Review

Development of Novel Drug Delivery System (DDS) Technologies for Proteomic-Based Drug Development

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Received June 24, 2004

With the success of human genome projects, the focus of life science research has shifted to the functional and structural analyses of proteins, such as disease proteomics. These structural and functional analyses of expressed proteins in the cells and/or tissues are expected to contribute to the identification of therapeutically applicable proteins for various diseases. Thus, pharmaco-proteomic based drug development for protein therapies is most noticed currently. However, there is a clinical difficulty to use almost bioactive proteins, because of their very low stability and pleiotropic actions *in vivo*. To promote pharmaco-proteomic based drug development for protein therapies to various diseases, we have attempted to establish a system for creating functional mutant proteins (muteins) with desired properties, and to develop a site-specific polymer-conjugation system for further improving the therapeutic potency of proteins. In this review, we are introducing our original protein-drug innovation systems mentioned above.

Key words phage display; PEGylation; proteomic; protein therapy; drug delivery system (DDS)

The completion of the human genome project has revealed that humans have about 30000 genes, and more than half of them encode unknown proteins. They include a number of proteins that are thought to be integrally related to pathological disorders and other biological phenomena. Thus, they are anticipated to be potentially useful as protein drugs and/or as targets for drug innovation and development. In the post-genome era, the focus of life science research has shifted to the functional and structural analysis of proteins, such as disease proteomics and structural genomics. Proteomics comprises the comprehensive analysis of all proteins with regard to their temporal and special, or qualitative and quantitative aspects. Structural genomics provides information about the relationship between protein function and 3D-structure. Thus, many scientists expect that these approaches will offer tremendous potential for discovery of novel drug targets and unique lead compounds for drug development.

In recent years, cytokine or antibody therapy has also received attention for advanced drug therapies in the twenty-first century, and attempts are being made to develop a wide variety of therapeutic proteins for diseases including cancer, hepatitis, and rheumatism.[1—9] For this reason, pharmaco-proteomic based drug development for protein therapies has received even greater attention as a future technology for the creation of therapeutic proteins.

It is a well-known fact that the later half of the 20th century brought about the identification of a number of bioactive proteins, anticipated as "dream" protein drugs for refractory diseases. However, as many cases in the past demonstrate, these proteins are limited in their clinical application because of unexpectedly low therapeutic effects.[5,6,10—12] The reason for this limitation is that these proteins are immediately degradated by various proteases *in vivo* and rapidly excreted from blood circulation. Therefore, frequent administration at an excessively high dose is required to obtain their therapeutic effects *in vivo*, which causes disturbance of homeostasis and unexpected side effects. Additionally, cytokines generally show pleiotropic actions through a number of receptors *in vivo*, making it difficult to elicit the desired effect without simultaneously triggering side effects as well. From this standpoint, creation of novel technologies that overcome the problems peculiar to bioactive proteins is absolutely essential for advancement of pharmaco-proteomic based drug development for protein therapies. These technologies are suitable for Drug Delivery Systems (DDS) that seek to maximize the therapeutic potency using proteins. It can thus be called a crossover, bridging the gap between basic research in the post-genome era and 21st century drug therapies.

Thus, our group's research has the following three goals (details of which will be taken up in separate sections to follow): 1) development of a powerful system that rapidly allows creation of functional muteins with a higher receptor affinity and receptor specificity based on the phage display technique,[13] 2) establishment of a novel polymer-conjugation system that can improve the stability of proteins *in vivo* and selectively increase their therapeutic effects,[13,14] 3) design of functional polymeric carriers (modifiers) with DDS capabilities such as targeting and controlled release.[15] We are now attempting to fuse[1—3] to create a protein-drug innovation system as the basis of DDS technology to further promote the pharmaco-proteomic based drug development for protein therapies. In this review, we will describe this protein-drug innovation system in as much detail as space permits.

CREATING A SYSTEM FOR PRODUCING ARTIFICIAL FUNCTIONAL MUTEINS WITH ADVANCED MEDICINAL APPLICATIONS

Seeking therapeutic applications of bioactive proteins, many research institutes for biotechnology have experimented in the past with the creation of muteins that had selectivity and high affinity for specific receptors by substituting amino acids with site-directed mutagenesis, as typified by the Kunkel method.[16—22] However, the creation of

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muteins by such point mutation approaches requires simulation of the mutant's 3D-structure and generation of individual mutants by replacement of the bioactive protein amino acids individually through a trial and error process. Additionally, each mutein's functional capabilities must be assessed individually to see if it achieves the desired goal. For these reasons, traditional approaches not only required a great investment of time and effort to create desired muteins, but also put limitations on the variety of mutational types that could be produced, making it difficult to achieve results as expected.

In recent years, the phage display system has been developed for the rapid and exhaustive determination of high affinity ligands for given targets (*i.e.*, a target molecule, particle and/or cell) by utilizing information on it's life cycle.[23] The advantages of a phage display system are 1) easily preparing a library consisting of structural variants of a polypeptide as diverse as over one hundred million and 2) isolating several molecules binding to a targeted ligand from this library in a few weeks. Thus, phage libraries displaying polypeptides have extensively been applied to identification of specific molecules with high affinity for targeted ligands. The phage display method is thus expanding its range of applications as a standard technology for quickly and thoroughly screening out bond molecules.[24—28] However, at present it appears to only be utilized as a way of identifying antibodies and peptides with high affinity for specific targets. There appear to have been no cases of exposing bioactive proteins like cytokines on phage surfaces.

Given the background outlined above, we first used the phage display system to quickly and efficiently create functional muteins with advanced medical value. Functional muteins with improved affinity and specificity for target receptors were selected from more than 10^8 types of structural mutant proteins displayed on phage particles. This is a neo-Darwinist molecular evolution strategy for pharmaco-proteomic based drug development for protein therapies. An example in previous structure-activity relationship research using site-directed mutagenesis like the alanine scan is that lysine residues (including Lys11, Lys65/Lys90) were generally thought to be indispensable in the formation of TNF-α (Tumor Necrosis Factor-α) 3D-structures (*i.e.*, trimeric structures) and receptor bonding. Because lysine residues have critical roles in protein structure or ligand-receptor binding, the replacement of lysine residues with amino acids other than basic ones such as arginine was assumed to be impossible without loss of bioactivity in point mutation analysis without being limited to TNF-α. In fact, no such muteins existed with which all lysine residues were substituted for other amino residues with full bioactivity (*i.e.*, a lysine-deficient mutant) until recently. However, we succeeded in creating lysine-deficient mutant TNF-αs that retained its original bioactivity, with some of them displaying 13 times the bioactivity within an *in vivo* therapeutic window of wild-type TNF-α (wTNF-α) Fig. 1. Our results have shaken the fixed concept mentioned before. Analysis using BIAcore and ultracentrifugation suggested that lysine-deficient mutant TNF-αs have affinities for TNF-R1 and TNF-R2 equivalent to that of wild-type TNF-α. We also confirmed through ultracentrifugation and gel filtration chromatography that they formed trimeric structures. These results lead us to believe that we have,

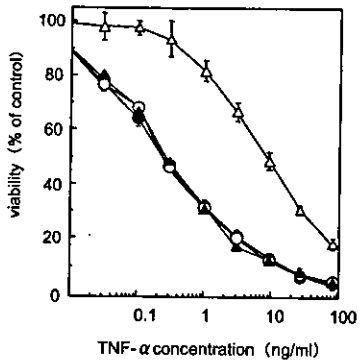

Fig. 1. *In Vitro* Specific Bioactivity of Randomly Mono-PEGylated wTNF-α (Ran-PEG-wTNF-α) and Site-Specifically Mono-PEGylated Lysine-Deficient Mutant TNF-α ([sp-PEG-mTNF-α-Lys(−))

○ wTNF-α, △ ran-PEG-wTNF-α, ● mTNF-α-Lys(−), ▲ sp-PEG-mTNF-α-Lys(−).

through affinity selection using TNF-R1 and anti-TNF neutralizing antibody, successfully isolated lysine-deficient mutant TNF-αs with full bioactivity and correct tertiary structure from the phage library consisting of 10^8 variant TNF-α with replacement of all lysine residues with other residues. Using these technologies, we have already created a number of functional TNF-α muteins and improved their affinity and *in vivo* stability. We also constructed lysine-deficient libraries of other cytokines, receptor proteins, and antibodies to create lysine-deficient muteins with full activity. Our system for applying the phage surface method to the construction of artificial functional muteins has not only shown the tremendous power for the DDS-based pharmaco-proteomic based drug discovery and development for protein therapy technology, but also redefined the concepts of protein engineering and the correlation between protein structure and activity, findings that have not been previously obtained by mutational technologies like the alanine scan. Remarkable progress in proteomics and structural genomics will accelerate the implementation of pharmaco-proteomic based drug development for protein therapies through combination with our neo-Darwinist molecular evolution strategy in the near future.

At the same time, progress in bioinformatics has provided a greater understanding of the relationship between amino acid sequences and structures of proteins and their functions, which will allow the prediction of structures and functions of unknown proteins, given an amino acid sequence. Thus, this will enable us to design amino acid sequences with a desired function and structure and efficiently simulate organic substances that mimic their forms and functions. To improve bioinformatics, structural muteins having a vast amount of diversity with regard to a wide variety of proteins must be created, and information on their functional properties such as ligand-receptor binding and bioactivity must be accumulated to determine the structure–activity relationship. Our group's "system of creating functional muteins" was capable of creating a library of more than 10^8 structural mutants in a single week, making it an important technology for the gathering of such information. In addition to the functional assessment of artificial functional TNF-α and other proteins, as described above, we are currently working on X-ray crystallography as well, in hopes that the combination of these two

forms of technology will contribute to new developments in bioinformatics in the near future.

A NEW METHOD FOR SITE-SPECIFIC POLYMER-CONJUGATION

Following the 1980's, attachment of water-soluble polymers like PEG (polyethylene glycol) to the surface of bioactive proteins (polymer-conjugation) has been devised in the field of protein drug development including DDS to overcome problems in their clinical application.[29—36] Covalent conjugation of proteins with PEG is specifically referred to as PEGylation. Polymer-conjugation of bioactive proteins decreases their renal excretion rate due to the increased molecular size. In addition, since the water-soluble polymers cover the protein surface, the attack from proteases is blocked by steric hindrance, resulting in prolongation of the *in vivo* half-life Fig. 2. By a similar effect of steric hindrance, antigenecity and immunogenicity in immune response decrease, resulting in prolongation of *in vivo* clearance and stabilization. All of these advantages lead to an increase in internal stability, in turn making it possible to decrease both administration doses and times. Polymer-conjugation has been recognized as one of the most efficient ways of improving therapeutic potency of proteins among several DDS technologies for pharmaco-proteomic based drug development for protein therapies. However, bioconjugated proteins described to date have mostly been limited to enzymes such as Adenosine Deaminase and Superoxide Dismutase (SOD) acting on low-molecular-weight materials.[37—39] In our previous study, the specific activity of bioconjugated SOD was not associated with the molecular weight of PEG and was determined only by the modification rate of active sites Fig. 3. These results suggest that SOD-substrate complex formation was possible without steric hindrance by water-soluble polymeric modifiers attached to SOD because of the low molecular weight of the substrate (superoxide anion). On the other hand, IL-6 (Interleukin-6),[40] which requires binding to high molecular receptors for the development of activity, displayed decreases in specific activity with an increase in the modification rate by water-soluble macromolecules, and the degree of this decrease was more notable when the molecular weight of the modifying macromolecule was larger Fig. 3. For this reason, in bioactive proteins consideration should be given to severe loss of activity derived from inhibition of binding to receptor molecules caused by steric hindrance formed in the polymeric modifier, in addition to a decrease in the specific activity due to modification of the active sites. Therefore, polymer-conjugation of bioactive proteins like cytokines that need binding with macromolecular receptors is advantageous in that it blocks the attacks of proteases by steric hindrance more effectively when the molecular weight of the modifying is larger but is overwhelmingly disadvantageous in that it also inhibits receptor binding, causing a reduction in the specific activity. Furthermore, the increase in the molecular weight by water-soluble polymeric modifiers improves the retention of the proteins in the circulation as a result of a decrease in the renal excretion rate, but this simultaneously causes marked restriction of their transport from the circulation to tissue. Thus, polymer-conjugation of physiologically active proteins that need binding with macromolecular receptors to produce their activity do good as well as harm.

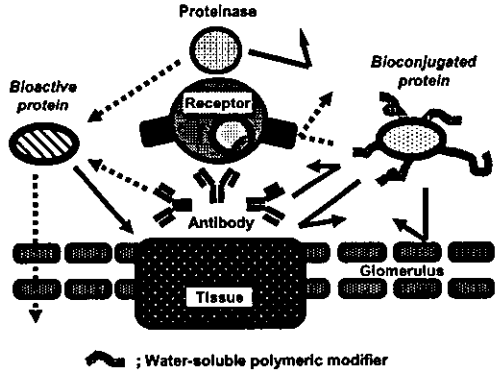

Fig. 2. Conjugation of Proteins with Water-Soluble Polymeric Modifiers

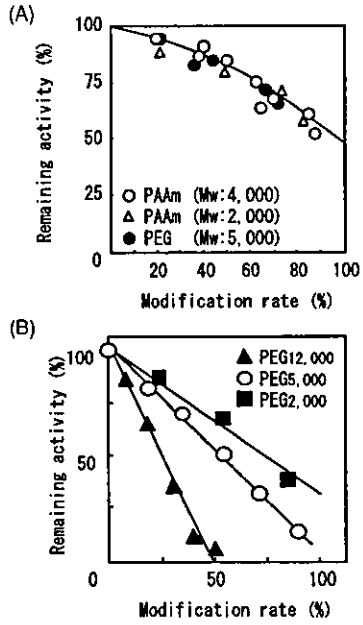

Fig. 3. Chemical Conjugation of Superoxide Dismutase (SOD) and Interleukin-6 (IL-6)

(A) Effects of degree of modification with water-soluble polymeric modifiers on SOD-activity. (B) Effects of degree of PEG-modification on bioactivity of PEGylated IL-6.

With these problems described above in mind, we attempted to improve the polymer-conjugation system using TNF-α and IL-6 as model proteins (therapeutic effect: antitumor effect for TNF-α, thrombopoietic effect for IL-6).[13—15,40—44] In summary, a loss in bioactivity could not be avoided but we: 1) selected the optimal modifying molecule according to the purpose of polymer-conjugation and properties of individual physiologically active proteins and 2) accumulated basic data such as the molecular weight-modification rate-activity relationship in detail and then determined the optimal polymer-conjugation conditions to a) increase plasma half-life and stability, b) control behavior in the body (well-balanced tissue transport), and c) selectively enhance desirable therapeutic activities of bioactive proteins without increasing their side effects. This c) selective enhancement of therapeutic effects in bioactive proteins was mainly brought about by a decrease in given dosages with increased *in vivo*

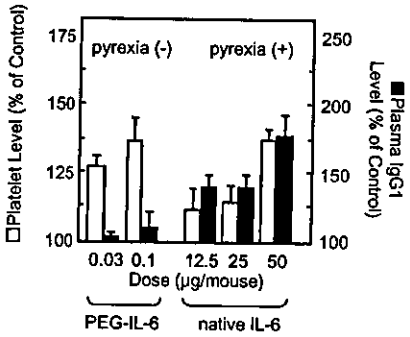

Fig. 4. PEGylation of IL-6 Effectively Increases Its *in Vivo* Thrombopoietic Potency

stability and by prevention of transport to tissues that were affected by the side effects. For example, we succeeded in increasing the anti-tumor effect of TNF-α and the thrombopoietic effect of IL-6 by 100 fold and 500 fold, respectively, without any side effects Fig. 4. From this standpoint, polymer-conjugation of bioactive proteins that requires binding to high molecular receptors for the development of activity has been attempted globally, and PEGylated Interferon-α has been used in clinical treatment of hepatitis C.[31,33,35] However, this approach is limited by the frequent substantial loss of protein specific activity associated with polymer-conjugation. Lysine amino groups of proteins are often used as substrates for polymer-conjugation because they are highly reactive and the bionconjugation reaction is mild enough to minimize disruption of the protein structure. This polymer-conjugation, however, is nonspecific and occurs at the N terminus as well as all internal lysine residues, some of which may be in or near protein active sites. Such bioconjugated proteins thus consist of positional isomers with polymeric modifiers at various sites that have distinct activities and other characteristics. Such bioconjugated isomer mixtures can therefore have inconsistent therapeutic effects. To overcome this problem, site-specific polymer-conjugation can be achieved through a free thiol in the engineered proteins.[14,45] This efficacy of this approach is compromised, however, by an extremely low yield of bioconjugated proteins and a substantial loss of activity owing to the introduction of a free thiol residue.

Based on these points, we successfully developed a novel polymer-conjugation system fused to a "neo-Darwinist model of molecular evolution." In other words, we first applied the phage display system to create fully bioactive lysine-deficient mutant proteins and then carried out site-specific polymer-conjugation to improve therapeutic potency. Regarding this site-specific polymer-conjugation, the maximal yield of bioconjugated protein was >90% with superior molecular uniformity because the N terminus of the protein is specifically bioconjugated.

An example in TNF-α is where amino groups of all six internal lysine residues are also targets for polymer-conjugation, and studies using site-directed mutagenesis analysis have reported that Lys11 and Lys90 are essential for the protein's homotrimer formation as an active form and for interaction with its receptor, respectively.[46–48] Therefore, traditional polymer-conjugation as mentioned has caused a substantial loss of specific activity. Indeed, this is supported by our results, in which randomly mono-PEGylated wild-type TNF-α (ran-PEG-TNF-α) was heterogeneous at the molecular level and had <10% of the specific bioactivity of unmodified wild-type TNF-α.

On the other hand, the N terminus of TNF-α is not indispensable for function because a deletion mutant of TNF-α lacking eight residues at the N terminus retains full bioactivity. In such instances, site-specific mono-PEGylated TNF-α (sp-PEG-TNF-α) was uniform at the molecular level and had similar bioactivity (80%) to that of unmodified mTNF-α. Additionally, this sp-PEG-TNF-αs displayed greater antitumor therapeutic potency than ran-PEG-TNF-α because of an increase in plasma half-life. Thus, we are now attempting to study the clinical application of sp-PEG-TNF-αs. On the other hand, we are currently trying to construct lysine-deficient forms of IFN-α, TNF-R1, antibody specific to TNF-α (scFv), and IL-6 with full activity and site-specific PEGylation of their N termini.

DESIGNING OF FUNCTIONAL POLYMERIC CARRIERS WITH DDS CAPABILITIES

The fate and distribution of conjugates between polymeric carriers and drugs is determined by their physicochemical properties, such as electric charge and hydrophilic balance. Therefore, we have been trying to design polymeric carriers with useful functions such as targeting and controlled release capability, which can closely regulate their behavioral characteristics *in vivo*, to achieve optimization of drug therapy by polymer conjugation.

For example, we have previously reported that PEG and PVP are useful and powerful polymeric carriers for improvement of the plasma half-lives of proteins. Furthermore, the copolymer of divinyl ether and maleic anhydride (DIVEMA) has antitumor activity (induction of INF-γ and activation of immunocompetent cells)[44] and the PVP introduced lauric acid selectively accumulated in the spleen.[43] These novel polymeric modifiers will be used to increase plasma half-lives of conjugated bioactive proteins and to provide useful DDS functions for control of the behavior of proteins *in vivo*, which cause further increase their therapeutic activity and safety by isolation of desirable activity for clinical use.

From a series of research, we have synthesized a novel polymeric drug carrier, polyvinylpyrrolidone-co-dimethyl maleic anhydride (PVD), which accumulated in the kidney and displayed controlled-release in response to changes in pH.[15] PVD binds to an amino group of a protein by forming an amide bond through its acid anhydride group at a pH of over 8.0 and then reversibly dissociates from the amino group, changing in pH to a slightly acidic form. In inflammatory tissue and tumor tissue, the pH is lower than normal. Therefore, if PVD is used in nephritis and renal cancer, PVD is expected to accumulate in the kidneys and gradually release the drugs. We found that about 80% of the dose of PVD selectively accumulated in the kidneys 24 h after intravenous injection, and about 40% remained 96 h after beginning the treatment Fig 5. PVD was selectively taken up by renal proximal tubular epithelial cells and no cytotoxicity was noted. Higher doses did not produce toxicity in the kidneys or other tissues. Additionally, PVD-modified superoxide dismutase accumulated in the kidneys after intravenous ad-

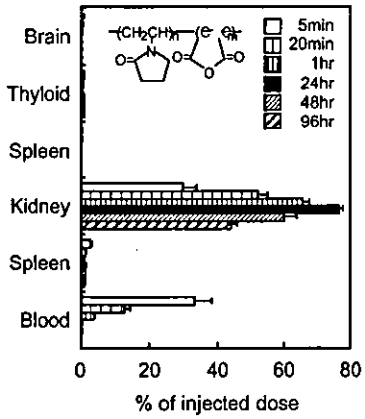

Fig. 5. Tissue Distribution of PVD after i.v. Injection in Mice

ministration and accelerated recovery from acute renal failure in a mouse model.

Foreshadowing the aging of society, renal disease is a serious problem that will increase worldwide.[49] Dialysis and renal translation are two treatment options. Although they are life-saving, they are expensive and do not restore normal health. Therefore, new strategies must be developed to treat patients with chronic kidney disease. From this standpoint, we are attempting to construct a new strategy for these patients by fusing the two systems mentioned, the mutein-creation system using a phage display technique and the site-specific polymer-conjugation system.

CONCLUSION

In this review, we presented the protein-drug innovation system as the basis of DDS technology in three approaches. We believe that our system will play a critical role in pharmaco-proteomic based drug development for protein therapies as translational research. Recently, gene shuffling[50—52] and artificial gene encoding systems[53] have received a great deal of attention. These approaches can create artificial proteins constructed with amino acids or sequences that do not exist in nature, though they have unfortunately not been able to produce bioactive proteins that can be applied to clinical settings. These technologies can be adapted to our systems for the screening, creation, stabilization, or functioning of non-natural proteins. We are now trying to construct a new strategy for production of artificial proteins by applying gene shuffling to the phage display system.

Making progress in pharmaco-proteomic based drug discovery and development for protein therapie requires the production of a wide variety of proteins and protein structural mutant types, development of a high-throughput screening of their function (*i.e.*, on the status and strength of ligand-receptor bonds), and thorough evaluation of the relationship between these factors and a protein's structure. Additionally, pharmaco-proteomic based drug discovery and development for protein therapie will not be achieved until bioinformatics allow the prediction of the functions and structures of unknown proteins using genome sequencing information. Our strategy, as outlined above, may become an essential basic technology for large-scale and high-throughput analysis of protein functions.

We are confident that our research has taken a number of important steps towards understanding the relationship between a protein's function (*i.e.*, medicinal value) and its structure. And we sincerely hope that the results of our research will contribute to the building of a pharmaco-bioinfomatics database that can be used to effectively set parameters for functional artificial proteins in the future.

Acknowledgments This study was supported in part by a Grant-in-Aid for Scientific Research (No. 15680014 and No. 16023242) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, in part by a Health and Labor Sciences Research Grants from the Ministry of Health, Labor and Welfare of Japan, in part by Health Sciences Research Grants for Research on Health Sciences focusing on Drug Innovation from the Japan Health Sciences Foundation, in part by Takeda Science Foundation, and in part by Senri Life Science Foundation.

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Featured Article

Functionalization of Tumor Necrosis Factor-α Using Phage Display Technique and PEGylation Improves Its Antitumor Therapeutic Window

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ABSTRACT

Purpose: **In this study, the optimization of antitumor therapy with tumor necrosis factor-α (TNF-α) was attempted.**

Experimental Design: **Using the phage display technique, we created a lysine-deficient mutant TNF-α (mTNF-K90R). This mutant had higher affinities to both TNF receptors, despite reports that certain lysine residues play important roles in trimer formation and receptor binding.**

Results: **The mTNF-K90R showed an *in vivo* therapeutic window that was 13-fold higher than that of the wild-type TNF-α (wTNF-α). This was due to the synergistic effect of its 6-fold stronger *in vitro* bioactivity and its 2-fold longer plasma half-life derived from its surface negative potential. The reason why the mTNF-K90R showed a higher bioactivity was understood by a molecular modeling analysis of the complex between the wTNF-α and TNF receptor-I. The mTNF-K90R, which was site-specifically mono-PEGylated at the NH_2 terminus (sp-PEG-mTNF-K90R), had a higher *in vitro* bioactivity and considerably longer plasma half-life than the wTNF-α, whereas the randomly mono-PEGylated wTNF-α had 6% of the bioactivity of the wTNF-α. With regard to effectiveness and safety, the *in vivo* antitumor therapeutic window of the sp-PEG-mTNF-K90R was 60-fold wider than that of the wTNF-α.**

Conclusions: **These results indicated that this functionalized TNF-α may be useful not only as an antitumor agent but also as a selective enhancer of vascular permeability in tumors for improving antitumor chemotherapy.**

INTRODUCTION

With the success of the human genome project, the focus of life science research has shifted to the functional and structural analyses of proteins, such as the fields of proteomics and structural genomics. These analyses of proteins, including newly identified proteins, are expected to contribute to the identification of therapeutically applicable proteins for various diseases. Thus, pharmacoproteomic-based drug discovery and the development of protein therapies has currently attracted a great deal of attention (1–3). However, it is clinically difficult to use most bioactive proteins, such as tumor necrosis factor-α (TNF-α), as antitumor agents because of their very low stability and pleiotropic action *in vivo* (4, 5).

TNF-α was reported to exert a strong cytotoxicity to various kinds of tumor cells but not to normal cells *in vitro* and to cause hemorrhagic necrosis of certain transplanted solid tumors (6). Thus, TNF-α has been considered a promising new drug for cancer therapy. On account of its short plasma half-life, a continuous infusion or frequent administration at high doses of TNF-α was required to sustain its plasma level to obtain significant antitumor effects. Additionally, TNF-α was found to have unexpected toxic side-effects in phase I studies (7). These severe toxicities of TNF-α prevented the administration of dosages required for replicating the antitumor activity observed in preclinical studies. For this reason, the clinical application of TNF-α as a systemic antitumor agent has been limited, although intratumoral administration of TNF-α showed marked antitumor effects in phase I studies (8, 9). Recently, TNF-α has been

Received 4/20/04; revised 8/1/04; accepted 8/18/04.
Grant support: Grant-in-Aid for Scientific Research (No. 15680014 and No. 16023242) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, in part by a Health and Labor Sciences Research Grants from Ministry of Health, Labor, and Welfare of Japan, in part by Health Sciences Research Grants for Research on Health Sciences Focusing on Drug Innovation from the Japan Health Sciences Foundation, in part by Takeda Science Foundation, and in part by Senri Life Science Foundation.
Note: H. Shibata and Y. Yoshioka contributed equally to the work.
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clinically applied to locoregional combination therapy with Melphalan, and this therapy showed a marked antitumor effect in patients with in-transit melanoma metastases (10–13). This clinical approach using TNF-α as a selective destruction agent against tumor endothelial cells and a selective enhancer of tumor vascular permeability for effective accumulation of antitumor chemotherapeutic agents is currently an attractive topic for optimization of cancer chemotherapy. This was approved by the European Agency for the Evaluation of Medicinal Products (14, 15). Additionally, fusion proteins composed of TNF-α and antitumor antibodies have been designed as systemic antitumor agents with tumor-targeting capabilities for improving TNF-α therapy (16–19). Thus, the creation of a functionalized TNF-α having both superior antitumor effectiveness and safety will be useful for obtaining not only the required antitumor effects but also selectively enhancing the vascular permeability of tumor vessels by its systemic administration.

To additionally promote these clinical applications of TNF-α, it is necessary to develop a system to create mutant proteins (muteins) with desired properties, such as superior bioactivity, and a drug delivery system to selectively enhance the desirable therapeutic activities of TNF-α and its muteins without increasing their side effects. A protein-drug innovation system that fuses these two systems will demonstrate the tremendous potential of TNF-α therapy.

We have attempted previously to create useful muteins by the substitution of amino acids using a site-directed mutagenesis method, as typified by Kunkel's method (20, 21). For instance, it was observed that a point mutation of basic residues that resulted in neutral or acid residues in immunotoxins lowered their isoelectric point, thus resulting in their reduced *in vivo* toxicity without loss of antitumor therapeutic activity. This was probably due to desirable changes in their pharmacokinetic properties that were derived from the surface negative electrostatic potential. However, the creation of muteins by such point mutation approaches takes a vast amount of time and effort to obtain the desired muteins, whereas the lowering of the isoelectric point of bioactive proteins may be an attractive approach to improve their therapeutic potency.

One of the most useful ways of enhancing the plasma half-lives of proteins is to conjugate them with polyethylene glycol (PEG) and other water-soluble polymeric modifiers (22–28). The covalent conjugation of proteins with PEG (PEGylation) increases their molecular size and steric hindrance, both of which depend on the properties of the PEG attached to the protein. This results in the avoidance of their renal excretion and in improvement of their proteolytic stability, whereas decreasing their immunogenicity and hepatic uptake. We have also reported that the optimal PEGylation of bioactive proteins could selectively improve their *in vivo* therapeutic potency and reduce side effects (22, 24–26). However, the PEGylation of proteins was mostly nonspecific and targeted at all of the lysine residues in the protein, some of which may be in or near an active site. As a result, the PEGylation of proteins was accompanied by a significant loss of their specific activities *in vitro* (27, 28). Thus, the clinical application of PEGylated proteins has been limited until today.

To overcome the problems of PEGylation mentioned above, using TNF-α as a model, we attempted recently to develop a novel strategy for site-specific mono-PEGylation for improvement of its *in vivo* antitumor potency (29). We isolated a bioactive lysine-deficient mutant TNF-α (mTNF-α) from phage libraries expressing mTNF-αs, in which all of the lysine residues were replaced with other amino acids. This lysine-deficient mTNF-α was site-specifically mono-PEGylated at its NH_2 terminus with PEG. This site-specifically mono-PEGylated mTNF-α showed increased antitumor therapeutic potency, compared with the unmodified wild-type TNF-α (wTNF-α) and randomly mono-PEGylated wTNF-α. However, the *in vitro* bioactivity of this site-specifically mono-PEGylated mTNF-α and its affinity to both the TNF-receptor I (TNF-RI) and TNF-receptor II (TNF-RII) decreased significantly compared with those of the wTNF-α. Thus, if it was possible to create a lysine-deficient mTNF-α with stronger *in vitro* bioactivity and higher receptor-affinity, a site-specific PEGylation of such a super mutant TNF-α (smTNF-α), which has a lower isoelectric point than the wTNF-α, may synergistically enhance the antitumor activity *in vivo* without adverse side effects.

In this study, we attempted to create a lysine-deficient smTNF-α with superior *in vitro* bioactivity and a lower isoelectric point using the phage display technique with a few modifications. To our knowledge, there are no reports regarding the creation of lysine-deficient mutant proteins with a higher bioactivity. The molecular modeling of TNF-α revealed the mechanism of higher bioactivity of the obtained smTNF-α. The obtained smTNF-α was site-specifically PEGylated at its NH_2 terminus. The mono-PEGylated smTNF-α thus obtained showed a higher *in vitro* bioactivity and longer plasma half-life than the unmodified wTNF-α and a previously reported site-specifically mono-PEGylated mTNF-α. Furthermore, its antitumor therapeutic window was 60-fold wider compared with that of the wTNF-α. Thus, this study will open new avenues for antitumor therapy using TNF-α as an antitumor agent and an enhancer of tumor vascular permeability. Additionally, this study may allow for the development of a novel protein-drug innovation system to promote pharmacoproteomic based drug discovery and development of protein therapies.

MATERIALS AND METHODS

Library Construction of Lysine-Deficient mTNF-αs and Selection of smTNF-α with Higher Bioactivity. Plasmid pY02-TNF encoding the wTNF-α, in which the COOH terminus of TNF-α was fused to the NH_2 terminus of the M13 phage g3p, was used as a PCR template for constructing the cDNA library of lysine-deficient mTNF-αs. A three-step PCR amplification was carried out using oligonucleotides containing the sequence "NNS" (where N and S represent G/A/T/C and G/C, respectively) at the wTNF-α codons for Lys11, Lys65, Lys90, Lys98, Lys112, and Lys128. The NNS can encode all 20 of the amino acids. The third PCR products were digested with restriction enzymes and ligated with the phagemid vector pY02. A phage library displaying lysine-deficient mTNF-αs was prepared, and phage clones displaying the smTNF-αs with a higher bioactivity were selected using BIAcore 2000 (BIAcore, Uppsala, Sweden) with TNF-RI, as described elsewhere (29). The bioactivity of each smTNF-α displayed by the phage clone was

Table 1 Amino acid sequence and the biological properties of the lysine-deficient smTNF-α

	Residue positions									
	11	65	90	98	112	128	pI	K_d (× 10 ^{10}M)	LC_{50} (ng/ml)	LD_{50} (μg protein/kg)
wTNF-α	Lys	Lys	Lys	Lys	Lys	Lys	7.44	2.01	0.17	390
mTNF-K90R	Ala	Ser	Arg	Ala	Leu	Thr	4.96	1.53	0.03	510
mTNF-K90P	Ala	Ser	Pro	Ala	Leu	Thr	4.76	1.51	0.14	Not determined

NOTE. The pI of smTNF-α was calculated using a program in the Genetics Computer Group (Madison, WI). K_d values for smTNF-αs and human TNF receptor-I were determined using surface plasmon resonance (BIAcore).

examined by a cytotoxicity assay using mouse LM cells, a cell line derived from L929 cells (30).

Expression and Purification of Recombinant TNF-αs. Plasmids pYas1-TNF, pYas1-K90R, and pYas1-K90P encoding the human wTNF-α and smTNF-αs (mTNF-K90R and mTNF-K90P), under the control of a T7 promoter, were prepared. Three recombinant TNF-αs were produced in *Escherichia coli* BL21(DE3) harboring these expression plasmids as described previously (24, 29). Endotoxin levels were determined to be <300 pg/mg each in the wTNF-α and smTNFαs. The electrostatic potential surface was generated using GRASP (31). The TNF-α crystal structure, PDB entry 1TNF, was used for the calculation. The electrostatic potential ranged from −7.5 kT (red) to 7.5 kT (blue). The isoelectric point of each TNF-α was calculated using a program in the Genetics Computer Group (Madison, WI) package that is available online.[7]

PEGylation of TNF-αs. Methoxy-PEG-succinimidyl propionate (mPEG-SPA; Mr 5000) was purchased from Shearwater Polymers (Huntsville, AL). The wTNF-α and mTNF-K90R in PBS were reacted with a 5-fold (wTNF-α) or 50-fold (mTNF-K90R) molar excess of mPEG5K-SPA, in terms of total primary amino groups of TNF-α, at 37°C for 30 minutes. After this, ε-aminocaproic acid (10 times molar excess in terms of the mPEG5K-SPA) was added to stop the reaction. The specific bioactivities of the mono-PEGylated forms of TNF-α were examined by a cytotoxicity assay using LM cells (30).

***In vivo* Studies.** All of the animal experiment protocols were in accordance with the Guide for Laboratory Animal Facilities and Care. These protocols have been approved by the committee of the Pharmaceutical School, Osaka University. The antitumor effects of TNF-αs were assessed in mice bearing Meth-A fibrosarcoma. The Meth-A cells were implanted intradermally (2×10^5 cells per site) in 5-week-old female BALB/c mice. On day 7, when the tumor diameter reached 7 mm, the TNF-α molecules were administered by a single i.v. injection. The antitumor potency was estimated from the tumor volume and tumor hemorrhagic necrosis 24 hours after the injection. The tumor volume was calculated using the formula described by Haranaka *et al.* (32). For the pharmacokinetic assay, normal female BALB/c mice were injected i.v. with 1 μg each of various TNF-αs. Blood samples were drawn at different times after the injection. The concentrations of TNF-α in the blood samples were measured by ELISA. For assessment of *in vivo* toxicity (LD_{50}; the dose that kills half of the animals tested), groups of 4 to 6 female BALB/c mice were injected i.v. with increasing doses of various TNF-αs.

RESULTS

Selection of a Lysine-Deficient smTNF-α with Higher Bioactivity. To create a lysine-deficient smTNF-α with higher bioactivity, a phage library displaying mTNF-αs with randomized sequences in place of the six lysine codons was prepared. The phage library was subjected to several rounds of panning against human TNF-RI using a BIAcore biosensor, and the clones were screened for TNF-specific bioactivity by a cytotoxicity assay using LM cells. In this screening, we succeeded in obtaining two smTNF-αs (mTNF-K90R and mTNF-K90P). DNA sequencing analysis of the mTNF-K90R and mTNF-K90P indicated that these proteins lacked all six of the lysine residues, which replaced similar amino acids (Table 1). The isoelectric point of the mTNF-K90R and mTNF-K90P was lowered from 7.44 (wTNF-α) to 4.96 and 4.76, respectively.

Properties of the Novel Lysine-Deficient smTNF-αs. The recombinant human wTNF-α, mTNF-K90R, and mTNF-K90P were prepared by a general recombinant protein technology. The purified mTNF-K90R and mTNF-K90P were analyzed by SDS-PAGE that revealed a single band of ~17 KDa as well as the wTNF-α. Using gel filtration chromatography, we confirmed that the mTNF-K90R and mTNF-K90P formed a trimeric structure, similar to that of the wTNF-α and natural human TNF-α, in an aqueous solution (data not shown). We assessed the changes in the surface electrostatic potential of both the mTNF-K90R and mTNF-K90P. Fig. 1 shows the electrostatic potential mapped onto the molecular surface of the wTNF-α, mTNF-K90R, and mTNF-K90P with red designating negative and blue designating positive potential values. The surface areas of negative potential were found to increase on the mTNF-K90R and mTNF-K90P due to the lack of lysine residues. The K_d value of the mTNF-K90R and mTNF-K90P to TNF-RI was 1.3-fold stronger than that of the wTNF-α, as shown in Table 1. The affinity of this smTNF-α to TNF-RII was also higher than that of the wTNF-α (data not shown). By means of the *in vitro* cytotoxicity assay using mouse LM cells in the presence of actinomycin D at the concentration of 2 μg/mL, the LC_{50} values (specific bioactivity) of the wTNF-α, mTNF-K90R, and mTNF-K90P were found to be 0.17 ng/mL, 0.03 ng/mL, and 0.14 ng/mL, respectively. We constructed >10 mutants of the mTNF-K90R in which K90 replaced any other residues by site-directed mutagenesis. The mTNF-K90R had the highest *in vitro* bioactivity and affinity to both TNF-RI and TNF-RII. The *in vivo* antitumor activity of the mTNF-K90R against mouse

[7] Internet address: http://molbio.info.nih.gov/molbio/gcglite/protform.htm.

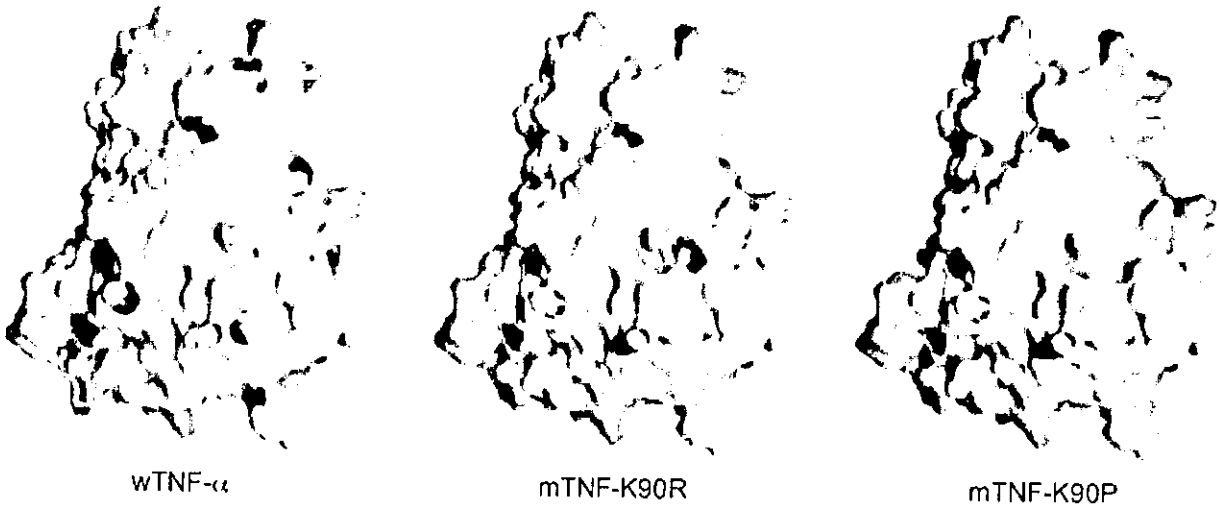


Fig. 1 Electrostatic potential mapped to the molecular surface of the wTNF-α, mTNF-K90R, and mTNF-K90P. The electrostatic potential ranges from -7.5 kT (red) to 7.5 kT (blue). Both the mTNF-K90R and mTNF-K90P were modeled manually based on the wTNF-α structure using the graphic program O (46). The images were produced using GRASP (31).

Meth-A solid tumors after a single i.v. injection was assessed by evaluating tumor hemorrhagic necrosis as an index (Fig. 2*A*). The wTNF-α showed weak hemorrhagic necrotic effects in a dose-dependent manner. In contrast, the mTNF-K90R at a dose of 0.3 μg per mouse expressed antitumor effects superior to those of the wTNF-α at a dose of 3 μg per mouse. The LD_{50} values for the wTNF-α and mTNF-K90R were 390 and 510 μg protein/kg, respectively (Table 1). These results indicated that the mTNF-K90R had 10-fold stronger *in vivo* antitumor activity and 1.3-fold weaker toxicity than the wTNF-α, whereas the *in vitro* bioactivity of the mTNF-K90R was 6-fold stronger. Thus, the pharmacokinetics of the mTNF-K90R after the i.v. injection of a single dose of 1 μg per mouse were measured (Fig. 3). We found that the plasma half-life of the mTNF-K90R was 2-fold longer than that of the wTNF-α.

Site-specific PEGylation of mTNF-K90R. The wTNF-α and mTNF-K90R were modified with 5 kDa of activated PEG. We confirmed by SDS-PAGE that a single PEG molecule was attached selectively to the NH_2 terminus of the mTNF-K90R, whereas PEG molecules were introduced randomly at multiple positions in the wTNF-α (data not shown). TNF-α molecules conjugated with one PEG molecule were purified by gel filtration- high-performance liquid chromatography. The purified site-specific mono-PEGylated mTNF-K90R (sp-PEG-mTNF-K90R) and randomly mono-PEGylated wTNF-α (ran-PEG-wTNF-α) were examined for their specific bioactivity (Fig. 4). The sp-PEG-mTNF-K90R had 60% of the specific activity of the mTNF-K90R, whereas the ran-PEG-wTNF-α had only 6% of the specific activity of the wTNF-α. Surprisingly, the sp-PEG-mTNF-K90R had higher *in vitro* bioactivity than the wTNF-α.

Antitumor Therapeutic Window of the sp-PEG-mTNF-K90R. To clarify the antitumor therapeutic window of the sp-PEG-mTNF-K90R, its *in vivo* antitumor activity and toxicity were assessed (Fig. 2). As shown in Fig. 2*A*, the antitumor activity of the ran-PEG-wTNF-α was similar to that of the wTNF-α. In contrast, the sp-PEG-mTNF-K90R at a dose of 1 μg per mouse induced marked tumor hemorrhagic necrosis compared with the mTNF-K90R at a dose of 3 μg per mouse. These results indicated that the antitumor activity of the sp-PEG-mTNF-K90R was >3-fold higher than that of the mTNF-K90R, which had *in vivo* antitumor effects that were 10-fold stronger than the wTNF-α. Thus, the sp-PEG-mTNF-K90R had an antitumor potency that was >30-fold higher than that of the wTNF-α and ran-PEG-wTNF-α. A single i.v. injection of the sp-PEG-mTNF-K90R at a dose of 1 μg per mouse completely inhibited solid tumor growth (Fig. 2*B*). The LD_{50} values of the sp-PEG-mTNF-K90R and ran-PEG-wTNF-α were 780 and 1,290 μg protein/kg, respectively. These results indicated that the *in vivo* toxicity of the sp-PEG-mTNF-K90R was ~1.5-fold, 2.0-fold, and 0.6-fold lower than that of the mTNF-K90R, wTNF-α, and ran-PEG-wTNF-α, respectively. Thus, the therapeutic window of the sp-PEG-mTNF-K90R expanded by >5-fold, 60-fold, and 18-fold compared with that of the mTNF-K90R, wTNF-α, and ran-PEG-wTNF-α, respectively. The pharmacokinetics of the sp-PEG-mTNF-K90R after i.v. injection were also assessed (Fig. 3). The plasma half-life of the sp-PEG-mTNF-K90R was 49 minutes, which was longer than that of the mTNF-K90R (24 minutes), wTNF-α (12 minutes), and ran-PEG-wTNF-α (24 minutes).

DISCUSSION

Several therapeutically useful bioactive proteins have been identified in the postgenome era. However, as elucidated in the clinical trials of various proteins in the past, protein therapy still has many problems derived from the *in vivo* drawbacks of proteins, such as their *in vivo* low stability (short plasma half-life) and complicated actions. One way to circumvent this problem may be to synthesize functional muteins by traditional techniques of amino acid substitution, such as site-directed mutagenesis, based on the structural simulation data of a mutein

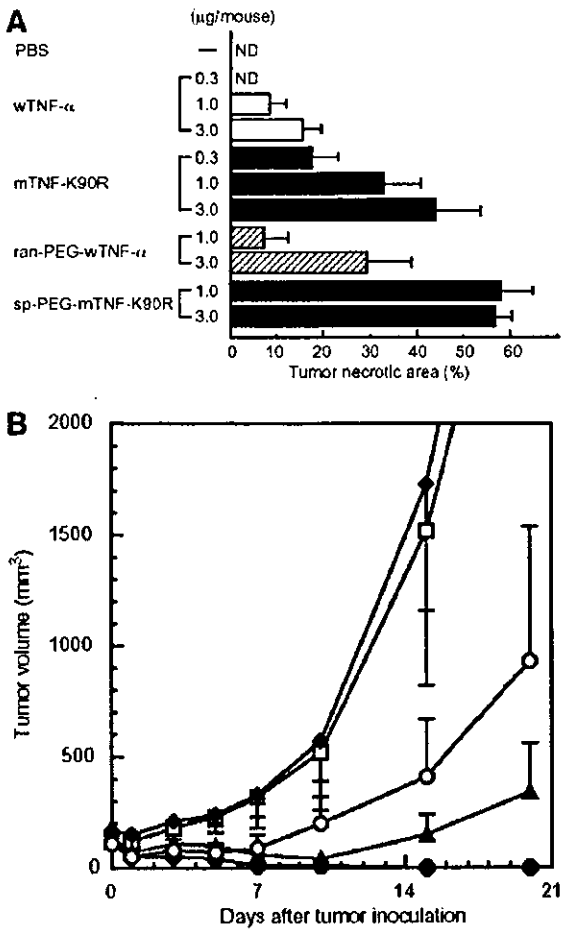
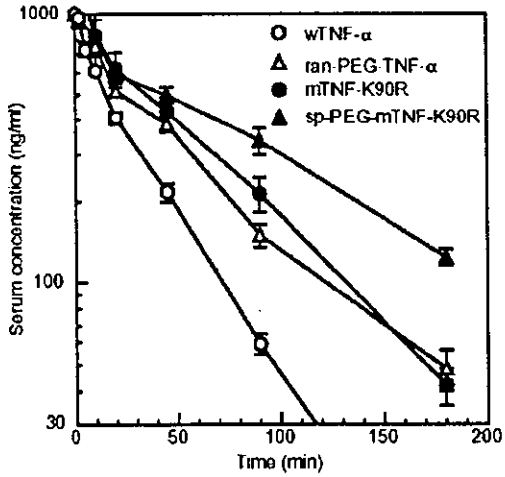


Fig. 2 Antitumor effects of various forms of TNF-α on mice bearing intradermally implanted Meth-A solid tumors. (a) Tumor necrotic effects of i.v.-administered TNF-α and mono-PEGylated TNF-α forms. Tumor hemorrhagic necrosis was scored 24 h after the injection. Each data value is the mean ± seconds.e., N.D., not detected. (b) Antitumor effect of the sp-PEG-mTNF-K90R on Meth-A solid tumors. At 7 d after the tumor inoculation, mice were treated with i.v. injections of PBS (□), 1 μg of the mTNF-K90R (○), 0.1 μg of the sp-PEG-mTNF-K90R (◆), 0.3 μg of the sp-PEG-mTNF-K90R (▲), 1 μg of the sp-PEG-mTNF-K90R (●). Each data value is the mean ± seconds.e.

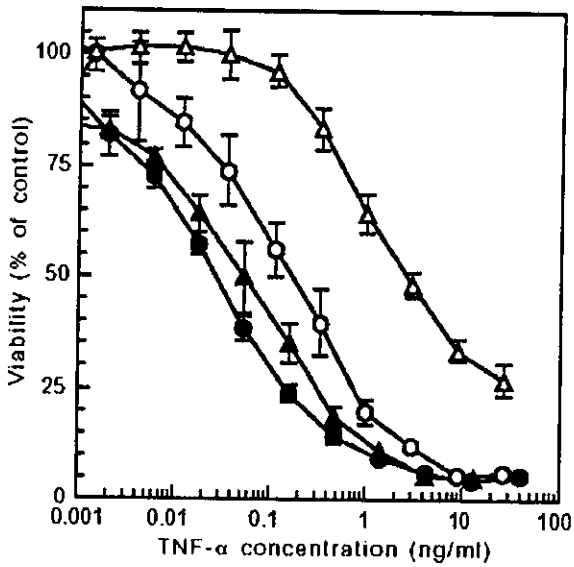
(21, 33, 34). However, it is difficult to obtain muteins with desired properties using these methods. This is because useful muteins must be identified only among several kinds of structural variant proteins that are produced by trial and error, after a great deal of time. Another effective method to overcome the *in vivo* drawbacks of proteins may be to modify the proteins with PEG (35, 36). However, the application of PEGylation has been limited to a small part of the protein, because the random introduction of PEG to ε-amino groups usually lowers the bioactivity of proteins markedly. Thus, the improvement of both the protein-drug innovation systems mentioned above is indispensable for the promotion of protein therapy. In this study, using TNF-α, we attempted to develop a novel protein-drug innovation system by fusing technology used to create clinically useful muteins and site-specific PEGylation.

Phage libraries displaying polypeptides, such as naive antibodies or random peptides, have extensively been applied for the identification of specific molecules with a high affinity for a target ligand (37–39). The advantages of a phage display system are easy preparation of a library consisting of structural variants of a polypeptide as diverse as over one hundred million and isolation of several molecules binding to a targeted ligand from this library in a few weeks. However, there are few studies on the application of the phage display technique for creation of therapeutically useful structural variants of a bioactive protein, such as muteins with a stronger bioactivity and longer plasma half-life. To create a lysine-deficient smTNF-α with a stronger *in vitro* bioactivity than the wTNF-α, a phage library displaying a lysine-deficient mTNF-α was prepared, and it consisted of $\sim 1 \times 10^8$ independent structural variants. After two rounds of biopanning against TNF-RI, the mTNF-K90R with an *in vitro* bioactivity that was 6-fold stronger was obtained, despite reports that some lysine residues were essential for its bioactivity (Fig. 2 and Table 1; refs. 40–43). This mTNF-K90R has an ~10-fold higher *in vivo* antitumor potency and 1.3-fold lower *in vivo* toxicity compared with that of wTNF-α. Therefore, the therapeutic window of the mTNF-K90R was extended by ~13-fold compared with that of the wTNF-α (Fig. 2*A* and Table 1). This improved therapeutic window of the mTNF-K90R was due to its stronger *in vitro* bioactivity as well as its longer plasma half-life (Fig. 3). We reported previously that lowering the isoelectric point of antitumor immunotoxins increased their therapeutic potency, probably due to the desirable changes in their pharmacokinetic properties derived from the surface negative electro-



	t1/2 (min)	AUC (×10³ng·min/ml)	CLtotal (μl/min)
wTNF-α	12± 2	28± 2	39±4
ran-PEG-wTNF-α	24± 4	45± 2	22±1
mTNF-K90R	24± 5	62± 7	17±2
sp-PEG-mTNF-K90R	49±16	85±10	12±1

Fig. 3 Pharmacokinetics of various forms of TNF-α after their i.v. injection. The concentration of TNF-α molecules in serum was quantified by ELISA. A standard curve was made for each form of TNF-α. Each data value represents the mean ± seconds.e.



		LC_{50} (ng/ml)
O	wTNF-α	0.17
Δ	ran-PEG-wTNF-α	2.85
●	mTNF-K90R	0.03
▲	sp-PEG-mTNF-K90R	0.05

Fig. 4 *In vitro* bioactivity of mono-PEGylated forms of TNF-αs. The specific activity of the mono-PEGylated forms of TNF-α was measured by a cytotoxic assay using LM cells in the presence of actinomycin D. Each data value represents the mean ± seconds.d. LC_{50} is the concentration of various PEGylated TNF-αs capable of killing 50% of the cells. Commercially available recombinant human TNF-α was used as a standard.

static potential (21). Thus, the longer half-life of the mTNF-K90R may partially result from the effects of lowering the isoelectric point.

Our data are the first to report the creation of a lysine-deficient mutant protein with a higher bioactivity and enhanced therapeutic antitumor effects. It is interesting to examine, from the standpoint of structure, why the mTNF-K90R had a stronger *in vitro* bioactivity and higher affinity to TNF-RI than the wTNF-α, when all of the lysine residues in the mTNF-K90R are replaced with other amino acids. Site-directed mutagenesis analysis of TNF-α suggested that among six lysine residues, Lys65 and Lys90, in particular, were involved in the interaction with its receptor (40–43). It was shown that the TNF-α mutant, in which Lys65 was replaced by Ala65 (K65A), bound the receptor better than the wTNF-α, whereas the K65W mutant showed a remarkably reduced binding to the receptor. In the wTNF-α structure, it was predicted by molecular modeling of the complexes between TNF-α and TNF-RI that Lys65 would repel against Lys78 in TNF-RI (Fig. 5*B*). This predicted model explains that the increased binding affinity of K65A would be due to the absence of short contacts between Ala65 in K65A and Lys78 in TNF-RI, and the decreased affinity of K65W may result from steric interference between Trp65 in K65W and Lys78 in TNF-RI. In the mTNF-K90R, Lys65 was replaced by Ser65. We considered that the substitutions of Lys65 with a small amino acid, such as Ser65 in the mTNF-K90R, would enable these proteins to bind receptors because of the loss of interference between Lys65 in TNF-α and Lys78 in TNF-RI. Surprisingly, Lymphotoxin-α (TNF-β), which can bind TNF-RI with similar affinity to TNF-α, has Ser65 instead of Lys65 in TNF-α (44). In the wTNF-α structure, Lys90 forms a hydrogen bond with Glu135 (Fig. 5*C*). This interaction is likely to stabilize the loop structure containing residues 84 to 89, which are involved in the receptor binding in the model. The loop is located near the trimer interface, and receptor binding studies have also suggested that the loop is essential for receptor binding. In the mTNF-K90R, Arg90 is also likely to be involved in hydrogen bonding with Glu135. The interaction would contribute to the stabilization of the loop structure. To additionally investigate the relationship between structure and activity, we are attempting to reveal the crystal structure of the mTNF-K90R.

The PEGylation of proteins is mostly nonspecific and may occur at all of the lysine residues, some of which may be within or near an active site. The resultant PEGylated proteins show markedly lower bioactivity. Additionally, the PEGylated proteins are composed of positional isomers with PEG at various sites, which have distinct activities and other characteristics (45). The NH_2 terminus site-specific mono-PEGylation was found to improve the plasma half-life of the mTNF-K90R without a marked reduction of its *in vitro* bioactivity, whereas the ran-PEG-wTNF-α had 6% of the bioactivity of the wTNF-α. Additionally, the sp-PEG-mTNF-K90R with superior molecular uniformity showed a much higher *in vitro* bioactivity than the wTNF-α and a site-specific PEGylated mutant TNF-α reported previously. Our data are the first to report the creation of a PEGylated protein with a higher bioactivity than the unmodified parent protein. Thus, these results indicate that this site-specific PEGylation system has solved the problems of previous random PEGylation systems. The sp-PEG-mTNF-K90R had a 3-fold higher *in vivo* antitumor effect and 1.5-fold lower toxicity compared with the mTNF-K90R; thus, the therapeutic window of the sp-PEG-mTNF-K90R was enhanced by 4.5-fold and 60-fold compared with that of the mTNF-K90R and wTNF-α, respectively. This may be due to the longer plasma half-life of the sp-PEG-mTNF-K90R. However, the replacement of the lysine residues in the mTNF-K90R may increase the immunogenicity of the protein, which must be investigated, although on the other hand, PEGylation is known to considerably decrease protein immunogenicity (24). When TNF-α is used as a systemic antitumor agent, its dose must be restricted to only 1/5 to 1/25 of the amount necessary to obtain sufficient antitumor activity, due to its adverse side-effects. The therapeutic window of the sp-PEG-mTNF-K90R was found to be 60-fold wider than that of the wTNF-α. TNF-α in combination with Melphalan achieved improved tumor response using local perfusion in transit melanoma metastases and limb salvage in soft-tissue sarcoma patients. This is because TNF-α selectively injured tumor endothelial cells and enhanced the vascular permeability of tumor vessels (10–15). The sp-PEG-mTNF-K90R can be used not only for systemic administration but also in combination with chemotherapy. In addition, some researchers reported that

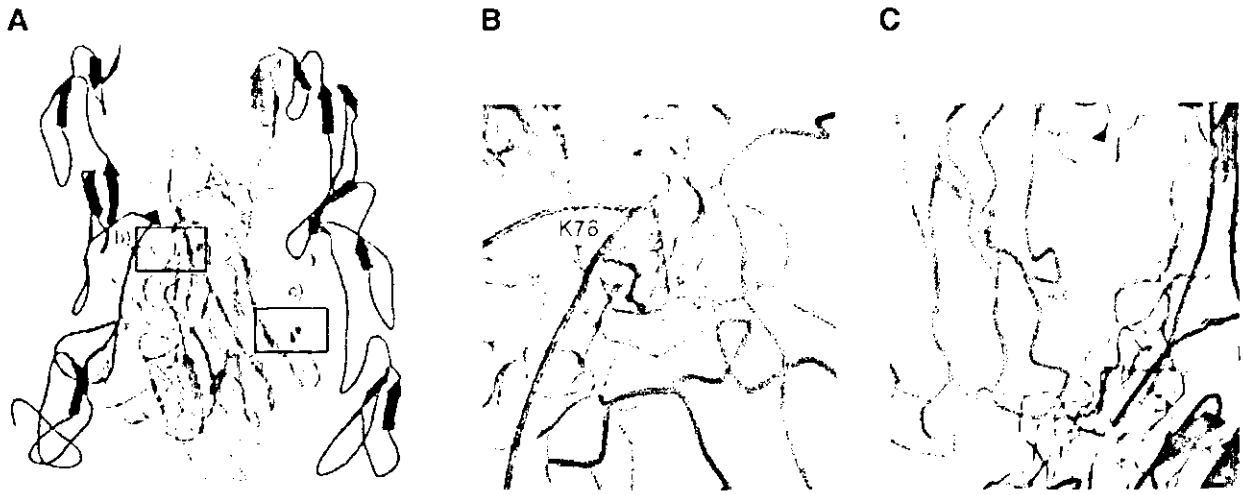


Fig. 5 Ribbon diagrams of the complex between the wTNF-α and TNF-RI. These images are based on the crystallographic structure of the complex between TNF-β and TNF-RI (PDB code: 1TNF; 44). The images were drawn using Bobscript (47) and Raster3D (48). (a) The wTNF-α is in red, green, and cyan. TNF-RI is in blue. (b, c) An enlargement of the interface boxed in (a). Here, wTNF-α is in red and TNF-RI is in blue.

fusion of TNF-α with a targeting ligand such as an antibody and peptide, against tumor or tumor endothelial cells, was useful for therapy (16–19). Currently, we are attempting to apply this system for creating muteins and for site-specific PEGylation of the fusion TNF-α.

In this study, we showed the advantage of a mutein creation system using the phage display technique and a site-specific PEGylation system to produce lysine-deficient muteins with a superior bioactivity for the promotion of pharmacoproteomic-based protein-drug discovery and development. The fusion of these two systems may be, at present, the best way to design optimal protein-drugs for therapy.

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蛋白療法の最適化にかなうあらたな薬物送達戦略

Novel drug delivery systems for optimization of protein therapies

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◎疾患プロテオミクス研究などで得られた情報を有効活用し，疾病治療に有効なペプチド・蛋白性医薬品を創製しようとするプロテオーム創薬への期待が高まっている．しかし，蛋白質の生体への適用には，いぜんとして蛋白質の生体内安定性を向上させ，かつその多様な *in vivo* 生理作用のなかから目的とする治療作用のみを選択的に発現させうる創薬テクノロジーの確立が必須となっている．本観点から著者らは最近，ファージ表面提示法を駆使した"医薬価値に優れた機能性人工蛋白質の迅速創出システム"とともに，"蛋白質の医薬品としての有効性と安全性を高めうる高分子バイオコンジュゲーション法"などをあらたに確立した．本稿では，この蛋白療法の最適化にかなう"プロテオーム創薬のためのDDS基盤テクノロジー"について紹介させていただく．

Key word：ファージ表面提示法，バイオコンジュゲーション，人工蛋白質，薬物徐放，ターゲティング

ヒトゲノム解読が完了し，約3万種の遺伝子で"ヒトの設計図"が描かれていること，そのうち約半数の遺伝子は未知蛋白質をコードしていることなどが明らかとなった．これらのなかには多様な生命現象や疾病に深く関与する蛋白質，すなわち医薬品シーズや創薬ターゲットとなりうる蛋白質が多数含まれるものと考えられている．そのため，創薬を指向したポストゲノム研究は，疾患状態における多種多様な蛋白質の時空間的・質量的な発現様式と疾患の発症・増悪・治癒との連関を網羅的に解析しようとする疾患プロテオミクスや，蛋白質の機能と立体構造を体系的に理解しようとする構造ゲノミクスなどへと集約されつつある．

一方で近年，癌や肝炎，リウマチなどに対するサイトカイン療法や抗体療法といった蛋白療法への期待がふたたび高まってきた．そのため，疾患プロテオミクス情報などを有効活用し，疾病治療に有効な蛋白質を創製しようとするプロテオーム創薬に大きな期待が寄せられている．しかし，一般に蛋白質は体内安定性が乏しいため，医薬品として適用しようとする際には大量頻回投与を余儀なくされてしまう．また，サイトカインなどの生理活性蛋白質は複数種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現してしまう．そのため生理活性蛋白質の臨床応用はいまだ著しく制限されており，そのほとんどは医薬品化されていない．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，有効かつ安全な蛋白療法を確立するためには，上述した生理活性蛋白質固有の問題点を克服しうる創薬テクノロジーの確立がいぜんとして不可欠となっている．この創薬テクノロジーはいわば蛋白療法の最適化をめざしたdrug delivery system(DDS)そのものであり，ポストゲノム基礎研究と21世紀医療の架け橋として鍵を握るものと位置づけられよう．

本観点から著者らは，①レセプター親和性・特

異性などが高く医薬価値に優れた機能性人工蛋白質を迅速創製できるネオ・ダーウィニズム的分子進化戦略の構築[1]，②蛋白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の確立[1,2]，③DDS機能(標的指向能・薬物徐放化能など)を有した機能化高分子キャリアの設計[3]に焦点を絞り，上記三者を融合させた"プロテオーム創薬にかなうDDS基盤テクノロジー"の確立をはかっている．本稿では誌面の許す限り詳細に，上述した①～③のDDS基盤テクノロジーについて紹介させていただく．

医薬価値に優れた機能性人工蛋白質の創製システムの構築

蛋白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"生理活性蛋白質のアミノ酸置換体(機能性人工蛋白質)"を創製するため，Kunkel法などの点突然変異法を用いた構造変異体の作製を精力的に試みている[2,4]．しかし，点突然変異法ではひとつひとつのアミノ酸を置換した変異体を作製し，個々の変異体を別々に精製し機能評価しなければならないため，莫大な時間と労力を要するうえ，有効な変異体の作製はきわめて困難であった．

その点，著者らは最近，ファージ表面提示法(「サイドメモ」参照)を独自に改良することにより10^8種類以上もの多様性を有した構造変異蛋白質(生理活性蛋白質のアミノ酸置換体)を一挙にcombinatorial biosynthesisし，この構造変異体ライブラリのなかからレセプター親和性や特異性などが向上した"医薬価値に優れた機能性人工蛋白質"を迅速かつ効率よく同定できる"プロテオーム創薬のための分子進化戦略"を確立した[1]．

たとえば，従来の点突然変異法を用いた構造-活性相関研究により，腫瘍壊死因子(TNF-α)のLys11やLys65・Lys90はその立体構造(三量体)形成やレセプター結合に必須といわれていた．これはTNF-αに限らず，一般にリジン残基は多くの場合，生理活性蛋白質の高次構造形成やリガンドーレセプター結合などに必須の役割を担っているため，他のアミノ酸への置換は致命的な活性低下を招いてしまうことが従来の点突然変異解析では常識となっていた．事実これまで，蛋白質中のリジン残基すべてを欠損させえた例(リジン欠損体)は皆無であった．しかし，著者らはこの既成概念を覆す知見，すなわちLys11やLys65・Lys90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，wild型TNF-α(wTNF-α)と同等さらには10倍以上もの生物活性を有するリジン欠損TNF-αを創製することにはじめて成功した(図1)．このwTNF-αと同等以上の生物活性を有する種々のリジン欠損TNF-αは，BIAcoreを用いたTNFレセプターIやTNFレセプターIIへの結合性評価によりwTNF-αと同等以上のレセプター親和性を有していること，超遠心解析やゲル濾過解析から三量体を形成していることも確認している．

これらの知見はTNF-α分子中の全6個のリジン残基を他のアミノ酸へ一挙かつ網羅的に置換した20^6(6,400万)種類もの構造変異TNF-α(アミノ酸置換体)を表面提示したファージライブラリを作製したうえで，TNFレセプターIや抗TNF中

サイドメモ

ファージ表面提示法

近年，バクテリオファージの生活環を巧みに利用し，ターゲット(分子・粒子・細胞)への高親和性結合分子を網羅的かつ迅速に探索・同定しうる基盤技術としてファージ表面提示法が考案された[5]．ファージ表面に数千万から数十億種類以上もの多様性に富んだランダムペプチドやナイーブ抗体，cDNA由来蛋白質などを発現させたファージライブラリを構築し，このライブラリのなかからターゲットへ高親和性に結合するファージを選択・回収，増幅するという操作(パンニング)を繰り返すことにより，ターゲットに対する高親和性結合分子を表面提示したファージのみを濃縮・選択できる．しかも得られたファージは目的の蛋白質をコードする遺伝子を内封しているため，その遺伝子配列をも同時に得られる．そのため，このファージ表面提示法は，さまざまな結合分子を迅速かつ網羅的にスクリーニングしうる基盤技術として，その応用範囲が急速に広がりつつある．

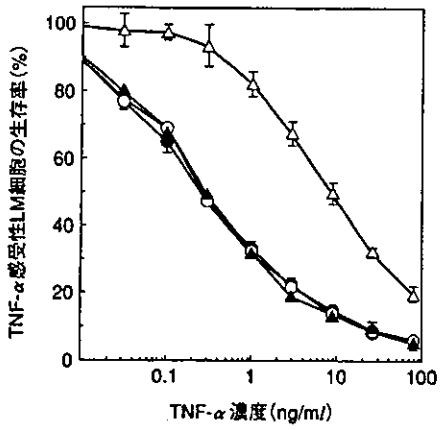

図1 リジン欠損TNF-αおよびその部位特異的バイオコンジュゲート体の *in vitro* 活性評価

TNF-αの *in vitro* 活性は，TNF-αに対して高感受性のマウスLM細胞(無血清培養可能なL929細胞派生株)を用いた細胞傷害性試験により評価した．

○：wTNF-α，△：ran-PEG-TNF-α，●：リジン欠損TNF-α，▲：sp-PRG-mTNF-α．

和抗体に対するアフィニティーバイオパンニングを行い，これら構造変異TNF-αの諸機能を高速解析することによって得られたものである．本方法を駆使することにより現在までに，レセプター指向性(選択性)や体内安定性に優れた機能性人工TNF-αも多数得ており，TNF-α以外の種々レセプター蛋白質や抗体についても活性を保持したリジン欠損体などを数多く得ている．したがって，著者らが確立した"ファージ表面提示法を駆使した機能性人工蛋白質の創製システム"は，他を圧倒する"プロテオーム創薬のための競争力(DDS基盤テクノロジー)"を提供するだけでなく，従来の点突然変異法(アラニンスキャン)による知見では得られなかった蛋白改変の概念や蛋白質の構造-活性相関概念をもあらたに提唱するものである．

あらたな部位特異的バイオコンジュゲーション法の確立

水溶性高分子で蛋白質を修飾するバイオコンジュゲーションは，蛋白質の生体内安定性を高めうる最適のDDSと世界的に認識されており，なかでもポリエチレングリコール(PEG)を用いたバイオコンジュゲーション(PEGylation)は数多くの蛋白質へ適用されようとしている[1-3,6,7]．また近年，PEG化インターフェロン-α(IFN-α)がC型肝炎の特効薬として使用されるなど，世界的に注目を集めている．この蛋白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蛋白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，結果として蛋白質の生体内半減期が延長される(図2)．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およ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，最終的に蛋白質の生体への投与量・回数を削減することが可能となる．

著者らもこれまで，抗腫瘍サイトカインとして期待されているTNF-αや血小板産生促進因子としてのインターロイキン-6(IL-6)などをモデル生理活性蛋白質として用い，このバイオコンジュゲーションの最適化をはかってきた[8-11]．その結果，蛋白質の生体内安定性や血中滞留性を飛躍的に向上させたうえで，目的とする治療作用と副作用とを選択分離し，目的作用のみを高めうることを明らかにしてきた．たとえば著者らは，PEG化TNF-αやPEG化IL-6の場合，副作用を増幅することなく目的とする抗腫瘍効果や血小板産生促進効果がそれぞれ100倍および500倍にも選択増強されることを認めている(図3)．

しかし，バイオコンジュゲーションによる蛋白性医薬品の実用化はIFN-αなどの例外を除き，アデノシン・デアミナーゼ，L-アスパラギナーゼ，スーパーオキシド・ディスムターゼなど，おもに低分子物質を基質とする酵素蛋白質に限られている．この最大の原因は，活性発現部位への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体の分子的・機能的不均一性にある．これまで汎用されてきたバイオコンジュゲーション法はアミノ基(リジン残基の有するεアミノ基およびN末端のαアミノ基)をターゲットとしたものであり，修飾高分子のアミノ基への結合はランダムでしかない．ほとんどの蛋白質においてリジン残基は活性発現に必須の役割を担っているため，

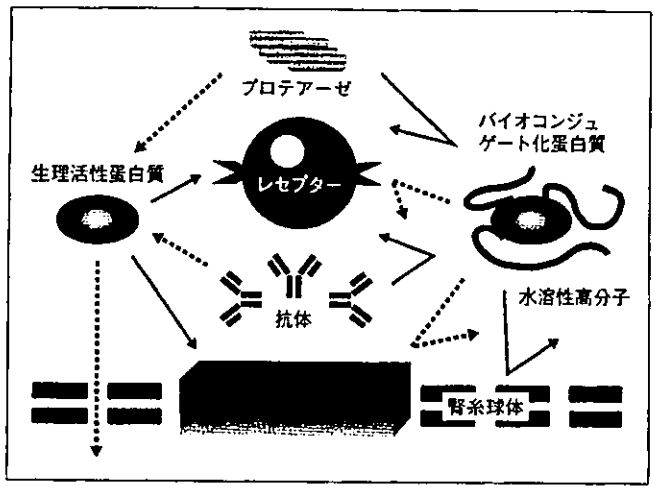

図 2　生理活性蛋白質の高分子バイオコンジュゲーションの特徴

生体に投与された生理活性蛋白質は，各種プロテアーゼにより分解されてしまうだけでなく，分子量数万以下のものは速やかに腎排泄されてしまうため，一般にその生体内安定性・滞留性はあまりにも乏しい．一方で高分子バイオコンジュゲーションは，高分子化による腎排泄速度の現象をもたらすだけでなく，蛋白質分子の表面を覆う修飾高分子により立体配位的にプロテアーゼからの攻撃をブロックするために，体内滞留時間の延長をもたらす．しかし，高分子レセプターとの結合を要するサイトカインなどのバイオコンジュゲーションは，同時に分子量増大に伴う組織移行性の低下や，蛋白質に結合した修飾高分子に由来する立体障害のために，レセプター親和性の低下(比活性の低下)を招いてしまう．

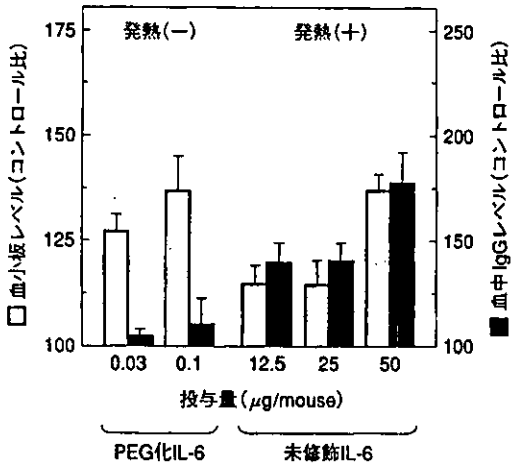

図 3　最適バイオコンジュゲーションによる IL-6 の血小板産生促進作用の選択増強

最適条件でバイオコンジュゲーションした PEG 化 IL-6 の *in vivo* における血小板産生促進作用は，未修飾 IL-6 の 500 倍にも増強されていた．また未修飾 IL-6 では，発熱や非特異的抗体産生の誘導といった強い副作用が観察されたが，PEG 化 IL-6 投与群ではこれらの副作用はほとんど認めなかった．

これらリジン残基への高分子導入により必然的に著しい比活性低下を招いてしまう．また，ランダムに修飾高分子が導入されるため，得られたバイオコンジュゲート体は蛋白質のさまざまな部位に種々個数の修飾高分子が結合した分子的に不均一な混合物となる．したがって，プロテオーム創薬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．

この点著者らは，前述したファージ表面提示法を駆使した“医薬価値に優れた機能性人工蛋白質を迅速創製できるネオ・ダーウィニズム的分子進化戦略”との融合アプローチにより，完全に活性を保持したリジン欠損機能性人工蛋白質を創製することによって，“N 末端アミノ基だけを標的とした部位特異的バイオコンジュゲーション”にはじめて成功した[1]．このリジン欠損機能性人工蛋白質に対する部位特異的バイオコンジュゲーションは

N末端アミノ基にのみ高分子導入されるため，分子的均一性に優れたバイオコンジュゲート体がほぼ100%の収率で得られる．たとえば，TNF-αの場合，上述したように，全6個のリジン残基のうちLys11やLys65・Lys90はその立体構造(三量体)形成やレセプター結合に必須と考えられており[12-14]，アミノ基に対するランダムなバイオコンジュゲーション法ではこれら活性発現や構造形成に関与するリジン残基までもが修飾されてしまうため，活性低下を避け得なかった．事実，wTNF-αのアミノ基に対するランダムPEGylationでは多様な修飾率(PEG導入率)のPEG化wTNF-αが得られてしまうが，そのなかから1分子のPEG導入体(ランダムモノPEG化wTNF-α：ran-PEG-TNF-α)の収率は20%程度，このran-PEG-TNF-αの残存活性はwTNF-αの約10%にまで減少していた(図1)．一方で，N末端側の8個のアミノ酸を欠損させてもTNF-αの活性は損なわれないことから，活性発現にN末端側は重要でないものと考えられている[15]．そのため，N末端アミノ基に対する部位特異的モノPEG化リジン欠損TNF-α(sp-PEG-mTNF-α)は80%以上の活性を保持しているなど，圧倒的な利点を有していることが判明した(図1)．

この分子的均一性や比活性，収率に優れた部位特異的PEG化リジン欠損TNF-αは，血中滞留性や抗腫瘍作用の選択的発現能に優れているうえ，従来法で作製したランダムPEG化TNF-αよりも著しく強い*in vivo*抗腫瘍効果を有していることも見出しており，現在臨床応用に向けた研究を推進中である．一方，N末端領域が活性発現に必須である蛋白質の場合でも機能性リジン欠損体を創製したうえで，活性発現とは無関係な領域にあらたなリジン残基を挿入することにより，αアミノ基とεアミノ基との反応性の違いを利用した部位特異的バイオコンジュゲーションが可能となることも判明している．

以上の革新的な部位特異的バイオコンジュゲーション法は，著者らが確立した機能性人工蛋白質の分子進化戦略との融合により機能性リジン欠損体を創製することによってはじめて可能となる．現在，種々の蛋白質に関して活性を十二分に保持したリジン欠損体創出を進めており，今後N末端アミノ基への部位特異的バイオコンジュゲーションの有用性をさらに追求していく予定である．

DDS機能を有した機能化高分子キャリアの設計

著者らは従来よりバイオコンジュゲート化蛋白質の生体内挙動や*in vivo*薬効発現強度が蛋白質表面を覆う修飾高分子の諸性質によって運命づけられることに着目し，バイオコンジュゲーション法のさらなるグレードアップを目的に，薬物徐放化能や標的指向能といったDDS機能を有する高分子キャリアの分子設計をはかってきた．たとえば，血中滞留性の向上を目的としたバイオコンジュゲーションにはPEGよりもポリビニルピロリドン(PVP)が圧倒的に優れた修飾高分子であること，新規合成したマレイン酸導入PVPやラウリル酸導入PVPがそれぞれIFN-γ誘導能(抗腫瘍免疫誘導能)や高度な脾指向能を有していることなどを明らかにしてきた[6]．これら新規修飾高分子を用いたバイオコンジュゲーションは単に蛋白質の生体内安定性を高めるだけでなく，高度な組織ターゲティング能やあらたな薬理活性を導入することにより，生理活性蛋白質の目的とする治療作用の選択的発現をさらに保証することを認めている．

このような一連の研究を通じて最近，著者らは腎への高度な薬物送達能とpH応答性薬物徐放化能を合わせもった高分子キャリア〔Poly(vinylpyrrolidone-co-dimethyl maleic anhydride)：PVD〕を新規合成することに成功した[3]．このPVDはpH8以上で蛋白質のアミノ基と結合し，pH7以下で結合蛋白質を徐々に解離する．一般に炎症組織や癌組織では正常組織よりも低pHであることからPVDを薬物キャリアとして適用した場合，病態組織でのみ効果的に蛋白質がpH応答的に徐放されることを意味している．このPVDをマウスに尾静脈内投与したところ，数時間後に投与量の約80%が腎へ選択的に集積し，4日後には40%に減少していた(図4)．このPVDは腎尿細管上皮細胞へのみ選択的に取り込まれるが，細胞毒性をまったく示さないうえ，大量投与しても腎を含め他の組織

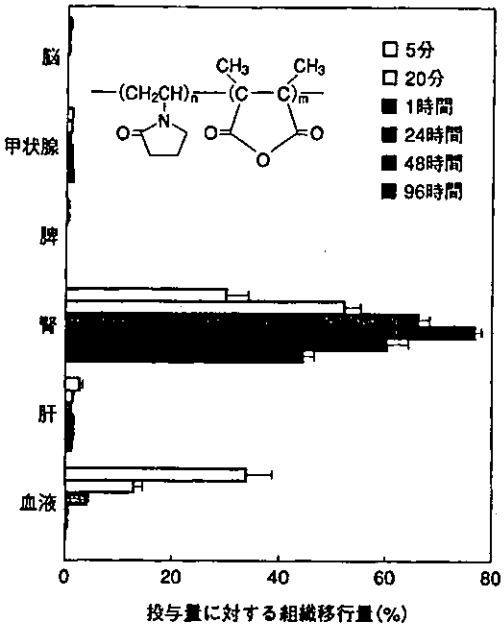

図 4　PVD の静脈内投与後の組織分布

PVD をマウスに尾静脈内投与したところ，わずか数分で投与量の約 30% が腎に集積し，投与後数時間～24 時間では投与量の約 80% もが腎へ選択的に滞留した．また徐々に尿中排泄され，投与後 96 時間後には約 40% が腎に滞留していた．

に何ら傷害を及ぼさない．さらに，PVD でバイオコンジュゲーションした抗炎症蛋白質(SOD)は生体内安定性に優れ，かつ静脈内投与後，選択的に腎へ高集積し，著しい腎炎治療作用を発揮することを見出した．

高齢化社会を迎え，腎不全をはじめとする腎疾患が世界的に深刻な社会問題となっている[16]．しかし，慢性腎疾患に対する治療は腎移植と透析に頼らざるをえないのが現状であり，患者の QOL (quality of life)の観点からも安全かつ有効な薬物療法の確立が待望されている[17]．本観点から現在，上述した“医薬価値に優れた機能性人工蛋白質を迅速創製できる分子進化戦略”による機能性人工蛋白質の創製や部位特異的バイオコンジュゲーションシステムとの融合により，あらたな腎疾患治療戦略の確立をさらに推進している．

おわりに

本稿で紹介した 3 段階の“プロテオーム創薬にかなう DDS 基盤テクノロジー”は，疾患プロテオミクス情報を有効活用したプロテオーム創薬の実現と安全かつ有効な蛋白療法の確立に向けて相乗的に機能するものと期待している．また，プロテオーム創薬を推進するためにはまず，多種多様な蛋白質とその構造変異体を網羅的に作製し，これらのレセプター・リガンド結合の様式・強度などをも含めた機能情報をハイスループットに評価可能な方法論の構築とその立体構造との連関を網羅的に評価することが必須となる．そのうえで，ゲノムシーケンス情報をもとにあらたに見出された蛋白性シーズなどの機能と構造を予測しうるバイオインフォマティクスが構築されて，ようやく真の意味でプロテオーム創薬が可能となってくる．この点，ファージ表面提示法を駆使した“医薬価値に優れた機能性人工蛋白質を迅速創製できる分子進化戦略”は，膨大な多様性をもった構造変異体を創出し，その機能解析を迅速に大量解析しうる最適の基盤テクノロジーとなりうる．

著者らの研究成果は，得られた数多くの機能性人工蛋白質の立体構造と機能特性との連関評価を通じて“機能(医薬価値)→構造”に関する知見の集積が可能となり，将来的に機能性人工蛋白質を合理的設計しうるファーマコ・バイオインフォマティクスの構築にも貢献しうるものと期待している．

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