

PEGylated recombinant human tumor necrosis factor alpha: preparation and anti-tumor potency¹

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KEY WORDS tumor necrosis factor; recombinant proteins; polyethylene glycol; peptides

ABSTRACT

AIM: To assess the merits of polyethylene glycol-modified recombinant human tumor necrosis factor alpha (PEG-rHuTNF- α). **METHODS:** The rHuTNF- α was modified with *N*-succinimidyl succinate monomethoxy polyethylene glycol (SS-PEG) of three different molecular weights. The PEG-rHuTNF- α was separated into fractions of various molecular weights by gel filtration chromatography. *In vitro* activities of various fractions were determined with L929 cell assay and *in vivo* anti-tumor potencies of main fractions were studied with respect to necrosis of S-180 solid tumor. **RESULTS:** The rHuTNF- α could be modified using SS-PEG under mild conditions. The main fraction of PEG₅₀₀₀-rHuTNF- α contained four PEG molecules, and PEG₁₂₀₀₀-rHuTNF- α and PEG₂₀₀₀₀-rHuTNF- α contained two PEG molecules, respectively. There was a higher activity when rHuTNF- α was coupled to less numbers of the same molecular weight PEG molecules. When PEG-rHuTNF- α was of the same molecular weight, rHuTNF- α modified with bigger molecular weight PEG molecules had a higher activity. PEG-rHuTNF- α was resistant to proteolysis, and over 70 % activity remained after 8 h, but the activity of rHuTNF- α was time-dependently diminished by incubation with bovine trypsin. PEG₅₀₀₀-rHuTNF- α (1500 IU per mouse) had a similar anti-tumor potency compared with rHuTNF- α (3000 IU per mouse). PEG₁₂₀₀₀-rHuTNF- α (1500 IU per mouse) had an in-

creased anti-tumor potency compared with rHuTNF- α (3000 IU per mouse). In particular, PEG₂₀₀₀₀-rHuTNF- α at a dose of 1500 IU per mouse had a higher anti-tumor potency than rHuTNF- α at a dose of 6000 IU per mouse. **CONCLUSION:** PEG-modified rHuTNF- α could be more suitable for therapeutic use.

INTRODUCTION

Tumor necrosis factor alpha (TNF- α), an anti-tumor cytokine produced by activated monocytes and macrophages, exhibits striking biological effects, such as direct cytotoxicity against various tumor cells, activation of immune anti-tumor response and inducement of hemorrhagic necrosis of certain transplanted solid tumors with selective impairment of tumor vascular endothelial cells^[1-3]. However, TNF- α having a very short plasma half-life can be rapidly cleared from the blood and excessively frequent and high doses are required for significant anti-tumor effects. In addition, it was found to have severe side effects in phase I - II studies, for example, tissue inflammation and injury, inhibition of gastric emptying, alteration in circulating leukocyte subsets, even a lethal endotoxic shock-like syndrome at very high doses^[4,5]. These drawbacks make effective systemic treatment with free TNF- α difficult.

In order to overcome these problems, TNF- α is modified with polyethylene glycol (PEG) or polyvinyl pyrrolidone (PVP). Tsutsumi *et al* studied the PEGylation of natural human TNF- α ^[6-11]. Kamada *et al* also studied its chemical modification with PVP^[12]. Their results showed that natural human TNF- α modified with PEG or PVP had a longer plasma half-life and better anti-tumor potency than unmodified one. Moreover, modified natural human TNF- α showed little adverse effects. Unfortunately, natural human TNF- α is very expensive and limited in source. On the contrary, recombinant human tumor necrosis factor- α (rHuTNF- α) is very rich in

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source with the development of gene-engineering technology. In addition, there are some differences between natural human TNF- α and rHuTNF- α , such as molecular weight, number of amino acids, chemical construction, bioactivity and toxicity, etc. Thus, it is very essential to study the PEGylation of rHuTNF- α .

The aim of the present work was to assess the merits of PEGylated rHuTNF- α . For this purpose, rHuTNF- α was modified using activated PEG, *N*-succinimidyl succinate monomethoxy polyethylene glycol (SS-PEG) of three different molecular weights. *In vitro* activities of their various fractions were determined, and *in vivo* anti-tumor potencies of the main fractions were studied.

MATERIALS AND METHODS

Materials Recombinant human tumor necrosis factor- α (rHuTNF- α , M_r 17 000) was donated by Shanghai Research Center of Biotechnology, Chinese Academy of Sciences. *N*-succinimidyl succinate monomethoxy polyethylene glycol (SS-PEG; M_r 5000, 12 000, and 20 000) was purchased from Nippon Oil and Fats Co (Tokyo, Japan). ϵ -Amino-caproic acid was purchased from Sigma Chemical Co (St Louis, MO). Other reagents and solvents were of analytical grade.

Animals and cells Kunming strain mice (Grade II, Certificate No 008, $n = 56$, $20 \text{ g} \pm 2 \text{ g}$, \uparrow) were supplied by the Shanghai Experimental Animal Center, Chinese Academy of Sciences (Shanghai, China) and divided randomly into 7 groups ($n = 8$). L929 cells were kindly supplied by Shanghai Research Center of Biotechnology, Chinese Academy of Sciences, and serially sub-cultured in RPMI-1640 medium with 10 % (v/v) fetal calf serum. Sarcoma-180 (S-180) cells were maintained intraperitoneally in Kunming mice.

Conjugation of PEG to rHuTNF- α PEG-rHuTNF- α was prepared using the typical procedure as previously described⁽⁹⁾. Briefly, rHuTNF- α was reacted with a 40-fold molar excess of SS-PEG at 25 °C for 30 min. Then, the reaction was stopped by adding 5-fold molar excess of ϵ -amino-caproic acid over the SS-PEG. PEG-rHuTNF- α was separated into fractions of various molecular weights by gel filtration chromatography (GFC; Sephadex G-150 column, 12.5 mm \times 100 cm, Shanghai; GFC-buffer; PBS 0.1 mol/L, pH 7.2). The molecular weight of separated PEG-rHuTNF- α was estimated by GFC analysis, and the numbers of PEG conjugated to rHuTNF- α was calculated from the molecular weight of PEG-rHuTNF- α .

Determination of protein concentrations The protein concentration of rHuTNF- α and PEG-rHuTNF- α was determined according to the method of Bradford with coomassie brilliant blue (R-250) protein assay dye reagent⁽¹³⁾. Different concentrations of bovine serum albumin (BSA) were used for the standard curve.

Assay of *in vitro* activities of rHuTNF- α and PEG-rHuTNF- α The *in vitro* activities of rHuTNF- α and PEG-rHuTNF- α were assayed as previously described⁽²⁾. Briefly, L929 cells, grown in RPMI-1640 medium with 10 % (v/v) fetal calf serum, were placed onto 96-well plates at 2.0×10^4 cells per 100 μL of the media per well. After overnight incubation, 50 μL of actinomycin D-containing medium was added to give a final drug concentration of 2 mg/L. Samples of rHuTNF- α and PEG-rHuTNF- α were immediately titrated at these targets, and incubation was continued overnight. After 22 h, 50 μL of crystal violet solution was added, and the remaining viable targets were allowed to incorporate the dye for 15 min. The rest of the dye was removed by washing once with PBS. Incorporated dye was solubilized with acidified ethanol, and the A_{540} for each well was determined using a multichannel scanning apparatus. The LD₅₀ of each sample was obtained from the titration curve of A_{540} and the rHuTNF- α dose, then the relative cytolytic activity (%) was calculated from the reciprocal of the LD₅₀ of the sample divided by that of native rHuTNF- α .

Proteolytic resistance of PEG-rHuTNF- α PEG-rHuTNF- α or rHuTNF- α were mixed with a 30-fold molar excess of bovine trypsin, and the mixture was incubated at 37 °C. At predetermined time, the proteolytic digestion was stopped and the mixture was diluted with minimal essential medium containing 1 % fetal calf serum (Filtron, Brooklyn, NY), then the L929 cytotoxicity was assayed.

***In vivo* antitumor activity of PEG-rHuTNF- α** S-180 cells were implanted intradermally into the armpit of Kunming mice. When the tumor nodules had grown to 10 – 12 mm in diameter after 7 d, rHuTNF- α and main fractions of three PEG-rHuTNF- α were given by iv. Anti-tumor effects against S-180 solid tumor were expressed according to the degree of tumor haemorrhagic necrosis at 24 h after iv. Tumor haemorrhagic necrosis was scored by the method previously described⁽¹⁴⁾. Briefly, the maximum necrotic response (score 3) indicates that 50 % or more of the tumor mass is necrotic, the moderate response (score 2), 25 % – 50 % necrotic, the minimal response (score 1), less than 25 %

necrotic, and no response (score 0) means no visible necrosis.

Statistical analysis Statistical evaluations of tumor haemorrhagic necrosis score were analyzed by *t*-test.

RESULTS

Preparation and *in vitro* activity of PEG-rHuTNF- α The protein was chemically modified by end point attachment with SS-PEG via the formation of an amino bond between lysine amino groups of protein and the terminal succinimidyl succinate group of SS-PEG (Fig 1).

The PEG-rHuTNF- α was separated into fractions of various molecular weights by GFC (Fig 2). The main fraction of PEG₅₀₀₀-rHuTNF- α contained four PEG molecules with M_r 37 000 (Fig 2A and Tab 1), and the yield was 46.5%. The main fraction of PEG₁₂₀₀₀-rHuTNF- α and PEG₂₀₀₀₀-rHuTNF- α contained two PEG molecules with M_r 41 000 (yield 54.3%) and 57 000 (Fig 2 B, C and Tab 1) (yield 63.7%), respectively. The coupling reactions between rHuTNF- α and PEG of different molecular weights were obviously different under the same conditions including concentration, temperature, and time. The rHuTNF- α coupled at least two PEG₅₀₀₀ molecules, and fractions coupling over five PEG₅₀₀₀ molecules were few. The rHuTNF- α could couple one PEG₁₂₀₀₀ or PEG₂₀₀₀₀ molecule, and fractions coupling over four PEG₁₂₀₀₀ or PEG₂₀₀₀₀ molecules were few. Their characterization including molecular weight, coupled numbers of PEG, and biological activity from differ-

ent fractions of PEG-rHuTNF- α were shown in Tab 1. The activity of PEG-rHuTNF- α was in relation to coupled numbers of PEG. There was a higher activity with less numbers of the same molecular weight PEG molecule. When PEG-rHuTNF- α was of the same molecular weight, the rHuTNF- α modified with a bigger molecular weight PEG molecule had a higher activity than that of the modified one with lower molecular weight PEG.

Tab 1. Characterization of PEG-rHuTNF- α . $n = 3$. $\bar{x} \pm s$.

	Molecular weight ¹⁾	Coupled number of PEG ²⁾	Remaining activity/% ³⁾
rHuTNF- α	17 000		100
PEG ₅₀₀₀ -rHuTNF- α Fr 1	27 000	2	71.1 \pm 4.6
PEG ₅₀₀₀ -rHuTNF- α Fr 2	37 000	4	49.3 \pm 2.4
PEG ₅₀₀₀ -rHuTNF- α Fr 3	42 000	5	31.6 \pm 2.7
PEG ₁₂₀₀₀ -rHuTNF- α Fr 1	29 000	1	71.7 \pm 3.8
PEG ₁₂₀₀₀ -rHuTNF- α Fr 2	41 000	2	58.7 \pm 4.9
PEG ₁₂₀₀₀ -rHuTNF- α Fr 3	53 000	3	34.6 \pm 3.6
PEG ₂₀₀₀₀ -rHuTNF- α Fr 1	37 000	1	65.7 \pm 4.2
PEG ₂₀₀₀₀ -rHuTNF- α Fr 2	57 000	2	47.8 \pm 2.1
PEG ₂₀₀₀₀ -rHuTNF- α Fr 3	77 000	3	28.9 \pm 1.6

1) Determined by GFC (protein standard). 2) Calculated from molecular weight. 3) Assessed by the growth inhibition L929 cell assay.

Proteolytic resistance of PEG-rHuTNF- α

There are no obvious differences between the activities of rHuTNF- α and PEG-rHuTNF- α after incubation without proteinase (Fig 3A). On the contrary, the activities of

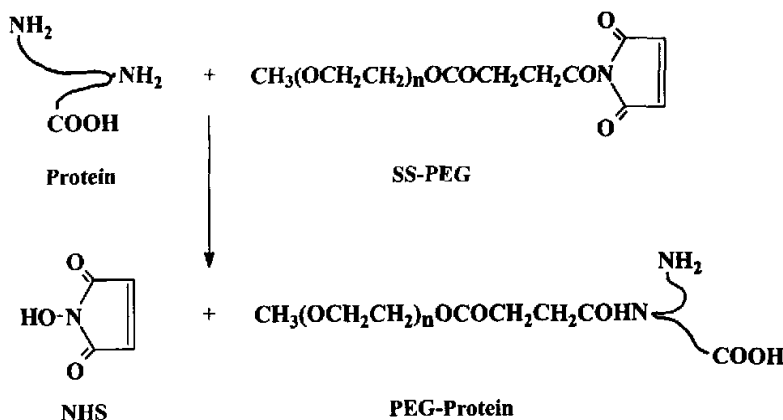


Fig 1. Reaction scheme of coupling succinimidyl succinate group of SS-PEG to lysine amino groups of protein.

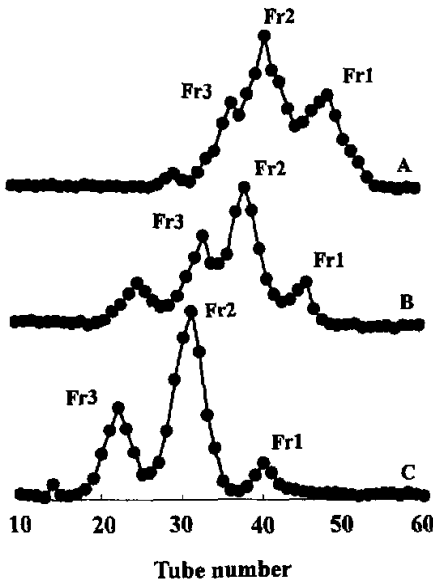


Fig 2. GFC profiles of PEG-rHuTNF- α . (A) PEG₅₀₀₀-rHuTNF- α ; (B) PEG₁₂₀₀₀-rHuTNF- α ; and (C) PEG₂₀₀₀₀-rHuTNF- α .

rHuTNF- α and PEG-rHuTNF- α after incubation with bovine trypsin were obviously different (Fig 3B). PEG-rHuTNF- α was much more resistant to proteolysis than rHuTNF- α , and over 70 % activity remained after 8 h.

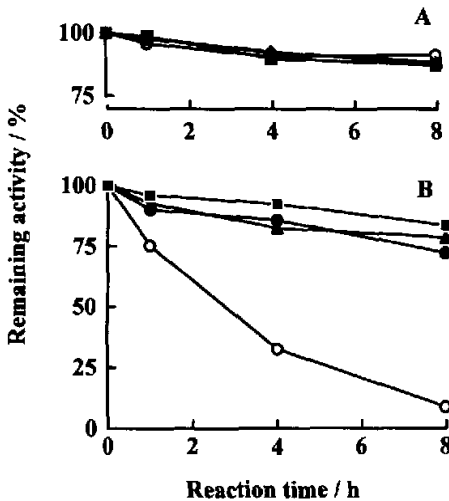


Fig 3. Resistance of rHuTNF- α and PEG-rHuTNF- α against proteinases. (A) no proteinase; (B) bovine trypsin. rHuTNF- α (○); PEG₅₀₀₀-rHuTNF- α (●); PEG₁₂₀₀₀-rHuTNF- α (▲); PEG₂₀₀₀₀-rHuTNF- α (■).

While the activity of rHuTNF- α time-dependently diminished by incubation with bovine trypsin, only a little activity remained after 8 h.

In vivo anti-tumor effect of PEG-rHuTNF- α

The anti-tumor effects of a single intravenous injection of PEG-rHuTNF- α on mice borne S-180 solid tumors were compared with that of rHuTNF- α (Fig 4). The rHuTNF- α was intravenously injected at three doses of 1500, 3000 and 6000 IU. PEG-rHuTNF- α was intravenously injected at a dose of 1500 IU. PEG₅₀₀₀-rHuTNF- α (1500 IU per mouse) had a similar anti-tumor potency compared with rHuTNF- α (3000IU per mouse) ($P > 0.05$). PEG₁₂₀₀₀-rHuTNF- α (1500 IU per mouse) had a increasingly induced tumor necrosis compared with rHuTNF- α (3000 IU per mouse) ($P < 0.05$). In particular, PEG₂₀₀₀₀-rHuTNF- α at a dose of 1500 IU per mouse had a higher tumor necrosis effect than rHuTNF- α at a dose of 6000 IU per mouse ($P < 0.05$).

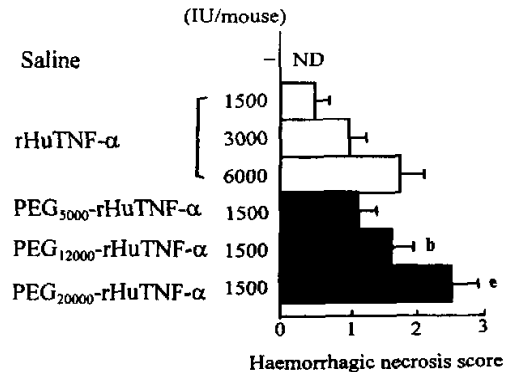


Fig 4. Tumor necrotic effects of rHuTNF- α and PEG-rHuTNF- α on mice borne S-180 solid tumors. $n = 8$. $\bar{x} \pm s$. ^b $P < 0.05$ vs rHuTNF- α 3000 IU. ^e $P < 0.05$ vs rHuTNF- α 6000 IU. ND, not detected.

DISCUSSION

The PEGylation of proteins and peptides has been considered as an important strategy to alter and improve their chemical and/or physiological properties in biological sciences. There are numerous advantages from PEG conjugates compared with their parent molecules, such as prolonging half-life⁽¹⁵⁻¹⁷⁾, decreasing immunogenicity⁽¹⁸⁾, improving solubility and increasing stability against denaturation in organic solvents from reinforcement of the protein hydration shell⁽¹⁹⁾. In the recent years, some of PEGylated proteins or peptides, such as

PEG-IFN^[20,21], PEG-IL-2^[22,23] and PEG-ADA^[24], have been used in clinical studies or practice and obtained good effects. The most often used modifiers for the conjugation of PEG with proteins are activated PEG, such as *N*-succinimidyl succinate monomethoxy PEG (SS-PEG), *N*-hydroxysuccinimide PEG (NHS-PEG) and succinimidyl carboxy-methylate PEG (SCM-PEG), in particular, SS-PEG being the most preferred^[25-27]. Thus, in this study, SS-PEG was chosen as a modifier of rHuTNF- α .

It has been reported that the PEG modification occurs most likely at some of the lysine ϵ -amino residues^[8]. The rHuTNF- α contains 157 amino acids including six lysine ϵ -amino residues, one at position 11, and the others at position 65, 90, 98, 112, 128, respectively. Lys-112 and Lys-128 are on the surface of its monomer. An intersubunit salt bridge between residues Lys-98 and Glu-116 is formed resulting from a rotation of the lysine side chain about the C α -C β bond, and another salt bridge between Lys-11 from one subunit and the terminal carboxylate (Leu-157) of the adjacent subunit is at the bottom of the trimer^[28]. In the present work, rHuTNF α was found at least coupled to two PEG₅₀₀₀, and the main fractions of PEG₁₂₀₀₀-rHuTNF- α and PEG₂₀₀₀₀-rHuTNF- α also contained two PEG₁₂₀₀₀ or PEG₂₀₀₀₀ (Fig 2 and Tab 1). These results indicate that PEG could easily conjugate with two lysines on the surface of rHuTNF- α monomer, but accurate position of every PEG molecule in PEG-rHuTNF- α was indistinct.

In vitro activity of PEG-rHuTNF- α relative to that of rHuTNF- α gradually decreased with an increase in the numbers of the same molecular weight PEG. More loss of activity of PEG-rHuTNF- α was observed with bigger molecular weight PEG when the rHuTNF- α was modified with the same numbers of PEG molecules (Tab 1). These findings suggest that PEG chains sterically inhibit rHuTNF-receptor binding and that some lysine amino residues of rHuTNF- α play an important role in bioactivity. In addition, because lysine modification with PEG is random and difficult to control, main fractions of PEG-rHuTNF- α were used in studies of proteolytic resistance and *in vivo* anti-tumor effect.

The rHuTNF- α was easily degraded by proteinases, on the contrary, PEG-rHuTNF- α had obvious resistance to proteolytic action of bovine trypsin (Fig 3). This conclusion was the same as reported for native TNF- α resulting from the steric hindrance caused by PEG chains attached to the protein^[11]. Of course, besides proteolysis, rHuTNF- α and PEG-rHuTNF- α also would be

affected by a lot of other factors *in vivo*, such as pH and mononuclear phagocyte system, etc.

PEG-rHuTNF- α showed greater anti-tumor potency than unmodified rHuTNF- α at the same dose. In particular, PEG₂₀₀₀₀-rHuTNF- α (1500 IU per mouse) showed a higher tumor necrosis effect than unmodified rHuTNF- α (6000 IU per mouse). In general, the vascular permeability of tumors is enhanced compared with normal tissues, and macromolecules with a molecular size similar to that of albumin markedly accumulate in tumor tissues^[29]. In addition, tumor vascular permeability is also selectively increased by TNF- α ^[30]. Thus, PEG₂₀₀₀₀-rHuTNF- α might be selectively distributed to tumor bearing tissues. In order to clarify these speculations, a more detailed study including the pharmacokinetics of PEG-rHuTNF- α is currently under investigation.

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聚乙二醇化重组人肿瘤坏死因子- α : 制备和抗肿瘤活性¹

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关键词 肿瘤坏死因子; 重组蛋白质类; 聚乙二醇; 肽类

目的: 评价聚乙二醇化重组人肿瘤坏死因子- α (PEG-rHuTNF- α) 的价值. **方法:** 用三种不同分子量的聚乙二醇(PEG)修饰重组人肿瘤坏死因子- α (rHuTNF- α); 用凝胶过滤色谱法分离 PEG-rHuTNF- α ; 按 L929 靶细胞法测定 rHuTNF- α 和 PEG-rHuTNF- α 的体外活性; 通过 S-180 实体瘤的坏死研究其体内活性. **结果:** rHuTNF- α 能在温和的条件下用 PEG 修饰, 用分子量为 5000 的 PEG 修饰重组人肿瘤坏死因子的主要产物

含有四个 PEG 分子, 分子量为 12 000 和 20 000 的 PEG 修饰的主要产物均含二个 PEG 分子. 用同一种分子量的 PEG 修饰所得 PEG-rHuTNF- α 的活性与所含 PEG 分子的个数有关, 所含 PEG 分子数愈少, 则活性愈高; 当 PEG-rHuTNF- α 的分子量相同时, 所含单个 PEG 分子的分子量愈大, 活性愈高. 当与胰蛋白酶混合存放时, rHuTNF- α 的活性随时间的延长而降低, 但所有 PEG-rHuTNF- α 对蛋白水解酶有明显的阻抑作用. 体内活性测定结果表明: PEG₅₀₀₀-rHuTNF- α (1500 IU/鼠) 与 rHuTNF- α (3000 IU/鼠) 有相近的抗肿瘤活性; PEG₁₂₀₀₀-rHuTNF- α (1500 IU/鼠) 与 rHuTNF- α (3000 IU/鼠) 比较有较高的诱导肿瘤坏死的效果; PEG₂₀₀₀₀-rHuTNF- α (1500 IU/鼠) 与 rHuTNF- α (6000 IU/鼠) 比较有更高的抗肿瘤活性. **结论:** 聚乙二醇化重组人肿瘤坏死因子可能更适于治疗使用.

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