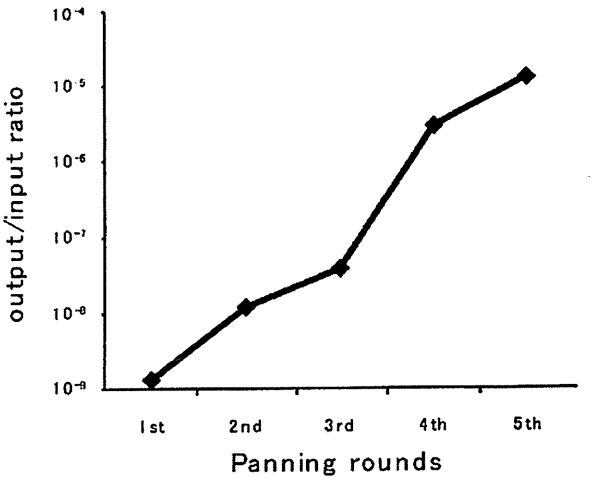

Fig. 5. Enrichment of Antibodies to Tumor Endothelial Cell Model by Subtraction Cell Panning

ELISA による抗原結合性評価の結果，Clone 1 ファージ抗体は，Colon26 へは結合性は示さない一方で，Colon26 CM-HUVEC へは HUVEC への結合性と比較して約 2 倍の抗原結合性を示した．さらにこのファージ抗体の抗原分子量を探索するため，Clone 1 ファージ抗体を用いて western blotting を行った．

Western blotting の結果，Clone 1 ファージ抗体は分子量約 45000 Da のタンパク質に対して結合性を示し，そのバンドの発光強度は ELISA での結果とほぼ相関していた．また，この抗原タンパク質は腫瘍組織血管に高発現しているとされている VEGFR2 とは異なる分子量を示していたことから，VEGFR2 以外の新規腫瘍組織血管マーカーである可能性がある．

さらに獲得抗体の *in vivo* での腫瘍組織集積性を評価するため，獲得ファージ抗体を担がんマウスへ静注し，全身灌流後，腫瘍組織を摘出した．その腫瘍組織内のファージ titer を計測することで獲得抗体の腫瘍組織集積能を評価した．*In vitro* における Colon26 CM-HUVEC への抗原結合性を示した Clone 1 ファージ抗体を担がんマウスへ投与したところ，有意差は得られなかった（$p = 0.08$）が Clone 1 ファージ抗体は野生型ファージと比べて約

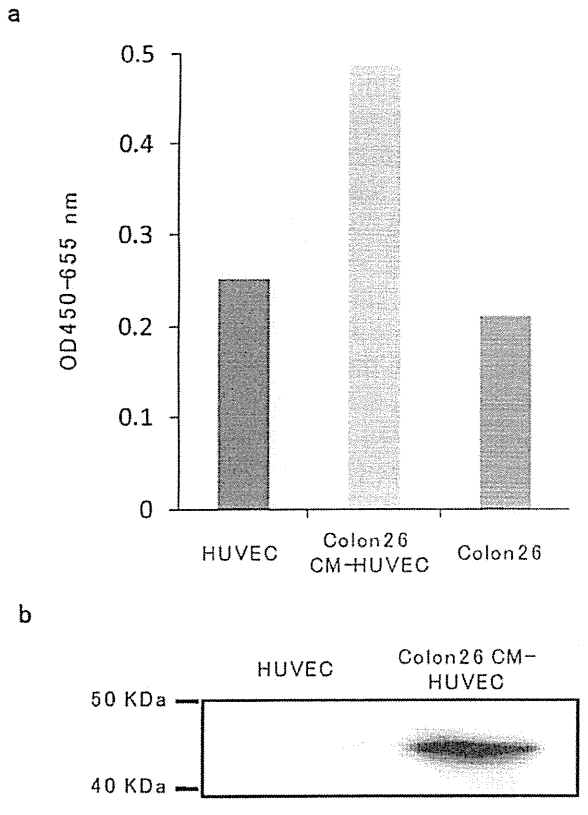

Fig. 6. Evaluation of Antibody Binding Activity *in Vitro*
(a: ELISA, b: Western blot). The binding specificities of the phage antibodies were assessed *in vitro* by using ELISA and Western blotting.

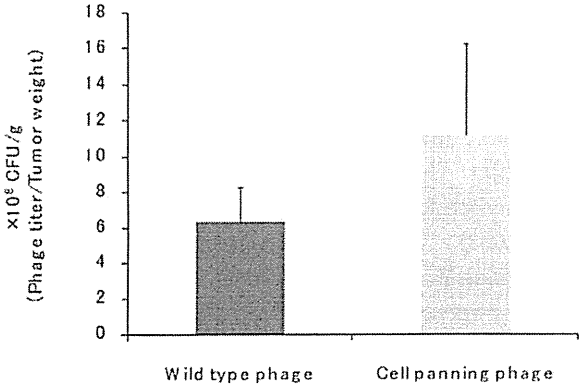

Fig. 7. Evaluation of Antibody Binding Activity in a Pilot Study *in Vivo*
The binding specificities of the phage antibodies were assessed *in vivo* by analyzing their accumulation on the tumor tissue. $n=3$, $p=0.08$.

1.8倍の腫瘍集積性を示す傾向にあった（Fig. 7）．この有意差が得られなかった原因は，抗体をファージ上に提示した状態で用いていることから，ファージが持つ非特異的吸着性の影響を受け，本来得られるべき差異がマスクされてしまったためとわれわれは推測している．そこで今後，より詳細に獲得した抗体の抗原認識性を評価するため，得られたClone 1の遺伝子をもとに，抗体のみを精製し，再検討する予定である．なお，Clone 2に関しても現在，同様に特性評価を行っている．

4. おわりに

本研究の結果，抗腫瘍組織血管抗体の創製に成功した．今後は免疫沈降法，質量分析法を駆使して各抗体の抗原タンパク質を同定し，それら抗原タンパク質の機能，生体分布評価を行う予定である．将来的に本研究により創製した抗体，その抗原が，がん治療，診断及び腫瘍組織血管研究の進展に大きく貢献することを期待している．

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Development of an antibody proteomics system using a phage antibody library for efficient screening of biomarker proteins

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ABSTRACT

Proteomics-based analysis is currently the most promising approach for identifying biomarker proteins for use in drug development. However, many candidate biomarker proteins that are over- or under-expressed in diseased tissues are found by such a procedure. Thus, establishment of an efficient method for screening and validating the more valuable targets is urgently required. Here, we describe the development of an "antibody proteomics system" that facilitates the screening of biomarker proteins from many candidates by rapid preparation of cross-reacting antibodies using phage antibody library technology. Using two-dimensional differential in-gel electrophoresis analysis, 16 over-expressed proteins from breast cancer cells were identified. Specifically, proteins were recovered from the gel pieces and a portion of each sample was used for mass spectrometry analysis. The remainder was immobilized onto a nitrocellulose membrane for antibody-expressing phage enrichment and selection. Using this procedure, antibody-expressing phages against each protein were successfully isolated within two weeks. The expression profiles of the identified proteins were then acquired by immunostaining of breast tumor tissue microarrays with the antibody-expressing phages. Using this approach, expression of Eph receptor A10, TRAIL-R2 and Cytokeratin 8 in breast tumor tissues were successfully validated.

These results demonstrate the antibody proteomics system is an efficient method for screening tumor-related biomarker proteins.

1. Introduction

Proteomics-based analysis is the most promising approach for identifying tumor-related biomarker proteins used in the drug development process [1–3]. The technological development of proteomics to seek and identify differentially expressed proteins in disease samples is expanding rapidly. However, in spite of the identification of many candidate biomarkers, the number of biomarker proteins successfully applied to drug development has been limited. The main difficulty is the lack of a methodology to comprehensively analyze the expression or function of many candidate proteins and to efficiently select potential biomarker proteins of interest. To circumvent this problem, an improved technology to efficiently screen the truly valuable proteins from a large number of candidates is desirable.

Monoclonal antibodies are extremely useful tools for the functional and distributional analysis of proteins [4–6]. For example, they can be applied to the specific detection and study of proteins through various techniques including ELISA, Western blotting, fluorescent imaging and tissue microarray analysis (TMA). Of all these techniques, TMA is particularly valuable because it enables the analysis of clinical expression profiles of antigens from many clinical samples [7–11]. However, the common hybridoma-based antibody production is a laborious and time-consuming method. Thus, it is impractical to create antibodies against many differentially expressed proteins identified by proteomics technologies, such as two-dimensional differential in-gel electrophoresis (2D-DIGE) [12–15]. Furthermore, a relatively large amount of antigen (several milligrams) is necessary to produce an antibody (i.e., immunization of animals or screening of positive clones). The

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production of protein on this scale often requires engineering the corresponding gene for heterologous expression, which may require some time to optimize. In this respect, phage antibody library technology is able to construct a large repertoire protein or peptide consisting of hundreds of millions of molecules. Monoclonal antibodies against target antigens are then rapidly obtained from the phage libraries displaying single chain fragment variable (scFv) antibodies *in vitro* [16–21].

However, the amount of protein in spots detected by 2D-DIGE analysis is generally very small (hundreds of nanograms). Therefore, a technology for generating monoclonal antibodies from such small amounts of antigen needs to be developed. There are no reports that describe the successful isolation of antibodies against small amounts of proteins obtained from differential proteome analysis.

Here, we report the establishment of a method for the efficient isolation of scFv antibody-expressing phages from a small amount of protein antigen prepared *via* 2D-DIGE spots using a high quality non-immune mouse scFv phage library [22]. We also describe an efficient method for screening and validating tumor-related biomarker proteins of interest from a number of differentially expressed proteins by expression profiling using TMA and scFv antibody-expressing phages.

2. Materials and methods

2.1. Non-immune mouse scFv phage library

Construction of the improved non-immune murine scFv phage library has been described previously [22]. The phage library was prepared from a TG1 glycerol stock containing the scFv gene library.

2.2. Affinity panning using BIAcore® and nitrocellulose membrane

Three different amounts (5000 ng, 50 ng or 0.5 ng) of KDR-Fc chimera (R&D systems Inc., Minneapolis, MN) or a portion of the proteins (1–5 ng) extracted from 2D-DIGE spots were immobilized on a BIAcore sensor chip CM3® (BIAcore, Uppsala, Sweden) or on a nitrocellulose membrane. BIAcore-based panning has been described previously [22]. Membrane-based panning was performed using the Bio-Dot Microfiltration Apparatus (Bio-Rad Laboratories, Hercules, CA). The membrane was incubated with blocking solution (10% skimmed milk, 25% glycerol) for 2 h and then washed twice with 0.1% TBST (Tris-buffered saline containing 0.1% Tween 20). The model phage library (anti-KDR scFv antibody-expressing phages: wild type phage = 1: 100) or the non-immune scFv phage library was pre-incubated with 90% blocking solution at 4 °C for 1 h and then applied to each well. After 2–3 h incubation, the apparatus was washed ten times with TBST. Bound scFv antibody-expressing phages were then eluted with 100 mM triethylamine. The eluted phages were incubated in log phase *E. coli* TG1 cells and glycerol-stocks prepared for further repeat panning cycles. Phage titer was measured by counting the number of infected colony cells on Petrifilm (3M Co., St. Paul, MN).

2.3. Colony direct PCR

After the panning, colonies of phage-infected TG1 were picked up at random as PCR templates. The gene inserts of 16 clones were amplified by PCR using the following primers: primer-156 (5′-CAACGTGAAAAAATTATTATTCGC-3′) and primer-158 (5′-GTAAATGA ATTTTCTGTATGAGG-3′), which anneal to the sequences of pCANTAB5E phagemid vector (GE Healthcare Biosciences AB, Uppsala, Sweden). The size of insert DNA sequence was analyzed by agarose gel electrophoresis.

2.4. Cell lines

Human mammary gland cell line 184A1 (American Type Culture Collection; ATCC, Manassas, VA) was maintained by MEGM Bullet Kit (Takara Bio, Shiga, JAPAN). Mammary gland-derived breast cancer cell line SKBR3 (ATCC) was maintained in McCoy's 5a plus 10% FBS. All cells were grown at 37 °C in a humidified incubator with 5% CO_2.

2.5. 2D-DIGE analysis

Cell lysates were prepared from human mammary gland cell line 184A1 and mammary gland-derived breast cancer cell line SKBR3, and then solubilized with 7 M urea, 2 M thiourea, 4% CHAPS and 10 mM Tris–HCl (pH 8.5). The lysates were labeled at the ratio 50 μg protein: 400 pmol Cy3 or Cy5 protein labeling dye (GE Healthcare Biosciences AB) in dimethylformamide according to the manufacture's protocol. For first dimension separation, the labeled samples (each 50 μg) were combined and mixed with rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 2% DTT, 2% Pharmalyte (GE Healthcare Biosciences AB)) and applied to a 24-cm immobilized pH gradient gel strip (IPG-strip pH 5–6 NL). The samples for the spot-picking gel were prepared without labelling by Cy-dyes. For the second dimension separation, the IPG-strips were applied to SDS-PAGE gels (10% polyacrylamide and 2.7% N,N′-diallyltartardiamide gels). After electrophoresis, the gels were scanned with a laser fluoroimager (Typhoon Trio, GE Healthcare Biosciences AB). The spot-picking gel was scanned after staining with Flamingo solution (Bio-Rad). Quantitative analysis of protein spots was carried out with Decyder-DIA software (GE Healthcare Biosciences AB). For the antigen spots of interest, spots of 1 × 1 mm in size were picked using an Ettan Spot Picker (GE Healthcare Biosciences AB). Proteins were extracted by solubilizing the picked gel pieces using 88 mM sodium periodide. Protein volumes were determined by BSA standard in Colloid Gold Total Protein staining (Bio-Rad).

2.6. In-gel tryptic digestion

Spots of 1 mm × 1 mm in size were picked using an Ettan Spot Picker and digested with trypsin as described below. The gel pieces were then destained with 50% acetonitrile/50 mM NH_4HCO_3 for 20 min twice, dehydrated with 75% acetonitrile for 20 min, and then dried using a centrifugal concentrator. Next, 5 μl of 20 μl/ml trypsin (Promega, Madison, WI) solution was added to each gel piece and incubated for 16 h at 37 °C. Three solutions were used to extract the resulting peptide mixtures from the gel pieces. First, 50 μl of 50% (v/v) acetonitrile in 1% (v/v) aqueous trifluoroacetic acid (TFA) was added to the gel pieces, which were then sonicated for 5 min. Next, we collected the solution and added 80% (v/v) acetonitrile in 0.2% TFA. Finally, 100% acetonitrile was added for the last extraction. The peptides were dried and then resuspended in 10 μl of 0.1% TFA before being cleaned using ZipTip™ μC_{18} pipette tips (Millipore, Billerica, MA). The tips were wetted with three washes in 50% acetonitrile and equilibrated with three washes in 0.1% TFA, then the peptides were aspirated 10 times to ensure binding to the column. The column and peptides were washed three times in 0.1% TFA before being eluted in 1 μl of 80% acetonitrile/0.2% TFA.

2.7. Mass spectrometry (MS) and database search

The tryptic digests (0.6 μl) were mixed with 0.6 μl α-cyano-4-hydroxy-trans-cinnamic acid saturated in a 0.1% TFA and acetonitrile solution (1:1vol/vol). Each mixture was deposited onto a well of a 96-well target plate and then analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/MS; autoflexII, Bruker Daltonics, Billerica, WI) in the Reflectron mode. The mass axis was adjusted with calibration peptide (BRUKER DALTNICS) peaks (M/z 1047.19, 1296.68, or 2465.19) as lock masses. Bioinformatic databases were searched to identify the proteins based on the tryptic fragment sizes. The Mascot search engine (http://www.matrixscience.com) was initially used to query the entire theoretical tryptic peptide as well as SwissProt (http://www.expasy.org/, a public domain database provided by the Swiss Institute of Bioinformatics, Geneva, Switzerland). The search query assumed the following: (i) the peptides were monoisotopic (ii) methionine residues may be oxidized (iii) all cysteines are modified with iodoacetamide.

2.8. Phage ELISA using nitrocellulose membrane

Phage ELISA using scFv antibody-expressing phages was performed as previously described [22]. Briefly, phage-infected TG1 clones were picked, monocloned in a Bio-Dot Microfiltration Apparatus and scFv antibody-expressing phages propagated. The supernatants containing scFv antibody-expressing phages were incubated with immobilized proteins (~1 ng) extracted from 2D-DIGE spots. scFv antibody-expressing phages bound to 2D-DIGE spots were visualized using HRP-conjugated anti-M13 monoclonal antibody (GE Healthcare Biosciences AB).

2.9. Immunohistochemical staining using scFv antibody-expressing phages

Human breast cancer and normal TMA (Super Bio Chips, Seoul, South Korea & Biomax, Rockville, MD) were deparaffinated in xylene and rehydrated in a graded series of ethanol. Heat-induced epitope retrieval was performed in while keeping Target Retrieval Solution pH 9 (Dako, Glostrup, Denmark) temperature following the manufacturer's instructions. Heat-induced epitope retrieval was performed while maintaining the Target Retrieval Solution pH 9 (Dako) at the desired temperature according to the manufacturer's instructions. After heat-induced epitope retrieval treatment, endogenous peroxidase was blocked with 0.3% H_2O_2 in TBS for 5 min followed by washing twice in TBS. TMA were incubated with 5% BSA blocking solution for 15 min. The slides were then incubated with the primary scFv antibody-expressing phages (10^{12} CFU/ml) for 60 min. After washing three times with 0.05% TBST, each series of sections was incubated for 30 min with ENVISION + Dual Link (Dako), washed three times in TBST. The reaction products were rinsed twice with TBST, and then developed in liquid 3,3′-diaminobenzidine (Dako) for 3 min. After the development, sections were washed twice with distilled water, lightly

counterstained with Mayer's hematoxylin, dehydrated, cleared, and mounted with resinous mounting medium. All procedures were performed using AutoStainer (Dako).

2.10. TMA Immunohistochemistry scoring

The optimized staining condition for breast tumor tissue microarray was determined based on the coexistence of both positive and negative cells in the same tissue sample. Signals were considered positive when reaction products were localized in the expected cellular component. The criteria for the staining were scored as follows: distribution score was scored as 0 (0%), 1 (1–50%), and 2 (51–100%) to indicate the percentage of positive cells in all tumor cells present in one tissue. The intensity of the signal (intensity score) was scored as 0 (no signal), 1 (weak), 2 (moderate) or 3 (marked). The total of the distribution score and intensity score was then summed into a total score (TS) of TS0 (sum = 0), TS1 (sum = 2), TS2 (sum = 3), and TS3 (sum = 4–5). Throughout this study, TS0 or TS1 was regarded as negative, whereas TS2 or TS3 was regarded as positive. Statview software was used in statistical analysis.

3. Results

3.1. Optimization of panning methods

To establish a method for the efficient isolation of antibodies against a small amount of protein antigen (nanogram-order or less) prepared from 2D-DIGE spots, 5000 ng, 50 ng or 0.5 ng of recombinant KDR proteins were first immobilized on a BIAcore sensor chip CM3® or on a nitrocellulose membrane using the Bio-Dot Microfiltration Apparatus®. Isolation of antibodies was assessed using a model phage library (anti-KDR scFv antibody-expressing phages: wild type phage = 1: 100) (Fig. 1). Enrichment of the desired clones in the output library was evaluated by analyzing the gene inserts of randomly-picked phage-infected TG1 cells by colony direct PCR. In the method using BIAcore®, enrichment was observed when 5000 ng of KDR was used for immobilization. By contrast, Membrane-based panning led to the successful enrichment of anti-KDR scFv antibodies from only 0.5 ng of KDR. These results demonstrated that membrane-based panning was suitable for the isolation of antibodies from very small amounts of antigen extracted from 2D-DIGE spot gel pieces.

3.2. 2D-DIGE analysis and identification of differentially expressed proteins

To identify breast tumor-related biomarker proteins and isolate monoclonal antibodies against them, we performed 2D-DIGE using breast cancer cell lines SKBR3 and normal breast cell lines 184A1 (Fig. 2). Quantitative analysis showed that 21 spots displayed increased or decreased expression levels in the cancer cell line compared with the normal cell line. MALDI-TOF/MS analysis of the spots subsequently identified 16 different proteins (Table 1).

3.3. Isolation of antibodies against each 2D-DIGE spot from the non-immune scFv phage library

The amount of protein extracted from the gel pieces ranged from several tens of nanogram to a few micrograms (Table 1). Because the membrane-based panning method facilitates the isolation of antibodies from 0.5 ng of protein (Fig. 1), we reasoned that this method could be used to isolate antibodies from the small amounts of proteins extracted from 2D-DIGE spot gel pieces. Thus a portion of the extracted proteins were immobilized onto nitrocellulose membranes by means of a Bio-Dot Microfiltration Apparatus, and membrane-based panning was performed using the non-immune scFv phage library [22] (Table 2). The results from this panning showed that the output/input ratio of phage titer (titer of the recovered phage library after the panning/titer of phage library before the panning) after the fourth round of panning against all spots increased approximately 20-fold–4000-fold in comparison to that obtained from the first round of panning. This elevated output/input ratio indicated the enrichment of the antigen-binding scFv antibody clones. To isolate monoclonal scFv antibodies to each spot, a total of 60 clones were randomly picked from the 4th panning output phage library and binding of the monoclonal scFv antibody-expressing phages to each antigen was tested by phage ELISA. As a result, several scFv antibody clones binding to each of the 16 antigens were isolated (Table 2). The antigenic specificity of isolated scFv antibodies was investigated by dot blot using various proteins as antigens. Some of the isolated scFv antibodies bound specifically to the antigen protein, but not to the His-tagged caspase-8, His-tagged importin-β, tumor necrosis factor receptor 1 (TNFR1)-Fc-chimera and KDR-Fc-chimera (data not shown). These results indicated the successful isolation of each spot-specific scFv antibody-expressing phages after only two weeks.

3.4. TMA analysis

The next stage in the process was to select the most valuable breast tumor-related biomarker proteins from a large number of

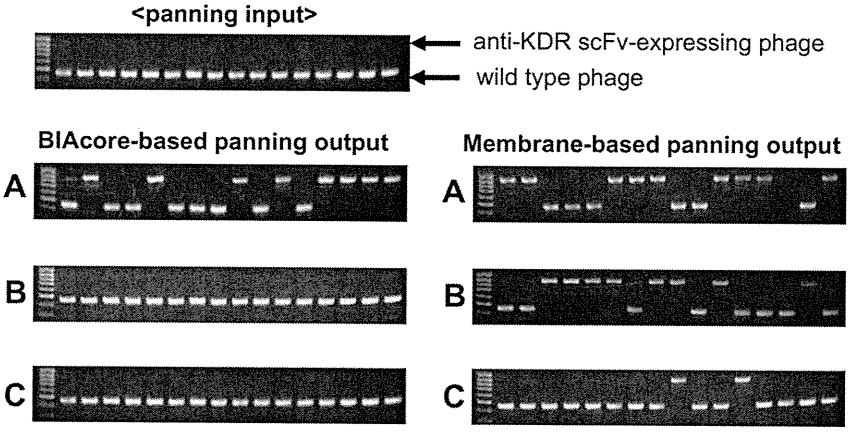

Fig. 1. Optimization of panning methods to isolate monoclonal antibodies from a very small amount of antigen. Model panning was performed using the BIAcore® or nitrocellulose membrane. The model library (anti-KDR scFv phage : wild type phage = 1: 100) was incubated with KDR ((A) 5000 ng, (B) 50 ng, (C) 0.5 ng) immobilized on a sensor chip or nitrocellulose membrane. The BIAcore-based panning method has been previously described [22]. After the binding step, the nitrocellulose membrane was washed ten times with TBST. The bound scFv antibody-expressing phages were eluted with triethylamine. The eluted scFv antibody-expressing phages were then incubated in log phase TG1 cells and individual TG1 clones were picked at random. Inserts of 16 phage clones were amplified by PCR. The gene sizes of inserts were analyzed by agarose gel electrophoresis.

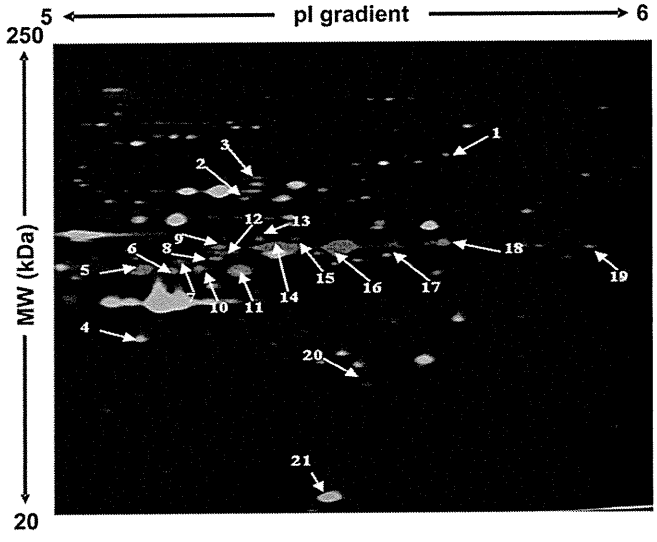

Fig. 2. 2D-DIGE image of fluorescently labeled proteins from SKBR3 and 184A cell. Breast cancer cell line (SKBR3) and normal breast cell line (184A1) were labeled using cy3 and cy5, respectively. The protein samples were then subjected to 2D electrophoresis. Spots that were over- and under-expressed in mammary cancer cells relative to normal cells were colored red and green, respectively. Yellow color spots show no change in expression.

identified candidate proteins. To this end, we immunostained TMA slides with 189 cases of breast tumors and 15 cases of normal breast specimens using the isolated spot-specific scFv antibody-expressing phages and screened the promising candidate biomarker proteins in terms of the expression profile in breast tumor tissues and normal tissues (Table 3). The result of the expression profile analysis showed that SPATA5, beta-actin variant, FLJ31438, PAK65, XRN1 and Jerky protein homolog-like were not expressed in normal and breast cancer tissue at all. By contrast, TRAIL-R2, Cytokeratin 8 and Eph receptor A10 were highly and specifically expressed (Fig. 3) in 63, 73 and 49% of breast tumor cases respectively, while the existing-breast cancer marker, Her-2, was expressed in 28% of breast tumor cases (Table 3). Thus, the relationship between the expression of each antigen and the Her-2 expression profile was analyzed. The level of expression of TRAIL-R2, Cytokeratin 8 and Eph receptor A10 in Her-2 positive cases were 77, 77 and 62%, and in Her-2 negative cases were 57, 67 and 44%, respectively (Table 4). Furthermore, the relationship between the expression of each antigen and clinical stage was analyzed in 187 of the 189 cases where all the clinical data was available. The level of expression of Cytokeratin 8 and Eph receptor A10 increased with progression of clinical symptoms (Table 5).

Table 1
Identification of 2D-DIGE spots by MALDI-TOF/MS.

Spot	Protein name	Accession number	MW (kDa)	pI	Protein volume (ng)	Expression ratio [cancer/normal] (fold)
#1	splicing factor YT521-B	Q96MU7	85	5.9	119	6
#2	IkappaBR	Q96HA7	63	5.5	104	6
#3	SPATA5	C9JT97	76	5.6	94	7
#4	skin aspartic protease	Q53RT3	37	5.3	610	0.1
#5	beta actin variant	P60709	42	5.3	99	15
#6	TRAIL-R2	O14763	48	5.4	100	18
#7	Cytokeratin-18	P05783	48	5.3	99	12
#8	TRAIL-R2	O14763	48	5.4	95	16
#9	RREB1	Q92766	52	5.3	109	10
#10	Cytokeratin-7	P08729	51	5.4	126	23
#11	Cytokeratin-18	P05783	48	5.3	497	13
#12	Cytokeratin-7	P08729	51	5.4	122	24
#13	FLJ31438	Q96N41	53	5.5	126	35
#14	Cytokeratin-7	P08729	51	5.4	406	36
#15	PAK65	Q13177	55	5.7	677	8
#16	Cytokeratin 8	P05787	54	5.5	694	32
#17	Cytokeratin 8	P05787	54	5.5	1143	72
#18	XRN1	Q8IZH2	54	5.8	353	8
#19	Jerky protein homolog-like	Q9Y4A0	51	6.0	130	22
#20	Eph receptor A10	Q5JZY3	32	5.7	119	9
#21	Glutathione S-transferase P	P09211	23	5.4	119	0.02

Table 2
Enrichment and isolation of antibodies to 2D-DIGE spots from non-immune libraries.

Spot	Protein name	Output/Input Ratio ($\times 10^{-7}$)/round				The number of isolated mAb.
		1st	2nd	3rd	4th	
#1	splicing factor YT521-B	6	7	16	480	4
#2	IkappaBR	6	7	15	500	3
#3	SPATA5	5	6	32	860	2
#4	skin aspartic protease	5	6	5	24	1
#5	beta actin variant	7	11	17	480	1
#6	TRAIL-R2	6	7	25	420	5
#7	Cytokeratin 18	5	11	62	260	4
#8	TRAIL-R2	5	27	41	1500	5
#9	RREB1	8	9	14	370	7
#10	Cytokeratin 7	6	7	3	2200	5
#11	Cytokeratin 18	6	8	15	84	2
#12	Cytokeratin 7	10	11	13	94	2
#13	FLJ31438	7	9	32	80	6
#14	Cytokeratin 7	4	7	46	280	5
#15	PAK65	7	11	51	580	9
#16	Cytokeratin 8	8	7	16	4100	6
#17	Cytokeratin 8	5	12	33	240	2
#18	XRN1	6	20	18	200	1
#19	Jerky protein homolog-like	7	10	49	940	3
#20	Eph receptor A10	8	6	57	3000	2
#21	Glutathione S-transferase P	7	8	110	1900	2

4. Discussion

Here, we aimed to develop a method of efficiently screening tumor-related biomarker proteins by proteome analysis. In

Table 3
Positive rate of identified proteins in breast cancer and normal breast tissues.

Protein name	Positive rate of antigens			
	Normal breast tissues		Breast cancer tissues	
Her-2	0/15	(0%)	53/189	(28%)
IkappaBR	3/15	(20%)	22/189	(12%)
SPATA5	0/15	(0%)	0/189	(0%)
beta actin variant	0/15	(0%)	0/189	(0%)
TRAIL-R2	0/15	(0%)	119/189	(63%)
RREB1	1/15	(7%)	83/189	(44%)
FLJ31438	0/15	(0%)	0/189	(0%)
PAK65	0/15	(0%)	0/189	(0%)
Cytokeratin 8	0/15	(0%)	137/189	(73%)
XRN1	0/15	(0%)	0/189	(0%)
Jerky protein homolog-like	0/15	(0%)	0/189	(0%)
Eph receptor A10	0/15	(0%)	93/189	(49%)

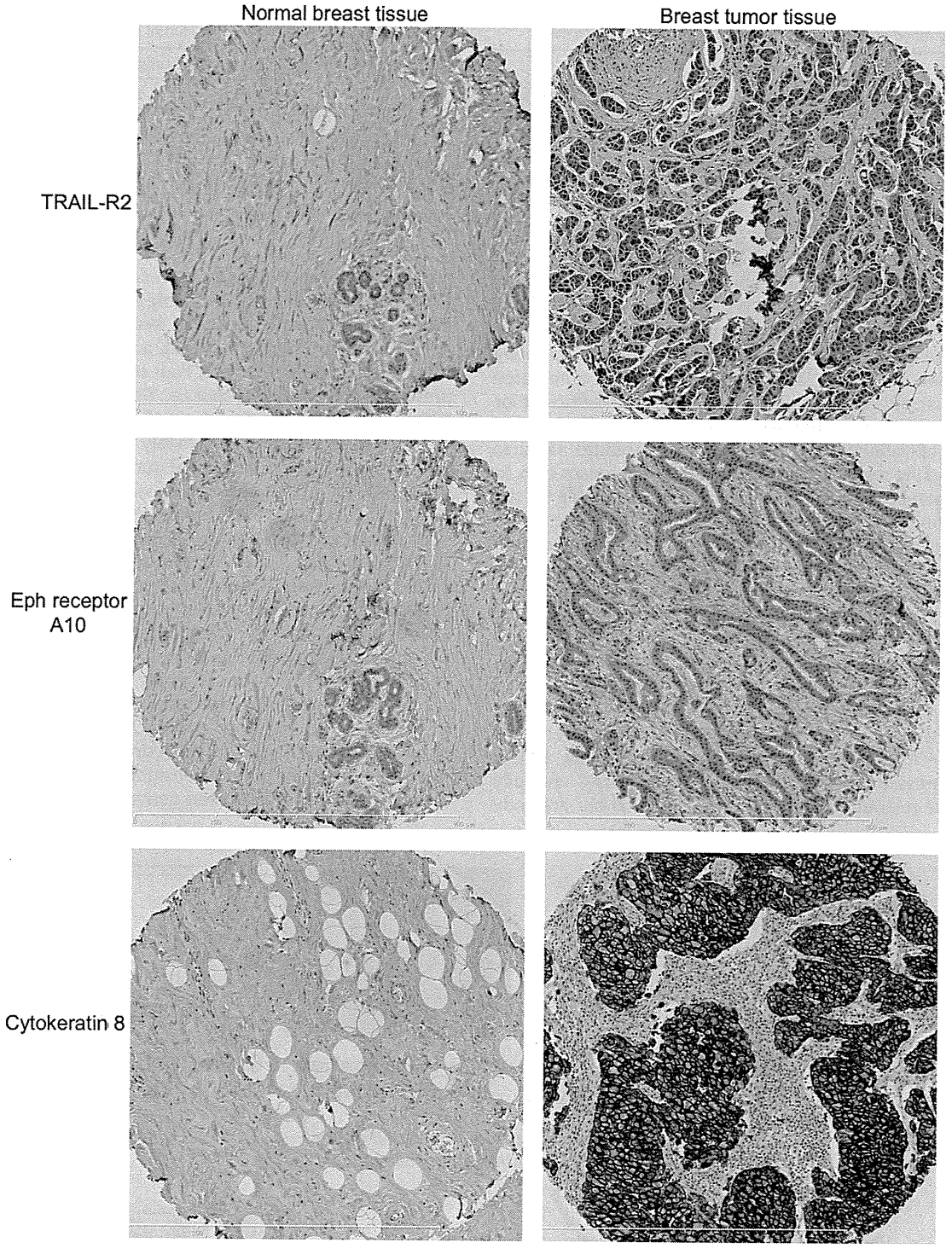

Fig. 3. Immunohistochemical staining of breast tumor and normal breast tissue microarray by scFv antibody-expressing phages. Typical images of breast cancer and normal breast tissue microarray stained by using scFv antibody-expressing phages to TRAIL-R2, Eph receptor A10 and Cytokeratin 8 are shown. Left panels are normal breast tissues and right panels are breast tumors. The tissue microarrays were counterstained by hematoxylin.

particular, we attempted to establish a means of isolating specific antibodies directly from small amounts of differentially expressed proteins obtained *via* 2D-DIGE analysis. To achieve this, we focused on a non-immune scFv phage library. Because the non-immune naïve scFv phage library has a huge repertoire of scFv on the surface of the phages, monoclonal antibodies to every antigen could be effectively isolated *in vitro*. Generally the diversity of the CDR3 domain, which is important for antigen-binding specificity, is estimated to be approximately twenty million [23]. Thus we reasoned that our previously constructed library, containing 2.4×10^9 scFv variants, has almost equal potential as the murine or human immune system [22]. Initially, in order to isolate monoclonal antibodies against very small amounts of antigen (hundreds of nanograms) recovered from the spots of 2D-DIGE analysis, we attempted to optimize the panning method using either a BIAcore® or nitrocellulose membrane. In the method using BIAcore®, the

Table 4
Positive rate of identified proteins in Her-2 positive and Her-2 negative cases.

Protein name	Positive rate of antigens in Her-2	
	Positive cases	Negative cases
TRAIL-R2	41/53 (77%)	78/136 (57%)
Cytokeratin 8	41/53 (77%)	91/136 (67%)
Eph receptor A10	33/53 (62%)	60/136 (44%)
TRAIL-R2 or Eph receptor A10	46/53 (87%)	100/136 (74%)

Table 5
Positive rate of identified proteins in clinical stage.

Protein name	Positive rate of antigens in clinical stage					
	Stage I		Stage II		Stage III	
Her-2	6/14	(43%)	17/87	(20%)	30/86	(35%)
TRAIL-R2	11/14	(79%)	51/87	(59%)	55/86	(64%)
Cytokeratin 8*	7/14	(50%)	58/87	(67%)	71/86	(83%)
Eph receptor A10*	4/14	(29%)	42/87	(48%)	47/86	(55%)

Man Whitney U test $^*P < 0.05$

enrichment of the desired clones was observed when immobilizing 5000 ng of KDR. By contrast, membrane-based panning led to the successful enrichment of clones from only 0.5 ng of KDR (Fig. 1). BIAcore-based panning has been recognized to be an effective method because the interaction of an antigen and a scFv antibody can be monitored in real time and the operation can be automated [24,25]. However, our results suggest that BIAcore® is inefficient for immobilizing very small amounts of antigen. This is because antigen immobilization using the BIAcore procedure requires a chemical coupling reaction with the surface of the sensor chip. In contrast, the membrane-based panning method is suitable for the isolation of antibodies against very small amounts of antigens. The suitability of this procedure when handling such small amounts of proteins presumably arises from the high efficiency of adsorption of antigens by the nitrocellulose membrane. These results show that monoclonal antibodies can be created from small amounts of proteins recovered from 2D-DIGE spots.

In breast cancer patients, the antibody targeting human epidermal growth factor receptor II (Her-2), is an effective drug [26,27]. However, because this receptor is over-expressed in only ~25% of breast cancer patients, anti-Her-2 antibody therapy is ineffective in ~75% of cases. Furthemore, approximately 30% of Her-2 over-expressed patients that received anti-Her-2 antibody therapy became tolerant [28–30]. Thus, we applied our antibody proteomics system to breast cancer samples for identification of the proteins to replace Her-2 as suitable therapeutic targets. Initially, 21 differentially expressed proteins between SKBR3 and 184A1 cells were found by 2D-DIGE analysis and 16 different proteins were identified by MALDI-TOF/MS. Four of the identified proteins were present in more than one spot i.e., TRAIL-R2 (spot 6, 8), Cytokeratin 18 (spot 7, 11), Cytokeratin 8 (spot 16, 17) and Cytokeratin 7 (spot 10, 12, 14). These proteins presumably display different pI and MW values due to posttranslational modification. Next, membrane-based panning against these spots was performed, and the output/input ratio of phage titer after the fourth round of panning increased from approximately 20-fold–4000-fold in comparison to that after the first round of panning. Moreover, we screened scFv antibody-expressing phages binding to each spot protein by phage ELISA and obtained each spot-specific scFv antibodies from all spots after approximately two weeks. Finally, it was necessary to select the most valuable proteins from a large number of differentially expressed proteins in breast cancer cells. Using the isolated spot-specific scFv antibody-expressing phages, we immunostained a TMA with 189 cases of breast cancer tissue and 15 samples of normal tissue. SPATA5, Beta actin, FLJ31438, PAK65 and XRN1 were not detected in either the tumor tissue or normal tissue. Thus, these proteins may have been derived from cell lines used in the

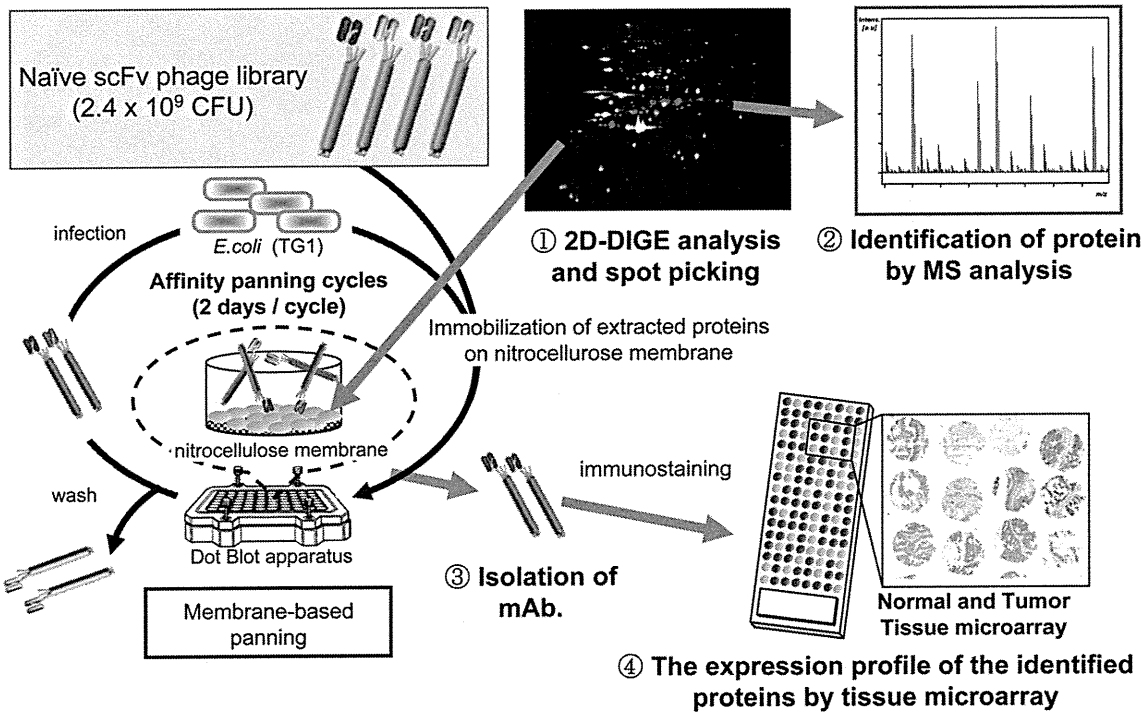

Fig. 4. Schematic illustration of the antibody proteomics system. Antibody proteomics system is an efficient method for screening tumor-related biomarker proteins. Because this system involves the direct isolation of monoclonal antibodies from 2D-DIGE spots without preparation of recombinant proteins, it enables the discovery and validation of tumor-related biomarker proteins by TMA analysis using the isolated scFv antibody-expressing phages.

proteome analysis or the antibodies against these proteins may not detect the antigen on formalin-fixed paraffin-embedded tissues. By contrast, TRAIL-R2, Cytokeratin 8 and Eph receptor A10 were specifically-expressed in over 40% of breast cancer tissues. We confirmed the immunohistochemical staining image generated by scFv antibody-expressing phages displayed a similar pattern to that generated by IgG type commercial antibody (data not shown). Interestingly, the expression rates of TRAIL-R2, Cytokeratin 8 and Eph receptor A10 were higher than the existing breast cancer marker, Her-2 (only about 25%). Moreover, the expression rates of TRAIL-R2 and Eph receptor A10 (cell membrane proteins) in Her-2 negative cases were over 40% and in Her-2 positive cases over 60%. This data indicates that TRAIL-R2 and Eph receptor A10 are promising alternative target candidates for anti-Her-2 antibody therapy ineffective patients, at least in terms of the expression profile. Further work is required to analyze the function of these proteins in more detail. Furthermore, by checking antigen expression profiles against clinical information, the expression rate of Cytokeratin 8 and Eph receptor A10 was found to have increased during progression of the clinical symptoms. These observations indicate that Cytokeratin 8 and Eph receptor A10 are promising diagnostic marker candidates for assessing the aggressiveness of breast cancer.

Recently, an anti-TRAIL-R2 antibody has been developed as an anticancer drug [31–33]. Moreover, Cytokeratin 8 has gained considerable attention as a cancer aggressiveness diagnostic marker [34–36]. These results demonstrate that this technology is able to select well-known drug-target markers (i.e., TRAIL-R2) and diagnostic markers (i.e., Cytokeratin 8) as well as unknown biomarker protein candidates (Eph receptor A10) from a large variety of differentially expressed proteins in cancer cells.

Our method employs a set of techniques for efficiently identifying biomarker candidates. Specifically, the method entails; 1) searching for differentially expressed proteins in disease samples, 2) identification of the proteins, 3) high throughput isolation of monoclonal antibodies against the proteins using a naïve scFv phage library, and 4) validation of the proteins by TMA analysis. This methodology is referred to as an "antibody proteomics system" (Fig. 4). We believe that the proteins identified using this approach will contribute to the drug development process. Indeed, the antibody proteomics system could become a platform technology for seeking tumor-related biomarker proteins by a proteomics-based approach.

5. Conclusions

In this study, we established the antibody proteomics system for efficiently screening and validating tumor-related biomarker proteins of interest by isolating specific antibodies directly from small amounts of proteins obtained *via* 2D-DIGE analysis. Applying this technique to the identification of breast tumor-related biomarker proteins, the expressions of Eph receptor A10, TRAIL-R2 and Cytokeratin 8 in breast tumor tissues were successfully validated from a large number of candidates. These results demonstrate that our original technology is an efficient and useful method for screening tumor-related biomarker proteins. Moreover, Eph receptor A10, TRAIL-R2 and Cytokeratin 8 identified in this study are promising breast tumor biomarkers for drug development.

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Appendix

Figure with essential color discrimination. Figs. 2–4 in this article have parts that are difficult to interpret in black and white. The full color images can be found in the on-line version, at doi:10.1016/j.biomaterials.2010.09.030.

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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

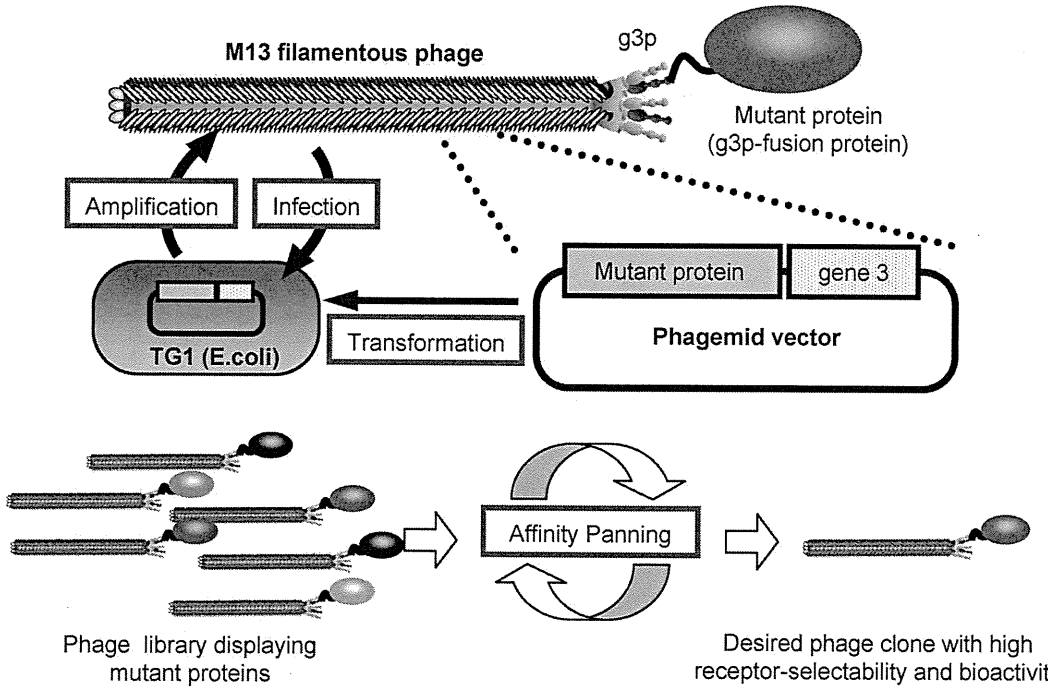

Fig. 1. Creation of Functional Mutant Proteins Using Phage Display

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

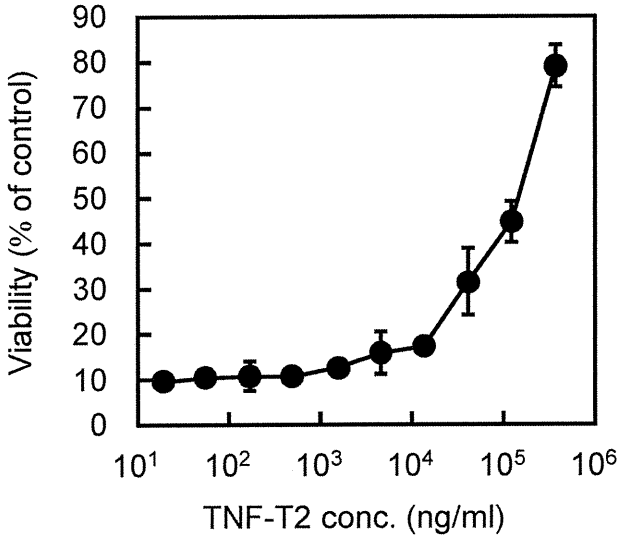

Fig. 2. Antagonistic Activities of the R1antTNF

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).

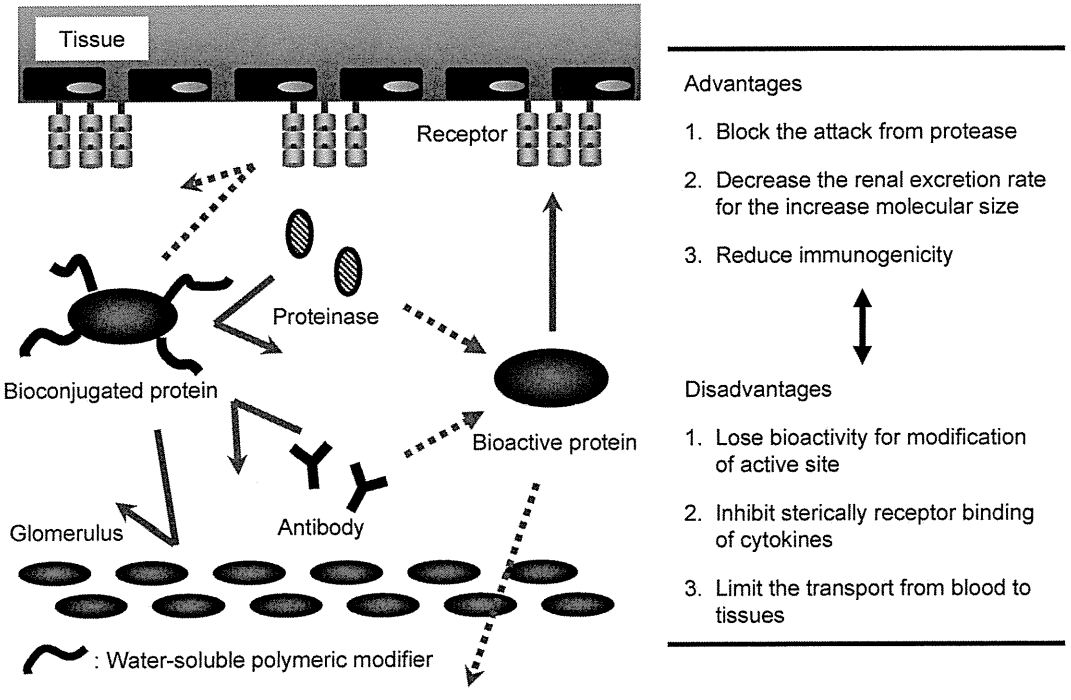

Fig. 3. Characteristics of Bioconjugation

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．

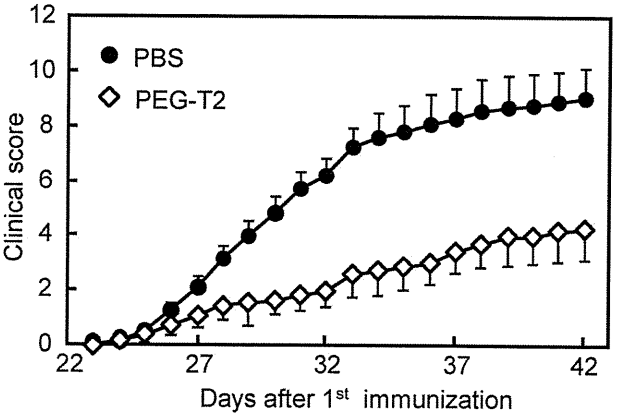

Fig. 4. Clinical Score of Collagen-induced Arthritis (CIA) Mice Treated with PEG-T2

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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Novel protein engineering strategy for creating highly receptor-selective mutant TNFs

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ABSTRACT

Tumor necrosis factor (TNF) plays important roles in host defense and in preventing tumor formation by acting via its receptors, TNFR1 and TNFR2, functions of which are less understood. To this end, we have been isolating TNF receptor-selective mutants using phage display technique. However, generation of a phage library with large repertoire (>10^8) is impeded by the limited transformation efficiency of *Escherichia coli*. Therefore, it is currently difficult to create a mutant library containing amino acid substitutions in more than seven residues. To overcome this problem, here we have used two different TNF mutant libraries, each containing random substitutions at six selected amino acid residues, and utilized a gene shuffling method to construct a randomized mutant library containing substitutions at 12 different amino acid residues of TNF. Consequently, using this library, we identified TNF mutants with greater receptor-selectivity and enhanced receptor-specific bioactivity than the existing mutants.

Introduction

Tumor necrosis factor-α (TNF) plays a critical role in host defense through regulation of cell survival, death, and inflammation by acting via one of its receptors, TNFR1 and TNFR2 [1]. Because excess or uncontrolled activity of TNF is often a cause for many immunological diseases and tumor development [2,3], biological drugs such as recombinant TNFs (for enhancement of TNF signaling) or anti-TNF monoclonal antibodies (Mab) and soluble TNF receptors (for neutralization of TNF signaling) have made significant impacts in the treatment of inflammatory diseases and in tumor therapy [4,5]. A number of published reports, however, suggested that neutralization of TNF resulted in increased risk of bacterial infection and several other side effects because of the blocking of signaling via both TNFR1 and TNFR2 [4,5]. Therefore, there is an urgent need to fully understand the biology of the TNF receptor mediated signaling pathway and to develop novel drugs for therapeutic use in TNF-related immunological diseases. For this purpose, TNF receptor-knockout mice were used to understand the relationship between the function of the TNF receptors and TNF-related diseases [8–10]. Recently, it was revealed that the two receptors 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 [1,6,7]. To elucidate the roles of TNFR1 and TNFR2, many researchers used an agonistic or an antagonistic TNF mutant that selectively binds to one of the receptors and initiates the biological activity of that specific receptor. These mutant TNFs might be a promising new class of TNF-related drugs without any side effect, and could as well serve as a tool for analyzing the receptor function. Development of these mutant TNFs has facilitated understanding of the molecular interaction between the TNF and its receptors, TNFR1 and TNFR2. In this context, it is noteworthy that several approaches have been undertaken to establish quantitative correlation between the receptor subtype specific-biological activity and the structural and kinetic binding parameters of a receptor-specific TNF mutant [8]. Mutational analysis of single amino acid residues revealed that amino acids at positions 15, 31–35, 84–87, 117, 119 and 143–148, which are clustered throughout in hotspots, greatly contributed to the biological activity of TNF [9,10].

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With a similar goal in mind, we previously constructed M13 bacteriophage libraries (Library I and Library II) displaying mutant TNFs randomized at amino acid positions 29, 31, 32, 145, 146 and 147 or at positions 84–89 of TNF, and succeeded in isolating agonistic- and antagonistic-mutant TNFs from these libraries [11,12]. Particularly, the TNFR1-selective antagonist mutTNF-T2 showed almost same therapeutic effect as the anti-TNF biologics in a hepatitis model [13].

Currently, generation of a phage-displayed library with large repertoire (>10^8) is impeded by the limited transformation efficiency of *Escherichia coli* (*E. coli*). As a result, it is difficult to construct a high quality mutant library ($20^6 = 6.4 \times 10^7$) that is randomized at more than seven different amino acid residues and includes almost all clones. To overcome this problem, here we have developed a novel protein engineering strategy (gene shuffling method) for creation of functional mutant proteins. To achieve our goal, we first constructed two types of phage libraries displaying mutant TNFs, in each of which six amino acids residues in the predicted receptor binding sites were replaced with other amino acid residues, and these phage display libraries were subsequently subjected to several rounds of panning against TNFR1 and TNFR2, respectively, using a surface plasmon resonance analyzer (BIAcore). After several rounds of panning, we obtained two libraries, each one containing enriched number of a TNF receptor-specific high affinity clones. Next, we utilized these enriched libraries to construct high quality TNF receptor-specific shuffling libraries using a gene shuffling method. Finally, panning of these shuffling libraries against TNFR1 and TNFR2, respectively, have allowed us to isolate TNF mutants with greater receptor-selectivity and enhanced receptor-specific bioactivity than the previously isolated TNFR1-selective mutant R1-5 and TNFR2-selective mutant R2-3 [12].

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, Sendai, Japan) and were maintained in RPMI 1640 medium (Sigma-Aldrich Japan, Tokyo, Japan) supplemented with 10% FBS and antibiotics cocktail (penicillin 10,000 units/ml, streptomycin 10 mg/ml, and amphotericin B 25 μg/ml; Nacalai tesque, Kyoto, Japan). hTNFR2/mFas-preadipocyte (mouse preadipocyte cell expressing a chimeric receptor, which consist of the extracellular and transmembrane domain of human TNFR2 and the intracellular domain of mouse Fas) cells were established previously in our laboratory [14] and were maintained in D-MEM (Wako Pure Chemical Industries, Osaka, Japan) supplemented with blasticidin S HCl (5 μg/ml Sigma-Aldrich Japan, Tokyo, Japan), 10% FBS, 1 mM sodium pyruvate, 5×10^{-5} M 2-mercaptoethanol, and antibiotic cocktail.

Selection of TNF receptor-selective mutants from the mutant TNF phage display library by panning. Human TNFR1 Fc chimera or human TNFR2 Fc chimera (R&D systems, Minneapolis, MN) was immobilized onto a CM3 sensor chip (GE Healthcare, Buckinghamshire, UK) as described previously [11,12]. The phage display library (1×10^{11} CFU/100 μl) was injected over the sensor chip at a flow rate of 3 μl/min on BIAcore. After binding, the chip was rinsed 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). Next, the phages in the eluted pool were amplified in the *E. coli* TG1. The panning, elution and amplification steps were repeated twice. Subsequently, single clones were isolated from the phage pool, and the DNA sequences of phagemids purified from the single clones were analyzed.

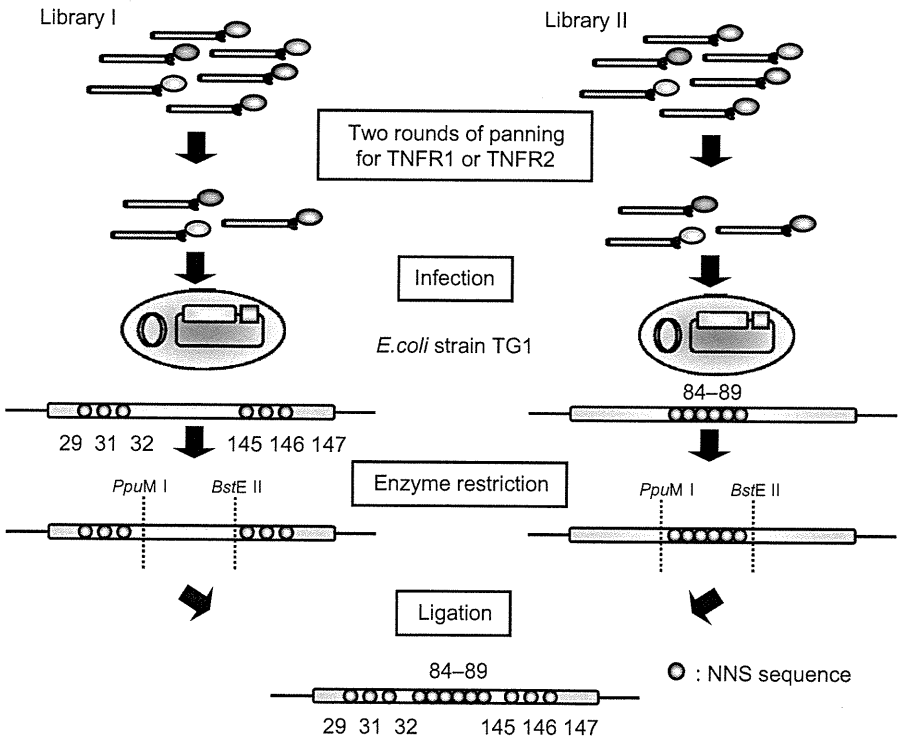

Fig. 1. Construction of the Gene Shuffling Library. Schematic description of the methods used to construct the Gene Shuffling Libraries A and B from two parent mutant TNF libraries, each one of which was created by replacing the codon of the amino acid residue at positions 29, 31, 32, 145, 146 and 147 (Library I) or at positions 84, 85, 86, 87, 88 and 89 (Library II) of TNF with the randomized codon NNS (where N and S represent G/A/T/C or G/C, respectively) to obtain all twenty amino acid substitutions at each position. NNS encodes all 20 different amino acids. Mutations were introduced by PCR using the lysine-deficient mutant TNF as the template as described in Materials and methods.