Review

Development of novel drug delivery systems using phage display technology for clinical application of protein drugs

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(Communicated by Shigekazu NAGATA, M.J.A.)

Abstract: Attempts are being made to develop therapeutic proteins for cancer, hepatitis, and autoimmune conditions, but their clinical applications are limited, except in the cases of drugs based on erythropoietin, granulocyte colony-stimulating factor, interferon-alpha, and antibodies, owing to problems with fundamental technologies for protein drug discovery. It is difficult to identify proteins useful as therapeutic seeds or targets. Another problem in using bioactive proteins is pleiotropic actions through receptors, making it hard to elicit desired effects without side effects. Additionally, bioactive proteins have poor therapeutic effects owing to degradation by proteases and rapid excretion from the circulatory system. Therefore, it is essential to establish a series of novel drug delivery systems (DDS) to overcome these problems. Here, we review original technologies in DDS. First, we introduce antibody proteomics technology for effective selection of proteins useful as therapeutic seeds or targets and identification of various kinds of proteins, such as cancer-specific proteins, cancer metastasis–related proteins, and a cisplatin resistance–related protein. Especially Ephrin receptor A10 is expressed in breast tumor tissues but not in normal tissues and is a promising drug target potentially useful for breast cancer treatment. Moreover, we have developed a system for rapidly creating functional mutant proteins to optimize the seeds for therapeutic applications and used this system to generate various kinds of functional cytokine muteins. Among them, R1antTNF is a TNFR1-selective antagonistic mutant of TNF and is the first mutein converted from agonist to antagonist. We also review a novel polymer-conjugation system to improve the in vivo stability of bioactive proteins. Site-specific PEGylated R1antTNF is uniform at the molecular level, and its bioactivity is similar to that of unmodified R1antTNF. In the future, we hope that many innovative protein drugs will be developed by combining these technologies.

Keywords: drug delivery system, phage antibody technology, phage display system, site-specific PEGylation

1. Introduction

In our post-genome era, a number of proteins thought to be involved in pathological disorders and other biological processes are considered potentially useful as therapeutic seeds or as targets for pharmaceutical development.[1–3] Therefore, comprehensive protein analyses in the healthy body and in disease conditions through disease proteomics, are now a focus of life science research and are expected to help identify proteins of therapeutic importance in various diseases. Against this background, potentially potent protein therapies that use cytokines or antibodies have recently received a great deal of attention. Indeed, attempts are currently under way to develop a wide variety of therapeutic proteins for treating conditions such as cancer, infectious diseases, and autoimmune disorders.[4–8]
Unfortunately, the clinical applications of protein drugs are still limited, except in the cases of drugs based on erythropoietin, granulocyte colony-stimulating factor, interferon-alpha, and antibodies. One difficulty lies in identifying proteins useful as therapeutic seeds or as targets for pharmaceutical development. The application of disease proteomics has enabled us to identify many candidate proteins that are differentially expressed in disease samples, but the most important issue that remains to be resolved is how to correctly identify the useful proteins from among the candidates. Another difficulty with the use of bioactive proteins, such as cytokines, as drugs is pleiotropic actions through a number of receptors in vivo, making it difficult to elicit the desired effect without simultaneously triggering undesirable secondary effects. Additionally, bioactive proteins generally have unexpectedly weak therapeutic effects. Often these proteins are degraded by various proteases in vivo and rapidly excreted from the circulatory system. Consequently, frequent administration of an excessively high dose of a protein is required to obtain its desired therapeutic effect in vivo, leading to disturbance of homeostasis and unexpected side effects. Thus, establishment of a series of novel DDS technologies that enhance the pathway from drug exploration to optimization and overcome the abovementioned problems is essential for the advancement of protein drug development.

From this perspective, our laboratory aims to develop a novel DDS platform to overcome problems with protein therapies by: (i) establishing a high-throughput system to validate many candidate proteins by applying a phage antibody library [i.e., an in vitro monoclonal antibody (mAb) development system] to the study of disease proteomics; (ii) developing a powerful system to rapidly create functional mutant proteins (muteins) with enhanced receptor affinity and receptor specificity by using a phage display technique; and (iii) creating a novel polymer-conjugation system to dramatically improve the in vivo stability of bioactive proteins. In this review, we describe these DDS technologies for advanced pharmaceutical applications.

2. Establishment of antibody proteomics technology: a high-throughput system for validation of multiple candidate proteins

Proteomics-based analysis is one of the most powerful approaches to identifying proteins useful for drug development. The technological development of proteomics to seek and identify small amounts of proteins that are differentially expressed in diseased samples and are thus candidate therapeutic seeds or targets is expanding rapidly. However, the number of 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 proteins of interest. To circumvent this problem, we need an improved technology to efficiently screen the truly valuable proteins from among large numbers of candidates. We have therefore focused on mAbs, which are essential tools for validation of proteins. However, the commonly used hybridoma-based mAb production requires preparation of recombinant proteins as antigens and is laborious and time-consuming, making it impractical for creating mAbs against many candidate proteins identified by proteomics-based analysis and forcing researchers to preferentially analyze proteins of their own interest. A phage antibody library system can rapidly produce mAbs against many antigens in vitro. We therefore applied this system to proteomics studies and developed an antibody proteomics technology that allows us to produce mAbs against many candidates to analyze their expression profiles or functions; this technology accelerates the identification of proteins potentially useful as therapeutic seeds or targets. This technology (Fig. 1) comprises (i) a search for disease-related proteins by proteomics-based analyses such as two-dimensional differential in-gel electrophoresis (2D-DIGE); (ii) identification of candidate proteins by using mass spectrometry analysis; (iii) isolation of mAbs against candidate proteins for which no commercially produced mAbs are available by using a phage antibody library; and (iv) validation of the proteins by using disease samples such as a tissue microarray, which is a glass slide containing many clinical samples and clinical information such as age, gender, clinical stage, and treatment history. The advantages of this technology are 1) high-throughput generation of mAbs against many candidate proteins by directly using small amounts of proteins extracted from 2D-DIGE gels by optimizing the mAb selection method, and 2) validation of candidate proteins using clinical samples, even if the candidate proteins have been detected using cell lines. We can comprehensively validate each candidate protein by analyzing the correlation between its expression profile and the clinical information.
We applied this technology to various kinds of tumors and successfully identified useful proteins for drug discovery, such as cancer-specific proteins, cancer metastasis–related proteins, and a cisplatin resistance–related protein. Here, we describe Ephrin receptor A10 (EphA10), which was identified as a novel breast cancer–related protein expressed in many breast cancer tissues but not normal breast tissues. EphA10 is a relatively uncharacterized protein: the only thing known about it before our report was that it is expressed in the testis at an mRNA level. Because EphA10 is a highly novel breast cancer–related protein, its production profile in normal and cancer tissues, as well as its function, need to be clarified to determine its potential as a drug target. We therefore analyzed the EphA10 production profile by using tissue microarrays and showed that EphA10 was produced in various subtypes of breast cancer — luminal A (54%), luminal B (68%), human epidermal growth factor receptor 2 (Her2)-enriched (64%), and triple negative breast cancer (TNBC) (67%) — but not in 35 kinds of normal tissue, except for the testis. The TNBC subtype, which lacks the production of estrogen receptor, progesterone receptor, and Her2, is refractory to current treatments because of an absence of molecularly targeted drugs. Therefore, there is considerable interest worldwide in developing therapeutics against TNBC. Our data suggest that EphA10 is an attractive drug target in breast cancer (particularly in patients with TNBC) and is a highly specific tumor antigen. To evaluate the utility of EphA10 as a drug target, we developed an anti-EphA10 mAb and administered it or a control mAb once a week in an intravenously xenografted mouse model. Tumor growth was significantly lower in the anti-EphA10 mAb–treated group than in the controls; this effect was dose dependent (Fig. 2). EphA10 is therefore a promising drug target for treating breast cancers, including TNBC.

This example shows that antibody proteomics technology is a powerful system for identifying proteins useful as therapeutic seeds or as targets for pharmaceutical development.

3. Development of a system to create artificial functional muteins for advanced pharmaceutical applications

Even though therapeutic seeds can be identified by proteome analyses such as antibody proteomics technology, the bioactive proteins generally have
pleiotropic actions through a number of receptors in vivo, making it difficult to elicit the desired effect without simultaneously triggering undesirable secondary effects. It is often desirable to alter the amino acid sequence to yield artificial functional muteins with enhanced receptor affinity and receptor specificity. Traditionally, most biotechnological research facilities have used site-directed mutagenesis, for example by the classic Kunkel method, to produce functional muteins. However, the creation of muteins by point mutations requires the generation of many individual mutants by replacing specific amino acids through a process of trial and error. The overall process of generating bioactive proteins with the desired properties is therefore time consuming and costly. Consequently, the variety of functional muteins is often limited, making it difficult to achieve the desired enhancement of therapeutic effect.

To solve these problems, phage display systems, including the phage antibody library described above, have recently been developed for constructing libraries of muteins displayed on a bacteriophage surface; these systems facilitate rapid screening against a given target (Fig. 3). From such libraries, the desired phages (e.g., phages displaying muteins with high affinity to target proteins) can be selected, isolated, and then expanded by application of a panning procedure. Moreover, the relevant gene sequence is readily determined because the selected phage contains the gene that encodes the desired protein. The range of applications of the phage display method as a standard technology for quick and efficient screening of molecules that bind to particular targets is constantly increasing.

From this perspective, we have been trying to create functional muteins for advanced pharmaceutical applications, using tumor necrosis factor-alpha (TNF) as an example. We previously constructed a phage library displaying mutated TNFs with substitutions of amino acids at the receptor binding sites predicted on the basis of a 3D structure of TNF receptor 1 (TNFR1) or 2 (TNFR2). We then successfully created various kinds of functional TNF muteins, such as those with enhanced affinity to TNFR1 or TNFR2, those with altered binding selectivity for the receptors, and lysine-deficient muteins with full bioactivity. Here, we describe the TNFR1-selective antagonistic mutant TNF (R1antTNF), which is the first mutein modified from agonist to antagonist (Fig. 4). Nowadays, TNF inhibitors such as anti-TNF mAbs have been used as effective drugs against autoimmune conditions, including rheumatoid arthritis. However, the drugs have serious side effects such as infections or increased cancer risk because they inhibit functions via both TNFR1 (induction of inflammation) and TNFR2 (protection against infection). Therefore, an agent that inhibits only TNFR1-mediated function is urgently needed. In this respect, R1antTNF can be useful because it displays TNFR1-selective binding and therefore selective inhibition of TNFR1-mediated biological activity in vitro (Fig. 4). R1antTNF may thus be an attractive therapeutic mutein. In fact, the therapeutic effects of R1antTNF in models of acute, potentially lethal hepatitis are as good as, or better than, those obtained by using conventional anti-TNF mAb therapy. By using these technologies, we have also been able to create many functional muteins for various proteins such as interferons, lymphotoxin-alpha, and LIGHT. This technology is versatile and can be used routinely to create functional muteins.
4. A new approach to site-specific PEGylation: creation of a polymer-conjugation system to improve the in vivo stability of bioactive proteins

We expect that R1antTNF will also have therapeutic effects in models of chronic inflammatory diseases, such as a model of collagen-induced arthritis and a model of experimental autoimmune encephalomyelitis. However, both R1antTNF and wild-type TNF (wtTNF) have short half-lives (about 10 min) in the plasma of mice. A method of extending the half-life of R1antTNF in the plasma is therefore needed.

Conjugation of water-soluble polymers such as polyethylene glycol (PEG) to bioactive proteins dramatically improves protein stability in vivo\(^{65-67}\) (Fig. 5). Bioconjugation of PEG to bioactive proteins—a process known as PEGylation—increases the molecular weight of the protein and decreases the rate of renal clearance. Additionally, because water-soluble polymers cover the protein surface, attack by proteases is generally blocked through steric hindrance, resulting in prolongation of the half-life in vivo. Moreover, steric hindrance decreases antigenicity and immunogenicity. Because of these advantages, it is possible to use the bioactive protein at a lower dose. Some bioactive proteins such as granulocyte-colony stimulating factor, interferon-alpha, asparaginase, and adenosine deaminase have been successfully PEGylated, and this process gives them greater therapeutic efficacy than their corresponding native forms.\(^{68-72}\) These findings suggest that PEGylation will become an important technique for expanding the clinical application of therapeutic proteins. However, in reality, the successful application of PEGylation is limited, because the polymers react with or mask the active site of the bioactive protein. PEGylation generally targets not only the N-terminal \(\alpha\)-amino group but also the \(\varepsilon\)-amino group of lysine residues, which are often important for the formation of multi-dimensional structures and in the binding between ligands and receptors. Therefore,
Introducing polymers at these sites can potentially reduce the biological activity of the protein. Indeed, PEGylated interferon, the use of which has raised hopes as a potential cure for hepatitis C, can be produced only as a heterogeneous mixture with only 10% to 30% of its anticipated activity.

Improvement in bioconjugation efficiency will require the creation of a system that maintains the current efficiency of polymer modification and simultaneously makes it possible to modify only specific sites within a protein.

We have developed a novel PEGylation system combined with a method of creating functional muteins. In our strategy, site-specific PEGylation to improve therapeutic potency is established by using a fully bioactive lysine-deficient mutant. Whereas conventional PEGylation of TNF causes a loss of bioactivity due to random introduction of PEG at the ε-amino groups of six lysine residues in monomeric TNF, our site-specific PEGylation introduces PEG only at the N-terminus via a lysine-deficient mutant TNF, without loss of bioactivity. R1antTNF was generated by using a phage library based on the lysine-deficient mutant of TNF. Consequently, the R1antTNF also lacked lysine residues. Interestingly, the N-terminus of TNF is dispensable for function, because a deletion mutant of TNF lacking eight residues at the N-terminus retains full bioactivity.

Thus, site-specific PEGylated R1antTNF (PEG-R1antTNF) was uniform at the molecular level and had similar bioactivity (80%) to that of unmodified R1antTNF. Furthermore, introducing PEG only...

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### Table: TNFR1 selectivity of R1antTNF

| Clone | TNFR1 Kd (nM) | TNFR2 Kd (nM) | Selectivity for TNFR1 |
|-------|---------------|---------------|-----------------------|
| wtTNF | 1.4           | 2.1           | 1.0                   |
| R1antTNF | 3.5           | 92900.0       | 17677.4               |

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**Fig. 4.** Antagonistic activities of the tumor necrosis factor receptor 1-selective mutein (R1antTNF). a) Dissociation constants for the interaction between TNFR1 and wild-type TNF (wtTNF) or R1antTNF were calculated by surface plasmon resonance analysis. Selectivity of R1antTNF for TNFR1 was normalized to that of wtTNF. b) LM cells, mouse fibroblast cells, were incubated with serial dilutions of R1antTNF only (open circles) or R1antTNF mixed with human wild-type TNF (wtTNF) (closed circles). The inhibitory effects of R1antTNF on the cytotoxicity of wtTNF were assessed by a methylene blue assay. The absorbance of cells without wtTNF was plotted as 100% viability.

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**Advantages of PEGylation**

1. Blocks protease attack
2. Decreases renal excretion rate by increasing molecular size
3. Reduces immunogenicity

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**Fig. 5.** Advantages of PEGylation. Chemical modification of bioactive proteins with polyethylene glycol (PEG) is known as PEGylation. PEGylation prolongs the half-lives of proteins and allows them to be used at lower doses.
to the N-terminal amino group made it possible to produce stable bioconjugated proteins with nearly 100% yield. In fact, PEG-R1antTNF had therapeutic effects in an experimental autoimmune encephalomyelitis model and a collagen-induced arthritis model (Fig. 6). This could have been due to the enhanced retention of R1antTNF in the circulatory system. Moreover, the prolonged retention might increase the availability of R1antTNF to block TNF/TNFR1 interactions in the general circulation or in the lesion areas, resulting in improved inhibitory activity. In future, we hope that PEG-R1antTNF will be a therapeutic option for managing chronic inflammatory diseases and that our site-specific PEGylation system will become a standard technology for clinical application of protein drugs.

5. Conclusions and prospects

Despite the enhancement of basic research in Japan, clinical application of protein drugs remains limited because of the poor nature of fundamental platforms for drug discovery. Here, we discussed a series of DDS technologies needed for protein drug development. Moreover, we have recently succeeded in developing functionalized polymer carriers or nano-carriers, which are able to regulate drug delivery into various tissues such as the placenta or brain, and into cellular organelles such as nuclei. Furthermore, we consider not only efficacy but also safety to be important in drug development. We have thus been promoting the fusion of sustainable nanotechnology with nano-safety science for assuring the safety of nanomaterials and nano-safety design study for enhancing safety of nanomaterials. We hope that many innovative protein drugs will be developed in Japan by combining these technologies.

Acknowledgments

We thank our staff and our collaborators in this long-term project, especially S. Nagata, T. Ise, S. Tsumoda, H. Kamada, Y. Mukai, and T. Mori (National Institutes of Biomedical Innovation, Health, and Nutrition); Y. Yoshioka, H. Takahashi, and K. Misato (Research Institute for Microbial Diseases, Osaka University); H. Shibata, H. Nabeshi, T. Yoshida, and Y. Abe (National Institute of Health Sciences); and T. Yoshikawa and K. Hagihisaoka (Graduate School of Pharmaceutical Sciences, Osaka University) for supporting this study and for their fruitful discussions. We also thank the staff and students of the Laboratory of Biotechnology and Therapeutics, Laboratory of Toxicology and Safety Science (Osaka University), Laboratory of Biopharmaceutical Research, Laboratory of innovative Antibody Engineering and Design, and Laboratory of Pharmaceutical Proteomics (National Institutes of Biomedical Innovation, Health, and Nutrition). Finally, we sincerely thank our former teachers, T. Mayumi (Emeritus Professor, Osaka University), T. Kawanishi (Director, National Institute of Health Sciences), K. Yamanishi (Director, The Research Foundation for Microbial Diseases, Osaka University), Y. Yoneda (Director, National Institutes of Biomedical Innovation, Health, and Nutrition).

This study was financially 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. This study was also supported in part by Health Labour Sciences Research Grants from the Ministry of Health, Labour, and Welfare of Japan; by Health Sciences Research Grants for Research on Publicly Essential Drugs and Medical Devices from the Japan Health Sciences Foundation; by a Global Environment Research Fund from the Ministry of the Environment; and by the Knowledge Cluster Initiative, The Nagai Foundation Tokyo, and the Takeda Science Foundation.
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(Received Feb. 1, 2016; accepted Mar. 29, 2016)
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