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Chemical Modification of Natural Human Tumor Necrosis Factor-α with Polyethylene Glycol Increases Its Anti-tumor Potency

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Natural human tumor necrosis factor-α (TNF-α) was chemically modified with an active ester of monomethoxy polyethylene glycol (PEG). The molecular weight of PEG-modified TNF-α depended on the reaction time as well as the initial molar ratio of PEG to TNF-α. The specific activity of modified TNF-α was gradually reduced with increase in the degree of PEG-modification, but the plasma half-life of TNF-α was increased by up to 40-fold. Modified TNF-α showed approximately 100 times greater anti-tumor potency than unmodified TNF-α. Covalent attachment of PEG to TNF-α thus increased the bioavailability of TNF-α, and may facilitate its potential therapeutic use.

Key words: TNF-α — PEG — Meth-A — Chemical modification — Anti-tumor potency

Tumor necrosis factor-α (TNF-α), a cytokine secreted by activated macrophages, was first described as a factor present in infected, endotoxin-injected mice that caused hemorrhagic necrosis of transplanted Meth-A fibrosarcoma.[1)] TNF-α has direct anti-tumor effects, stimulates a host immune anti-tumor response, and induces hemorrhagic necrosis of tumors via specific interactions with tumor-vascular endothelial cells.[2)] TNF-α is thus a promising new therapeutic agent with three distinct, synergistic, anti-tumor effects. However, it is rapidly cleared from the circulation, resulting in limited bioavailability.[3,4)] The high doses of TNF-α required for significant anti-tumor clinical effects often induce host toxicity, such as tissue inflammation and injury, and a lethal endotoxic shock-like syndrome.[2,5,6)] Nevertheless, the systemic administration of TNF-α in high doses caused the complete regression of various transplanted solid tumors.[5–7)] This suggests that increase in the bioavailability of TNF-α may enhance its clinical potency, thus facilitating more effective use of TNF-α as an anti-tumor drug.

In recent years, chemical modifications of biologically active proteins with poly(ethylene glycol) (PEG) have been found to prolong greatly the plasma half-life and can effectively reduce the immunogenicity, resulting in augmentation of bioavailability.[8–12)] In the present investigation, we attempted to prepare PEG-modified TNF-α (PEG-TNF-α) to see whether the anti-tumor potency would be increased. PEG-TNF-α exhibited an increased plasma half-life, and was efficacious in a mouse tumor model.

A typical procedure for preparation of PEG-TNF-α is as follows. Natural human TNF-α (Hayashibara Biological Laboratories Inc., Okayama) in 0.2 *M* phosphate buffer, pH 7.2, was reacted with a 60-fold molar excess of N-succinimidyl succinate PEG (Mw=5,000; Aldrich Chemical Company Inc., Wisconsin, USA) at room temperature for 30 min. The reaction was stopped by addition of a 5-fold molar excess of ε-amino caproic acid over the PEG. PEG-TNF-α was purified and separated into fractions of various molecular weights by gel filtration chromatography (GFC; TSKgel G3000SW$_{XL}$, Tosoh, Tokyo). The number-average molecular weight (Mn) was estimated by GFC analysis and the degree of PEG-modification was calculated from Mn. The modified TNF-α, in which 56% of the lysine amino groups were coupled with PEG, was termed MPEG-TNF-α (characterized in Table I; run 3). The bioactivity of TNF-α and PEG-TNF-α was measured by L-M cytotoxicity assay.[13)] The bioactivity of TNF-α was expressed in terms of the Japan Reference Unit (JRU).

TNF-α and MPEG-TNF-α were ^{125}I-labeled by the lactoperoxidase method,[14)] yielding [^{125}I]TNF-α and [^{125}I]MPEG-TNF-α with specific radioactivities of 23.8 mCi/mg protein. Meth-A fibrosarcoma cells were maintained intraperitoneally by serial passage in female BALB/c mice. Meth-A cells (4×10^5) were implanted intradermally in the abdomen of 5-week-old female mice

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Table I. Characterization of PEG-modified TNF-α

run	Number-average molecular weight[a]	Degree of PEG-modification[b] (%)	Specific activity[c] ($\times 10^4$ JRU/mg TNF-α)	Remaining activity (%)	
1	148,000	100.0	2.19	1.0	
2	122,000	71.0	30.8	14.1	
3	108,000	56.0	114	52.3	MPEG-TNF-α
4	84,000	29.0	163	74.5	
5	58,000	0	218	100.0	Native TNF-α

a) Determined by GFC.
b) Calculated from number-average molecular weight.
c) Assessed by L-M cytotoxicity assay.

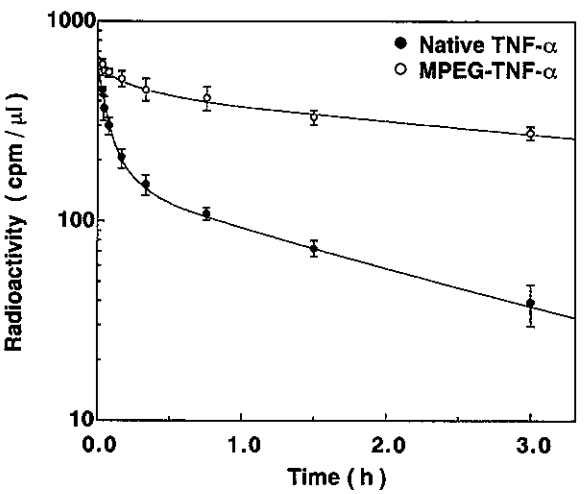

Fig. 1. Pharmacokinetics of PEG-TNF-α and unmodified TNF-α in tumor-bearing mice. Mice were used in groups of 4. Values are means±SE.

on day 0. On day 7, the pharmacokinetics of TNF-α and PEG-TNF-α after intravenous administration to tumor-bearing mice was studied at a dose of 20 ng protein/mouse. At selected time points after injection, blood was collected from the tail vein and radioactivity was measured.

The method for the evaluation of *in vivo* anti-tumor effects was as follows. Meth-A fibrosarcoma cells were maintained and implanted as described above. On day 7, MPEG-TNF-α or TNF-α was given by intravenous (i.v.) single injection. Drug efficacy against Meth-A solid tumor was expressed as mean tumor volume, and scores were assigned for tumor hemorrhagic necrosis. Tumor volume was assessed by the method of Haranaka *et al.*[15] Tumor hemorrhagic necrosis was scored according to the method described by Carswell *et al.*[1]

Natural human TNF-α was modified with N-succinimidyl succinate PEG via the formation of an amide bond between a lysine amino residue of TNF-α and the succinimidyl succinate group. PEG-TNF-α was separated into various Mn fractions by GFC. Table I shows the Mn, degree of PEG-modification and remaining bioactivity of PEG-TNF-α and TNF-α. The degree of PEG-modification depended on the reaction time as well as on the initial molar ratio of PEG to TNF-α (data not shown). A longer reaction time and a higher concentration of PEG relative to TNF-α resulted in a higher molecular weight of PEG-TNF-α. As can be seen in the table, the elevation of the degree of modification of lysine amino residues with PEG was accompanied with a decrease in bioactivity. Extensive PEG-modification resulted in the complete loss of *in vitro* bioactivity (Table I; run 1). These results strongly suggest that PEG chains sterically inhibit TNF-receptor binding and that some lysine amino residues play an important role in the bioactivities. These views are partly supported by the fact that Lys 11, which forms a salt bridge, fulfils a structural role.[16]

The effect of PEG-modification on the plasma half-life of TNF-α was studied with MPEG-TNF-α (characterized in Table I; run 3), in which relatively high bioactivity was maintained even at a high Mn. [^{125}I]TNF-α and [^{125}I]MPEG-TNF-α were biologically identical to the unlabeled protein (data not shown). Fig. 1 shows the pharmacokinetic data of TNF-α and MPEG-TNF-α after i.v. injections in Meth-A solid tumor-bearing mice. The serum concentration profiles of TNF-α and MPEG-TNF-α showed biexponential elimination. TNF-α rapidly disappeared from the circulation. The plasma half-life of TNF-α was 3.5 min and this value is in good agreement with those in previous reports.[3,4] The rapid clearance of TNF-α in mice via glomerular filtration in kidney, proteolysis and hepatic uptake was predicted. In contrast, chemical modification of TNF-α with PEG markedly increased its plasma half-life to 2.5 h. This decrease in the

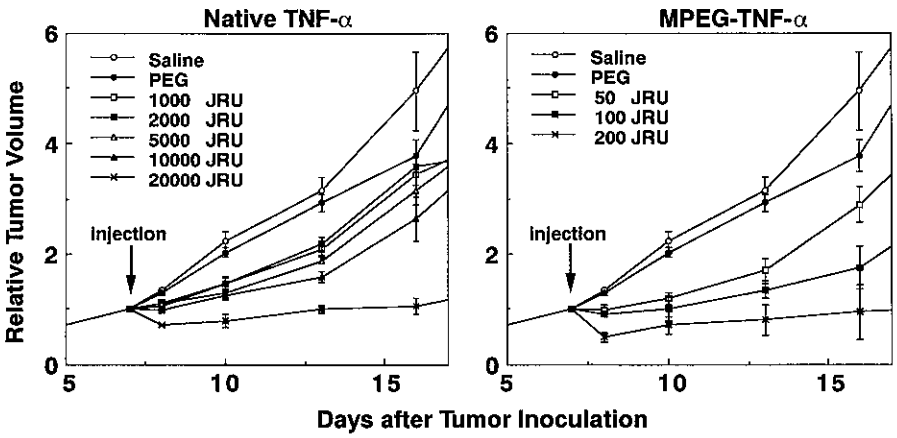

Fig. 2. Anti-tumor effect of PEG-TNF-α on tumor growth. PEG was administered with 10 μg/mouse. Mice were used in groups of 7. Values are means±SE.

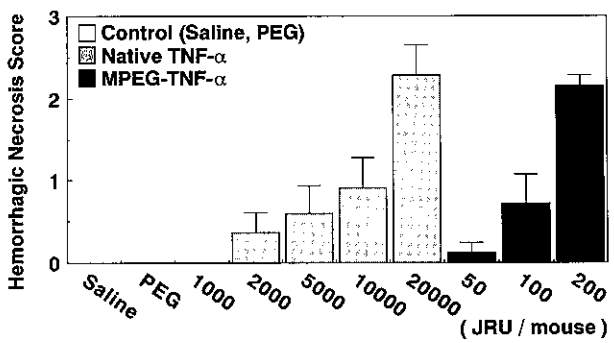

Fig. 3. Tumor necrotic effect of PEG-TNF-α on tumor-bearing mice. PEG was administered at 10 μg/mouse. Mice were used in groups of 7. Values are means±SE.

plasma clearance of PEG-TNF-α may be accounted for by the shielding of the proteolytic sites in TNF-α by PEG chains. PEG-modified proteins have been shown to be more resistant to proteolysis than the corresponding unmodified proteins.[17] In addition, the renal clearance of TNF-α is speculated to be prevented by increasing the molecular weight through covalent attachment of PEG. In fact, the glomerular filtration of PEG-modified interleukin-2 decreased with increase in its molecular weight.[18]

The anti-tumor effects of a single i.v. injection of TNF-α or MPEG-TNF-α on tumor-bearing mice was studied. PEG (10 μg/mouse) had no anti-tumor effect (Figs. 2 and 3). TNF-α and MPEG-TNF-α inhibited tumor growth in a dose-dependent manner (Fig. 2), and the necrotic scores were dose-dependently higher at 24 h after i.v. injection on day 7 (Fig. 3). The dose of 20,000 JRU of TNF-α induced the maximal anti-tumor response. However, 2 of the 7 mice died within 24 h after injection of TNF-α at a dose of 20,000 JRU, and the remaining five mice developed piloerection, tissue inflammation and decrease in the body weight during the experimental period (data not shown). MPEG-TNF-α was approximately 100 times more potent than TNF-α. As shown in Figs. 2 and 3, 200 JRU of MPEG-TNF-α had effects comparable to those of a dose of 20,000 JRU of TNF-α. This increase in the anti-tumor potency may be attributed to an increase in the plasma half-life. During the experimental period, all doses of MPEG-TNF-α were well tolerated and body weight increased (data not shown). PEG-modification of TNF-α substantially reduced the TNF-α-mediated toxicity. PEGylation of TNF-α thus enables us to decrease the therapeutic dose of TNF-α, resulting in a decrease in the side-effects.

In recent years, chemical modifications of cytokines typified by interleukin-2, interferon-γ and granulocyte colony-stimulating factor with PEG have been found to be effective to increase their plasma half-lives and stability, and to reduce their immunogenicity *in vivo*, resulting in augmentation of their bioavailability.[8,9,19] Our studies demonstrated that the modification of TNF-α with PEG increased its anti-tumor potency and also markedly reduced the toxic side-effects. PEG-TNF-α was no less efficacious in a mouse tumor model than other PEG-modified cytokines previously reported. PEG-TNF-α seems to have potential as a candidate anti-tumor therapeutic agent.

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