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Optimization of drug therapy based on molecular design of hybrid bioactive proteins with water-soluble polymers

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This study was conducted to optimize the drug therapy based on molecular design of hybrid bioactive proteins with water-soluble polymers (bioconjugation). We performed the covalent bioconjugation of natural human tumor necrosis factor alpha (TNF-α), recombinant human interleukin-6 (IL-6) and etc. with polyethylene glycol (PEG). In this paper, we showed using TNF-α as an example, the possibility that bioconjugation of cytokines overcomes their inherent problems in vivo. PEG-modified TNF-α (PEG-TNF-α) was markedly increased the anti-tumor potency compared to native TNF-α. In particular, modified TNF-α, in which 56% of lysine residures were coupled to PEG (MPEG-TNF-α) had anti-tumor activity approximately 100 times greater than unmodified TNF-α in the murine Meth-A fibrosarcoma model. However, plasma half-lives of PEG-TNF-αs prolonged with an increase in molecular size of modified TNF-α. These results suggested that optimal polymer modification in view of molecular size, remaining activity, and degree of modification could optimize the drug therapy based on bioconjugation.

For clinical application of bioactive proteins such as cytokines, frequent administration at a high dose is needed by reason of a short half-life *in vivo*. This markedly destroys the homeostasis, indusing unexpected side effects. In recent years, in the development of drugs considering the DDS, bioconjugation of water-soluble polymers to the surface of bioactive proteins has been devised [1]. Bioconjugation decreases the renal excretion rate due to the increased molecular size. In addition, since the water-soluble polymers cover the protein surface, the attack from proteinases was blocked by steric hindrance, resulting in prolongation of the half-life *in vivo*. However, the transport form blood to target tissues of bioconjugated proteins is limited by their high molecular weight, and receptor binding of proteins is sterically inhibited [2]. Therefore, in order to achieve optimum delivery of bioactive proteins utilizing polymeric modifiers conjugation for clinical application, we found that the hybrid proteins and peptides must be show desirable pharmacokinetical characteristics such as plasma clearence and tissue distribution [3]. For optimization of molecular design of hybrid proteins, we showed the importance of selecting the optimal polymeric modifiers by the purposes of bioconjugation and properties of each protein [4]. Furthermore, it is necessary to accumulate basic data on the balance of degree of modification, molecular size, and remaining activity in detail.

In this study, we attempted to optimize modification of tumor necrosis factor alpha (TNF-α) with polyethylene glycol (PEG). Bioconjugated TNF-α was separated into various molecular size to study the relationship between degree of modification, molecular size, and anti-tumor activity. These approach enable us to design hybrid bioactive proteins with water-soluble polymers suitable for clinical use.

1. MATERIALS AND METHODS

1.1 Conjugation of PEG to TNF-α

Natural human TNF-α (Hayashibara biological Laboratories, Okayama, Japan) in PBS (-) was allowed to react with a 60-fold molar excess of *N*-succinimidyl succinate monomethoxy polyethylene glycol (SS-PEG; MW=5000, Sigma, St Louis, MO, USA) at room temperature for 10 min. The reaction was stopped by addition of 5-fold molar excess of ε-amino caproic acid. PEG-modified TNF-α was separeted into several fractions by gel filtration chromatography (TSKgel G3000 SWXL, Tosoh, Tokyo, Japan). The specific activity of native TNF-α and PEG-modified TNF-α were estimated by cytotoxic activity against L-M cells, and were expressed in terms of the Japan reference unit (JRU) [5].

Table 1 Characterization of PEG-modified TNF-αs.

Number-Average Molecular Size	[a] Degree of Modification (%)	[b] Specific Activity (x 10^4 JRU / mg TNF)	[c] Remaining Activity (%)	Yield (%)	
148,000	100	2.19 ± 3.00	1.0	23.2	
122,000	71	30.8 ± 10.3	14.1	36.4	HPEG-TNF-α
108,000	56	114 ± 20.6	52.3	24.6	MPEG-TNF-α
84,000	29	163 ± 2.40	74.5	10.2	LPEG-TNF-α
58,000	0	218 ± 4.59	100.0	5.6	Native TNF-α

a; Determined by GFC (protein standard). b; Calculated from number-average molecular weight.
c; Assesed by growth-inhibit L-M tumor cell assay.

1.2 Pharmacokinetics of PEG-TNF-α

Native TNF-α and PEG-TNF-α were labelled with ^{125}I by the lactoperoxidase method. [^{125}I]TNF-α and[^{125}I]PEG-TNF-α were injected i.v. to Meth-A solid tumor-bearing mice. After i.v. administration, blood was collected from the tail vein at indicated time. The plasma half-lives of native TNF-α and PEG-TNF-αs were evaluated by curve fitting with the non-linear least-squares method.

1.3 Evaluation of anti-tumor activity in vivo

Meth-A fibrosarcoma cells were implanted intradermally to Balb/c mice. On day 7 after implantation of cells, native TNF-α and PEG-TNF-α were intravenously administerd. Anti-tumor effect was evaluated by means of haemorrhagic necrotic scores and lifespan [6, 7].

2. RESULTS

2.1 Characterisation of PEG-TNF-α

Native TNF-α was bioconjugated with PEG, and the synthetic PEG-modified TNF-α was separeted into several fractions by gel filtration chromatography. Table 1 shows the number average molecular size, degree of PEG-modification, and specific activity of separeted PEG-TNF-αs. The specific activity of PEG-TNF-αs decreased with an increase in the degree of PEG-modification. In particular, HPEG-TNF-α, in which 71% of lysine residures coupled to PEG, showed only 14.1% of specific activity. The major product on this condition as shown "materials and methods" was MPEG-TNF-α.

2.2 Pharmacokinetics of PEG-TNF-αs

The pharmacokinetics of PEG-TNF-αs after i.v. administration on Meth-A solid tumor-bearing mice were compared with that of native TNF-α. The plasma concentration profiles of native TNF-α and PEG-TNF-αs showed biexponential elimination (Fig.1). PEG-TNF-αs circulated longer than native TNF-α in the blood. The plasma half-lives of PEG-TNF-αs were higher with an increase of degree of PEG-modification (native TNF-α ; 3.2min, LPEG-TNF-α ; 45min, MPEG-TNF-α ; 117min, HPEG-TNF-α ; 136min).

2.3 Anti-tumor effect of PEG-TNF-α

The anti-tumor effects of PEG-TNF-αs on the Meth-A solid tumor-bearing mice were studied. Native TNF-α at a dose of 10,000 JRU slightly showed the haemorrhagic necrotic effect. PEG-TNF-αs increased the haemorrhagic necrotic effect on the Meth-A solid tumor-bearing mice (Fig.2). In particular, MPEG-TNF-α at a dose of 200 JRU showed dramatically anti-tumor effect. Although HPEG-TNF-α had a longer plasma half-life than MPEG-TNF-α, its anti-tumor effect was similar to native TNF-α. By contrast, anti-tumor effect of LPEG-TNF-α was slightly increased compared to native TNF-α. During the experimental period, sudden death and side effects such as body weight reduction and piloerection were observed in the mice administered native TNF-α. However, Administration at a dose of 200 JRU of MPEG-TNF-α could achieve the complete regression in two of seven mice without side effects (data not shown).

3. DISCUSSION

In recent years, various bioactive proteins were attempted to increase its biological activity

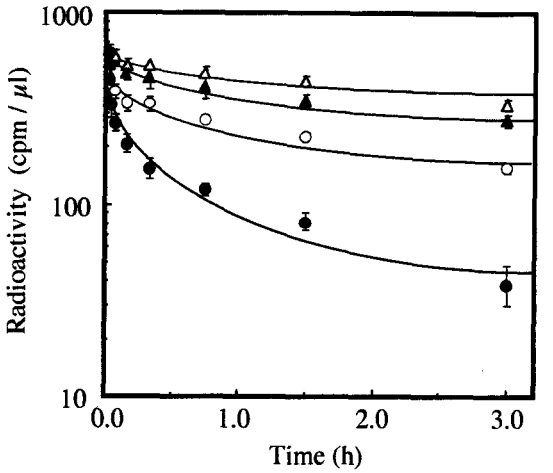

Fig. 1 Pharmacokinetics of native TNF-α (●) and PEG-TNF-αs (LPEG-TNF-α (○), MPEG-TNF-α (▲), HPEG-TNF-α (△)). Each value represents mean ± S.E.

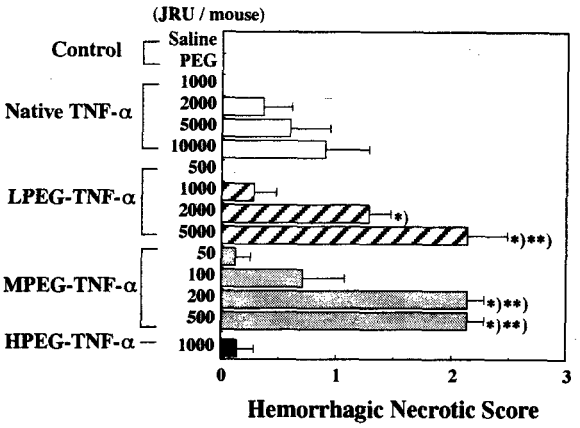

Fig.2 Tumor necrotic effects of native TNF-α and PEG-TNF-αs on Meth-A solid tumors. Each value represents mean ± S.E. *P<0.001, **P<0.05 significantly different from the group treated with 10,000JRU of native TNF-α.

based on bioconjugation. However, many of those approachs were applied to enzymes, bioconjugation of bioactive proteins such as cytokine based on the purpose of bioconjugation and properties of each cytokine has not been studied. In this study, we attempted to optimize the cytokine therapy based on bioconjugation.

PEG modification was allowed to couple the lysine residures of TNF-α to PEG via amido bond. Specific activity of PEG-modified TNF-α decreased with an increase of degree of PEG-modification (Table.1). The molecular size and degree of PEG-modification of PEG-TNF-α depended on the reaction time and initial molar ratio of PEG to TNF-α (data not shown). Resident time of PEG-modified TNF-α in blood were prolonged with an increased of degree of PEG-modification (Fig.1). In particular, HPEG-TNF, in which 71% of lysine amino groups of TNF-α were coupled to PEG, showed the longest half-life in PEG-TNF-αs. However, anti-tumor effect of HPEG-TNF-α was as potent as native TNF-α (Fig. 2). On the contrary, MPEG-TNF-α showed the highest anti-tumor activity on Meth-A solid tumor-bearing mice, and complete regression was obtained in two of seven at a dose of 200JRU of MPEG-TNF-α. These results lead to the conclusion that high degree of modification of bioactive proteins did not always achieve the highest biological activity *in vivo*, bioconjugated proteins must be designed to show desirable properties in view of molecular size, remaining activity, and degree of modification. Furthermore, we showed the importance of selecting the optimal polymeric modifiers by the purposes of bioconjugation and properties of each protein. From this viewpoint, we synthesized polyvinylpyrrolidone (PVP), and TNF-α and IL-6 were bioconjugated PVP to lysine residures of proteins. PVP-modified proteins showed the increase of those biological activities considering the balance of degree of PVP-modification, molecular size, and specific activity (data not shown). These results indicated that PVP was useful polymeric modifier as potent as PEG. These approach enable us to accumulate basic data of polymeric modifiers, and further progress in this study is required to design bioconjugated drugs suitable for clinical use.

4. ACKNOWLEDGEMENT

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