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Intravenous Administration of Polyethylene Glycol-modified Tumor Necrosis Factor-α Completely Regressed Solid Tumor in Meth-A Murine Sarcoma Model

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Complete regression of solid tumors was achieved by plural intravenous (i.v.) administrations of polyethylene glycol (PEG)-modified tumor necrosis factor-α (TNF-α), prepared by covalently modifying natural human TNF-α with N-succinimidyl succinate PEG. The anti-tumor efficacy of PEG-modified TNF-α (MPEG-TNF-α), in which 56% of the TNF-α-lysine residues were coupled with PEG, was compared with that of native TNF-α in the Meth-A murine fibrosarcoma model. MPEG-TNF-α and native TNF-α were given as i.v. injections twice a week for 2 weeks. The anti-tumor activity of MPEG-TNF-α was dose-dependent and was far superior to that of native TNF-α. Complete regression was observed in 3 of the 8 mice administered native TNF-α at the dose of 10,000 JRU (Japan reference unit), but 4 of the 5 remaining mice died during the therapeutic period. At 5,000 JRU of native TNF-α, no case of complete regression was observed. By contrast, complete regression was obtained in all 10 mice given 200 JRU of MPEG-TNF-α. No side-effects were observed at the dose of 500 JRU of MPEG-TNF-α, which was 2.5 times the minimal dose (200 JRU) of MPEG-TNF-α required for complete regression in all treated mice. MPEG-TNF-α appears to have potential as a candidate anti-tumor therapeutic agent.

Key words: PEG-modified TNF-α — Complete regression — Intravenous administration

Tumor necrosis factor-α (TNF-α) was originally identified as a tumoricidal cytokine causing hemorrhagic necrosis of transplanted solid tumors in mice.[1)] TNF-α exhibited striking cytotoxicity against various tumor cells *in vitro* whilst causing minimal harm to normal cells, and attracted attention as a potential anti-tumor drug.[2,3)] However, TNF-α was found to be rapidly eliminated from the blood circulation, and a very high dose was required to obtain a significant clinical anti-tumor effect.[4,5)] The systemic administration of TNF-α often induced toxic side effects. Thus, cancer therapy with TNF-α has been limited to local intratumoral administration.[6,7)] We previously reported that the chemical modification of TNF-α with polyethylene glycol (PEG) prolonged its plasma half-life, and increased its anti-tumor activity in the Meth-A murine solid tumor model.[8)] MPEG-TNF-α, in which 56% of the lysine-amino groups of TNF-α were coupled with PEG, was 100-fold more potent as an anti-tumor agent than native TNF-α, so that the therapeutic dose of TNF-α could be decreased, resulting in a decrease in the side-effects. We hoped that the use of MPEG-TNF-α *in vivo* would result in greater efficacy. In the present investigation, we attempted to regress completely Meth-A solid tumor in mice by plural i.v. administrations of MPEG-TNF-α alone.

Natural human TNF-α was kindly supplied by Hayashibara Biological Laboratories Inc. (Okayama). N-Succinimidyl succinate monomethoxy polyethylene glycol (SS-PEG; Mw = 5,000) was obtained from Sigma (St. Louis, Mo., USA). Female BALB/c mice (4–5 weeks old) were purchased from Shizuoka Laboratory Animal Center (Hamamatsu). L-M cells and Meth-A fibrosarcoma cells were generously provided by Mochida Pharmaceutical Co., Ltd. (Tokyo). Other reagents and solvents were of analytical grade.

The typical procedure for preparation of MPEG-TNF-α has been described elsewhere.[8)] The specific activities of native TNF-α and MPEG-TNF-α were measured by use of the L-M cytotoxicity assay.[9)] The specific activities of native TNF-α and MPEG-TNF-α were expressed in terms of the Japan Reference Unit (JRU).

The method for the evaluation of *in vivo* anti-tumor effects was as follows. Meth-A fibrosarcoma cells (4×10^5) were implanted intradermally in the abdomen of 5-week-old female mice (Day 0). Seven days later, native TNF-α and MPEG-TNF-α were given as i.v. injections twice a week for 2 weeks (Days 7, 10, 14 and 17). The drug efficacy against Meth-A was expressed in terms of the mean tumor volume and life span. Tumor volume

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Table I. Characteristics of Native TNF-α and MPEG-TNF-α

	Number-average molecular weight[a]	Degree of modification[b] (%)	Specific activity[c] ($\times 10^4$ JRU/mg TNF)	Remaining activity (%)
MPEG-TNF-α	108,000	56	114±20.6	52.3
Native TNF-α	58,000	0	218±4.59	100.0

A typical procedure for preparation of MPEG-TNF-α was as follows. TNF-α in 0.2 *M* phosphate buffer, pH 7.2, was allowed to react with a 60-fold molar excess of SS-PEG at room temperature for 10 min. The reaction was stopped by addition of a 5-fold molar excess of ε-aminocaproic acid over the SS-PEG. The resulting PEG-TNF-α was purified and separated into fractions of various molecular weights by gel filtration chromatography (GFC; TSKgel G3000SW$_{XL}$, Tosoh, Tokyo).

a) Determined by GFC (protein standard).

b) Calculated from number-average molecular weight.

c) Assessed by L-M cytotoxicity assay.

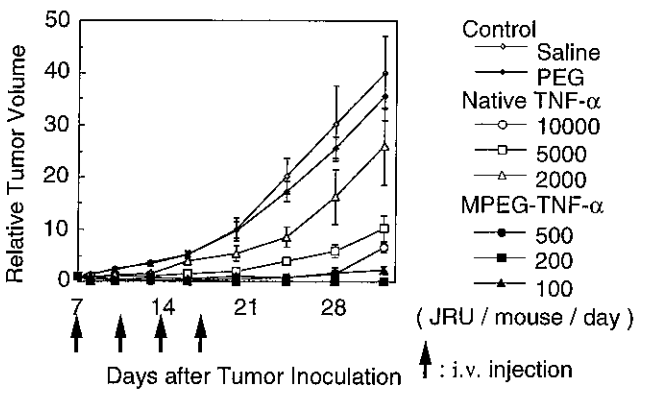

Fig. 1. Anti-tumor effects (in terms of tumor growth) of native TNF-α and MPEG-TNF-α by plural administration. Mice were used in groups of more than 7. Data were expressed as relative tumor volume by using the equation: relative tumor volume = mean tumor volume at a given time/mean tumor volume on day 7. Each value is the mean±SE.

was calculated by using the formula described by Haranaka *et al.*[10] The significance of differences was evaluated by using Student's *t* test.

The anti-tumor effects of plural i.v. injections of native TNF-α and MPEG-TNF-α on Meth-A solid tumor-bearing mice were studied. Table I summarizes the characteristics of native TNF-α and MPEG-TNF-α. Native TNF-α and MPEG-TNF-α inhibited tumor growth in a dose-dependent manner when administered by i.v. injection twice a week for 2 weeks (Fig. 1). Native TNF-α at the dose of 10,000 JRU induced the maximal anti-tumor response, and complete regression was obtained in 3 of the 8 mice (Table II). Four of the 8 mice given native TNF-α at the dose of 10,000 JRU died during the therapeutic period (Days 7 to 17). In addition, the three surviving mice developed piloerection, tissue inflammation and transient decrease in body weight during the experimental period (Fig. 2). At 2,000 and 5,000 JRU of native TNF-α, tumor growth was only slightly inhibited (Fig. 1), and all the treated mice died during the experimental period (Table II). The anti-tumor action of MPEG-TNF-α was more potent than that of the native TNF-α (Fig. 1). As shown in Fig. 1, MPEG-TNF-α at the dose of 100 JRU had an effect comparable to that of native TNF-α at the dose of 10,000 JRU. Complete regression was obtained in 3 of the 7 mice at the small dose of 100 JRU (Table I). These findings suggested that MPEG-TNF-α was 100-fold more potent than native TNF-α. In our previous study, we found that the plasma half-life of MPEG-TNF-α was approximately 40 times longer than that of unmodified TNF-α.[8] Thus, this increase in anti-tumor potency may be attributed predominantly to an increase in the plasma half-life. At the doses of 200 and 500 JRU, MPEG-TNF-α completely inhibited tumor growth, and complete regression was obtained in all 10 mice. In the completely tumor-regressed mice treated with MPEG-TNF-α, no trace of tumor was observed more than 150 days after tumor inoculation. During the experimental period, all doses of MPEG-TNF-α were well tolerated and body weight reduction was not observed (Fig. 2). Use of MPEG-TNF-α enabled us to decrease the therapeutic dose as compared with TNF-α, resulting in a decrease in side-effects. PEG (10 μg/mouse) had no anti-tumor effect (Fig. 1 and Table I), and the marked increase in the body weight of the saline control and PEG control animals was due to tumor growth (Fig. 2). The relative body weight change of negative control mice, that were not inoculated with Meth-A fibrosarcoma, was indistinguishable from that of MPEG-TNF-α-500 JRU-injected mice (Fig. 2).

The systemic administration of TNF-α at high doses often induces toxic side-effects.[4,5] Therefore, cancer therapy with TNF-α has been limited to intratumoral administration.[6,7] Our studies demonstrated that the i.v. administration of MPEG-TNF-α alone could induce

Table II. Anti-tumor Effects of Native TNF-α and MPEG-TNF-α in Terms of Survival Days after Tumor Inoculation

Run	Injection dose[a] (JRU/mouse/day)	Survival time[b] (days)		Complete regression[c]
Saline	0	32±1.1	(29, 29, 31, 32, 33, 33, 37)	0/7
PEG	0	31±1.2	(28, 28, 28, 32, 33, 34, 35)	0/7
Native TNF-α	10,000	67±24.8	(7, 7, 7, 16, 50, 150<, 150<, 150<)	3/8
	5,000	46±1.1[d]	(43, 43, 44, 48, 48, 48, 50)	0/7
	2,000	43±1.1[d]	(40, 40, 42, 43, 44, 44, 48)	0/7
MPEG-TNF-α	500	150<[d, e]	(150<, 150<, 150<, 150<, 150<, 150<, 150<, 150<, 150<, 150<)	10/10
	200	150<[d, e]	(150<, 150<, 150<, 150<, 150<, 150<, 150<, 150<, 150<, 150<)	10/10
	100	95±19.5[d]	(46, 48, 52, 59, 150<, 150<, 150<)	3/7

a) Native TNF-α and MPEG-TNF-α were i.v. injected on days 7, 10, 14, 17.
b) Days after tumor inoculation (mean±SE).
c) Complete regression was defined as no regrowth of tumor for at least 150 days.
d) Significant difference from the saline-control group ($P<0.01$).
e) Significant difference from the 10,000 JRU native TNF-α-treated group ($P<0.01$).

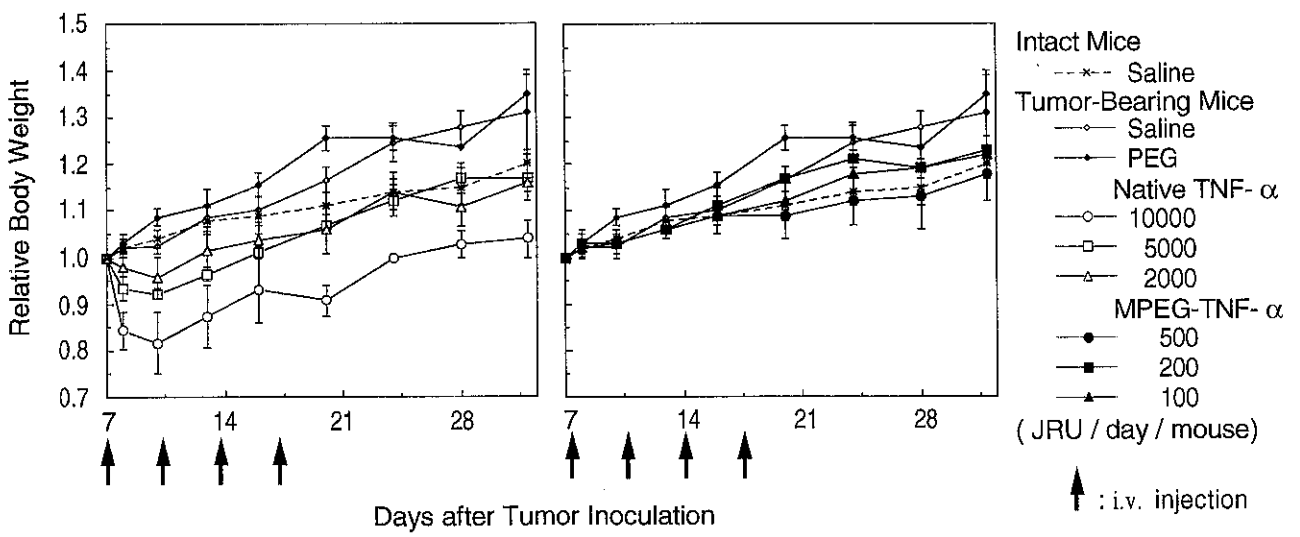

Fig. 2. Body weight of Meth-A tumor-bearing mice treated with native TNF-α and MPEG-TNF-α. Mice were used in groups of more than 7. Data were expressed as relative body weight by using the equation: relative body weight = mean body weight at a given time/mean body weight on day 7. Each value is the mean±SE.

complete regression of solid Meth-A tumors without any TNF-α-mediated side-effect. MPEG-TNF-α has high *in vivo* anti-tumor activity and was not any less efficacious in this mouse tumor model than other anti-tumor drugs, including other PEG-modified cytokines, previously reported. We intend to assess the anti-tumor effect of MPEG-TNF-α against various other tumors.

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