PEGylated Recombinant Human Tumor Necrosis Factor Alpha: Pharmacokinetics and Anti-tumor Effects

Ya-Ping Li,*,a,b Yuan-Ying Pei,a Zhao-Hui Zhou,b Xian-Ying Zhang, b Zhou-Hui Gu, b Jian Ding, b Xiu-Jian Gao, a and Jian-Hua Zhu,a

Department of Pharmaceutics, School of Pharmacy, Fudan University; a 138 Yí Xüe Yuan Road, Shanghai 200032, China, and Institute of Materia Medica, Shanghai Institute for Biological Science, Chinese Academy of Sciences; b 294 Tai Yuan Road, Shanghai 200031, China. Received December 6, 2000; accepted February 21, 2001

The aim of the present work was to investigate and assess the merit of PEGylated recombinant human tumor necrosis factor-α (rHuTNF-α) following our previous work. The rHuTNF-α was modified using activated polyethylene glycol (PEG), N-succinimidyl succinate monomethoxy polyethylene glycol (SS-PEG). The pharmacokinetics and anti-tumor effect were investigated. The experimental results showed that PEGylated rHuTNF-α could obviously alter in vivo behavioral characteristics of rHuTNF-α. Among the synthesized PEG-rHuTNF-α with different PEG molecules, PEG20000-rHuTNF-α demonstrated the longest circulating half-life (24.8 h) which was about 50 times longer than that of rHuTNF-α (28.8 min). In addition, there was much more PEG20000-rHuTNF-α distributed into tumor tissues than other PEG-rHuTNF-α or rHuTNF-α with time, and PEG20000-rHuTNF-α also showed the highest anti-tumor potency. These results indicated that PEG20000-rHuTNF-α was a useful long circulating molecule with selective localization in tumor tissues and enhanced anti-tumor activity of rHuTNF-α. 

Key words tumor necrosis factor-α; polyethylene glycol; anti-tumor potency

Tumor necrosis factor alpha (TNF-α) has attracted attention as a novel anti-tumor agent due to its striking biological effects, such as direct cytotoxicity against various tumor cells, activation of immune anti-tumor response and inducement of hemorrhaged necrosis of certain transplanted solid tumors.1—3) However, TNF-α can rapidly be cleared from the blood with very short plasma half-life, and high doses are required to obtain significant anti-tumor effects because of its very low in vivo stability. In addition, it was found to have severe toxic side effects in phase I—II studies, for example, tissue inflammation and injury, decrease in blood pressure, inhibition of gastric emptying, even a lethal endotoxic shock-like syndrome at very high doses.4,5) Therefore, when TNF-α was clinically used as a systemic anti-tumor agent, its dose was limited to 1/5—1/25 of the dose required for the development of anti-tumor effects.

In order to overcome these problems, it is a considerable strategy to utilize polyethylene glycol-conjugated pharmaceutic proteins which can improve their physicochemical, pharmacokinetic and biological properties to attain maximum clinical potency.6) In recent years, some of the PEGylated proteins or peptides, such as PEGylated interferon (PEG-IFN), PEGylated interleukin-2 (PEG-IL-2) and PEGylated adenosine deaminase (PEG-ADA), have been used in clinical practice or studies and obtained good effects.7—11) PEGylation of natural human TNF-α demonstrated that PEGylated natural human TNF-α had longer plasma half-life and better anti-tumor potency than free natural human TNF-α; in particular, PEGylated natural human TNF-α showed much lower side effects.12—18) 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 resources with the development of gene-engineering technology. However, there are some differences between natural human TNF-α and rHuTNF-α, such as molecular size and number of amino acids, etc. These differences can cause different properties between PEGylated natural human TNF-α and PEGylated rHuTNF-α. Thus, it is very essential to study PEGylated rHuTNF-α.

The purpose of the present work was to further investigate and assess the merits of PEGylated rHuTNF-α following our previous work.19) The pharmacokinetics and anti-tumor effects of PEGylated rHuTNF-α were investigated. The results of this study will provide fundamental information enabling us to design useful PEGylated rHuTNF-α with long circulation half-life and selective localization in tumor tissues.

MATERIALS AND METHODS

Materials Recombinant human tumor necrosis factor-α (rHuTNF-α, MW=17000) was obtained from Shanghai Research Center of Biotechnology, Chinese Academy of Sciences (Shanghai, China). N-succinimidyl succinate monomethoxy polyethylene glycol (SS-PEG, MW=5000, 12000 and 20000) was purchased from Nippon Oil and Fats (Tokyo, Japan). e-Amino-caproic acid and carrier-free Na125I were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Other reagents and solvents were of analytical grade.

Animals and Cells Female Kunming strain mice (20±2 g) were supplied by Shanghai Experimental Animal Center, Chinese Academy of Sciences (Shanghai, China ). The animals were acclimatized at a temperature of 25±2 °C and a relative humidity of 70±5% under natural light/dark conditions for one week before dosing. L929 cells were kindly supplied by Shanghai Research Center of Biotechnology, Chinese Academy of Sciences (Shanghai, China), and serially subcultured in a RPMI-1640 medium with 10% (v/v) fetal calf serum. Sarcoma-180 cells were maintained intraperitoneally in Kunming mice.

Conjugation of PEG to rHuTNF-α PEGylated rHuTNF-α (PEG-rHuTNF-α) was prepared using the procedure as described elsewhere.19) Briefly, rHuTNF-α was reacted with a 40-fold molar excess of SS-PEG at 25 °C for 30

* To whom correspondence should be addressed. e-mail: ypli@mail.shcnc.ac.cn © 2001 Pharmaceutical Society of Japan
min. Then, the reaction was stopped by adding 5-fold molar excess of ε-amino-caproic acid over SS-PEG. The resulting PEG-rHuTNF-α was separated into fractions of various molecular weights by gel filtration chromatography. The molecular weight of separated PEG-rHuTNF-α was estimated by GFC analysis with protein standard and the numbers of PEG conjugated to rHuTNF-α were calculated from the molecular weight of PEG-rHuTNF-α. In vitro activities of rHuTNF-α and PEG-rHuTNF-α were determined with L929 cells. The protein concentration of rHuTNF-α and PEG-rHuTNF-α was determined by the method of Bradford. Different concentrations of bovine serum albumin (BSA) were used for the standard curve. The main fractions of PEG-rHuTNF-α with different PEG molecules were used for investigating pharmacokinetics and anti-tumor activity described as follows.

Radioiodination of rHuTNF-α and PEG-rHuTNF-α The rHuTNF-α was labeled with $^{125}$I using the IODO-GEN procedure. Briefly, 60 μg of protein in 50 μl 0.1 M potassium phosphate buffer (pH 7.2) was layered over a freshly prepared film of IODO-GEN (100 μg) and incubated for 10 min at 4°C in the presence of 1 μCi of carrier-free Na$^{125}$I. The reaction mixture was brought up to 0.5 ml volume with PBS, and the unreacted iodine was removed by gel filtration chromatography on a Sephadex G-25 PD10 column equilibrated with PBS. The specific radioactivity of the product was assessed in an autogamma (Packard Instruments, CT, U.S.A.). The $^{125}$I-iodinated rHuTNF-α and PEG-rHuTNF-α were used for the study of pharmacokinetics as described below.

Pharmacokinetics of PEG-rHuTNF-α Four groups of female Kunming strain mice were used in this experiment, group 1 being treated with $^{125}$I-rHuTNF-α and groups 2—4 with the main fractions of $^{125}$I-PEG$_{5000}$-rHuTNF-α, $^{125}$I-PEG$_{12000}$-rHuTNF-α, and $^{125}$I-PEG$_{20000}$-rHuTNF-α, respectively. For administration, $^{125}$I-rHuTNF-α and $^{125}$I-PEG-rHuTNF-α were prepared into the same concentration of protein, and each animal was given intravenously at the dose of 0.5 μg of protein with a trace of $^{125}$I (1 μCi). After intravenous administration, blood was collected at 0.25, 0.5, 1, 2, 3, 6, 12 and 24 h from the tail vein and the radioactivity levels were measured. The pharmacokinetic parameters of rHuTNF-α and PEG-rHuTNF-α were calculated using the Practical Pharmacokinetic Program—Version 87.

In order to study the tissue distribution, eight groups of female Kunming strain mice with S-180 tumor nodules of 9—10 mm in diameter were used. The group 1—2 were treated with $^{125}$I-rHuTNF-α and groups 3—8 with the main fractions of $^{125}$I-PEG$_{5000}$-rHuTNF-α, $^{125}$I-PEG$_{12000}$-rHuTNF-α and $^{125}$I-PEG$_{20000}$-rHuTNF-α, respectively. Each animal was given intravenously at the dose of 0.5 μg of protein with a trace of $^{125}$I (1 μCi). The mice were dehematized of the abdominal aorta at 1 h and 6 h after intravenous injection. Tissues were collected and weighted and the radioactivities were measured.

Anti-tumor Activity of PEG-rHuTNF-α Eight groups of female Kunming strain mice (n=10) were used, and sarcoma-180 (S-180) cells were implanted intradermally into the armpit of the mice. After 7 d, when the tumor nodules had grown to 9—10 mm in diameter, rHuTNF-α and PEG-rHuTNF-α were given by intravenous injection once every 2 days for 8 d. The group 1 was treated with saline, groups 2—5 with rHuTNF-α at the doses of 0.1, 0.5, 1 and 2 μg per mouse, respectively, and the groups 6—8 with the main fractions of PEG$_{5000}$-rHuTNF-α, PEG$_{12000}$-rHuTNF-α and PEG$_{20000}$-rHuTNF-α at the dose of 0.5 μg protein per mouse, respectively. Anti-tumor effects against S-180 solid tumor were expressed according to mean relative tumor volume and survival days. Tumor volume was calculated by the formula as previously described.

Statistical analysis Statistical evaluations of tumor volume and survival days were analyzed by using Student’s t test.

RESULTS

The main fractions of PEG-rHuTNF-α with different PEG molecules were obtained by gel filtration chromatography in this paper through our previous work. The main fraction of PEG$_{5000}$-rHuTNF-α contained four PEG molecules with molecular weight 37000 and 49.3% remaining activity. The main fractions of PEG$_{12000}$-rHuTNF-α and PEG$_{20000}$-rHuTNF-α contained two PEG molecules with MW 41000 (58.7% remaining activity) and 57000 (37.8% remaining activity), respectively.

The plasma curves of rHuTNF-α and PEG-rHuTNF-α after intravenous injection were shown in Fig. 1. The radioactivities in plasma at 2 h after intravenous administration of PEG$_{5000}$-rHuTNF-α, PEG$_{12000}$-rHuTNF-α and PEG$_{20000}$-rHuTNF-α were about 3.5, 6.3 and 8.4 folds of that observed for free rHuTNF-α, respectively. All of PEG-rHuTNF-α exhibited delayed blood clearance. The much higher blood-associated radioactivity (99.5 cpm/μl) of PEG$_{20000}$-rHuTNF-α could be seen at 24 h compared to that of PEG$_{5000}$-rHuTNF-α (3.9 cpm/μl) or PEG$_{12000}$-rHuTNF-α (15.9 cpm/μl), but rHuTNF-α was quickly removed from the circulation system. The radioactivity—time curves for all of PEG-rHuTNF-α in mice were fitted with a two-compartment model and their pharmacokinetic parameters were shown in Table 1. The plasma half-lives of PEG$_{5000}$-rHuTNF-α, PEG$_{12000}$-rHuTNF-α and PEG$_{20000}$-rHuTNF-α were 6.3 h, 9.3 h and 24.8 h, respectively. The radioactivity—time curve of rHuTNF-α after intravenous injection in mice was fitted with a one-compartment model and its pharmacokinetic parameters were shown in Table 2. Its half-life was only 28.8 min.

The distribution profiles of $^{125}$I-labeled rHuTNF-α and PEG-rHuTNF-α were calculated using the Practical Pharmacokinetic Program—Version 87.
gradually with the molecular size of PEG-rHuTNF-PEG20000-rHuTNF-0.05) and obviously extended survival days (Table 3). PEG 12000-rHuTNF-rHuTNF-rHuTNF- had more obvious inhibitory action to S-180 solid tumor in normal tissues, but plasma levels of PEG-rHuTNF-rHuTNF-rHuTNF- was not obviously different that of rHuTNF-rHuTNF-rHuTNF- at the same time point. Their tumor accumulations were time-dependently increased. At 1 h after intravenous injection, tumor accumulation of PEG-rHuTNF-rHuTNF-rHuTNF- was not obviously different from rHuTNF-rHuTNF-rHuTNF-, but tumor accumulation of PEG-rHuTNF-rHuTNF-rHuTNF- dramatically increased at 6h, which was higher than that of rHuTNF-rHuTNF-rHuTNF- and increased with the attached PEG molecular weight.

The inhibitory action of PEG-rHuTNF-rHuTNF-rHuTNF- against S-180 solid tumors in mice was shown in Fig. 3. The rHuTNF-rHuTNF-rHuTNF- at doses of 1.0 and 2.0 μg per mouse induced the obvious anti-tumor response, but did not extend survival days and complete regression was at a dose of 2.0 μg per mouse (Table 3). In addition, 1 mouse and 3 mice died at the tenth day for rHuTNF-rHuTNF-rHuTNF- at doses of 1.0 and 2.0 μg per mouse, respectively. The other surviving mice developed piloerection and a transient decrease in body weight (data not shown). PEG5000-rHuTNF-rHuTNF-rHuTNF- had a similar tumor growth inhibitory action compared with rHuTNF-rHuTNF-rHuTNF- (2.0 μg per mouse) (p>0.05), but could extend survival days (Table 3). PEG12000-rHuTNF-rHuTNF-rHuTNF- had more obvious inhibitory action to S-180 solid tumor growth compared with HU1N3-rHuTNF-rHuTNF-rHuTNF- (2.0 μg per mouse) (p<0.05) and obviously extended survival days (Table 3). PEG6688-rHuTNF-rHuTNF-rHuTNF- had the highest anti-tumor potency with the tumor growth in most of the mice inhibited completely, and the survival days of mice were also the longest (36.6 days) (Table 3). For all PEG-rHuTNF-rHuTNF-rHuTNF-, the relative tumor volume in mice treated with PEG-rHuTNF-rHuTNF-rHuTNF- was decreased gradually with the molecular size of PEG-rHuTNF-rHuTNF-rHuTNF- increased. During the experimental period, all PEG-rHuTNF-rHuTNF-rHuTNF- were tolerated well, and body weight reduction and other rHuTNF-rHuTNF-rHuTNF- mediated side-effects were not observed (data not shown). Saline and rHuTNF-rHuTNF (0.1 μg per mouse) did not inhibit tumor growth (Fig. 3).

**DISCUSSION**

In general, PEGylated protein or peptide differs from the parent molecule in physicochemical, pharmacokinetic and biological properties. In this paper, it was found that in vivo behavior of rHuTNF-rHuTNF- was altered after being combined with PEG molecules. The radioactivity-time curves of PEG-rHuTNF-rHuTNF- were fitted with a two-compartment model, but that of rHuTNF-rHuTNF- was fitted with a one-compartment model. The plasma half-lives of PEG-rHuTNF-rHuTNF- were longer than that of rHuTNF-rHuTNF- and extended with molecular size of PEG-rHuTNF-rHuTNF-: the bigger molecular size, the longer half-life.

### Table 1. Pharmacokinetic Parameters of rHuTNF-rHuTNF-rHuTNF- in Mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>PEG5000-rHuTNF-α</th>
<th>PEG12000-rHuTNF-α</th>
<th>PEG6688-rHuTNF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1/2b (h)</td>
<td>1.41±0.17</td>
<td>0.99±0.06</td>
<td>0.54±0.15</td>
</tr>
<tr>
<td>T1/2a (h)</td>
<td>6.25±0.81</td>
<td>9.28±1.03</td>
<td>24.77±4.76</td>
</tr>
<tr>
<td>K12 (h-1)</td>
<td>0.18±0.05</td>
<td>0.22±0.02</td>
<td>0.78±0.19</td>
</tr>
<tr>
<td>K21 (h-1)</td>
<td>0.32±0.09</td>
<td>0.24±0.03</td>
<td>0.05±0.01</td>
</tr>
<tr>
<td>Vd (×10^{-2} µl)</td>
<td>2.09±0.17</td>
<td>5.98±0.36</td>
<td>10.06±0.38</td>
</tr>
<tr>
<td>Vc (µl)</td>
<td>2.95±0.22</td>
<td>2.41±0.39</td>
<td>2.27±0.49</td>
</tr>
<tr>
<td>AUC0-24h (h·cpm/µl)</td>
<td>704.04±60.4</td>
<td>2130.95±388.15</td>
<td>5887.55±206.31</td>
</tr>
</tbody>
</table>

### Table 2. Pharmacokinetic Parameters of rHuTNF-rHuTNF-rHuTNF- in Mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1/2a (h)</td>
<td>0.48±0.09</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>0.58±0.04</td>
</tr>
<tr>
<td>Vd (×10^{-2} µl)</td>
<td>1.80±0.29</td>
</tr>
<tr>
<td>Vc (µl)</td>
<td>510.78±48.67</td>
</tr>
<tr>
<td>AUC0-24h (h·cpm/µl)</td>
<td>668.51±72.06</td>
</tr>
</tbody>
</table>

125I-labeled PEG-rHuTNF-rHuTNF-rHuTNF- in S-180 solid tumor-bearing mice after intravenous administration were shown in Fig. 2. At 1 h after intravenous administration, rHuTNF-rHuTNF-rHuTNF- was distributed to normal tissues, successively, liver<spleen>lung<kidney>. The tumor accumulation of rHuTNF-rHuTNF-rHuTNF- was very low. At 6 h after intravenous injection, rHuTNF-rHuTNF-rHuTNF- was markedly eliminated from all tissues. There were no large differences in distribution of PEG-rHuTNF-rHuTNF-rHuTNF- compared with rHuTNF-rHuTNF-rHuTNF- in normal tissues, but plasma levels of PEG-rHuTNF-rHuTNF-rHuTNF- were markedly higher than that of rHuTNF-rHuTNF-rHuTNF- at the same time point. Their tumor accumulations were time-dependently increased. At 1 h after intravenous injection, tumor accumulation of PEG-rHuTNF-rHuTNF-rHuTNF- was not obviously different from rHuTNF-rHuTNF-rHuTNF-, but tumor accumulation of PEG-rHuTNF-rHuTNF-rHuTNF- dramatically increased at 6h, which was higher than that of rHuTNF-rHuTNF-rHuTNF- and increased with the attached PEG molecular weight.

In general, PEGylated protein or peptide differs from the parent molecule in physicochemical, pharmacokinetic and biological properties. In this paper, it was found that in vivo behavior of rHuTNF-rHuTNF- was altered after being combined with PEG molecules. The radioactivity-time curves of PEG-rHuTNF-rHuTNF- were fitted with a two-compartment model, but that of rHuTNF-rHuTNF- was fitted with a one-compartment model. The plasma half-lives of PEG-rHuTNF-rHuTNF- were longer than that of rHuTNF-rHuTNF- and extended with molecular size of PEG-rHuTNF-rHuTNF-: the bigger molecular size, the longer half-life.
The same phenomenon was also observed with natural tumor necrosis factor-\(\alpha\).\(^2\) The accumulated amount of PEG-rHuTNF-\(\alpha\) in tumors time-dependently increased, whereas the distribution of PEG-rHuTNF-\(\alpha\) to normal tissues decreased over time. At 6 h after intravenous injection, the order of the amount distributed in the tumor was PEG\(_{20000}\)-rHuTNF-\(\alpha\) > PEG\(_{12000}\)-rHuTNF-\(\alpha\) > PEG\(_{5000}\)-rHuTNF-\(\alpha\). It showed an obvious relationship between the amount of PEG-rHuTNF-\(\alpha\) distributed to tumor tissues and the molecular size of PEG-rHuTNF-\(\alpha\).

PEG\(_{20000}\)-rHuTNF-\(\alpha\) (molecular size, 57000) showed the highest anti-tumor potency among the synthesized PEG-rHuTNF-\(\alpha\). This result might result from its highest accumulation and prolongation of the plasma half-life, which was about 50 fold that of HuTNF-\(\alpha\). This difference could be caused by the steric hindrance of PEG chain. In addition, there was much more PEG\(_{20000}\)-rHuTNF-\(\alpha\) distributed to tumor tissues than other PEG-rHuTNF-\(\alpha\) with time, and PEG\(_{20000}\)-rHuTNF-\(\alpha\) also showed the highest anti-tumor potency. These results demonstrated that PEG\(_{20000}\)-rHuTNF-\(\alpha\) was a useful long circulating PEGylated rHuTNF-\(\alpha\) molecule with selective localization in tumor tissue and helpful in enhancing antitumor activity of rHuTNF-\(\alpha\).

**Acknowledgements** The work was supported by the National Natural Science Foundation of China, No 39870866. The authors thank Prof. Chang-Qing Chen (Shanghai Research Center of Biotechnology, Chinese Academy of Sciences, Shanghai, China) for supporting rHuTNF-\(\alpha\).

**REFERENCES**


---

**Table 3. Anti-tumor Effect of rHuTNF-\(\alpha\) and PEG-rHuTNF-\(\alpha\) on Survival Days after S-180 Tumor Inoculation**

<table>
<thead>
<tr>
<th>Run</th>
<th>Injection dose ((\mu g))</th>
<th>Survival time(^a) (d)</th>
<th>Complete regression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>0</td>
<td>19.1±3.1</td>
<td>0/10</td>
</tr>
<tr>
<td>rHuTNF-(\alpha)</td>
<td>0.1</td>
<td>18.4±4.6</td>
<td>0/10</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>21.3±2.4</td>
<td>0/10</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>22.1±4.1</td>
<td>0/10</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>15.9±5.7</td>
<td>1/10</td>
</tr>
<tr>
<td>PEG(_{5000})-rHuTNF-(\alpha)</td>
<td>0.5</td>
<td>27.9±4.3*</td>
<td>3/10</td>
</tr>
<tr>
<td>PEG(_{12000})-rHuTNF-(\alpha)</td>
<td>0.5</td>
<td>29.1±5.2**</td>
<td>5/10</td>
</tr>
<tr>
<td>PEG(_{20000})-rHuTNF-(\alpha)</td>
<td>0.5</td>
<td>36.6±6.7**</td>
<td>8/10</td>
</tr>
</tbody>
</table>

\(^a\) Days after tumor inoculation (mean±S.D.). *\(p<0.05\), **\(p<0.01\), significant difference from the saline group.