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Preparation and evaluation of a new releasable PEGylated tumor necrosis factor-α (TNF-α) conjugate for therapeutic application

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To design a releasable PEGylated TNF-α (rPEG-TNF-α), a cathepsin B-sensitive dipeptide (Val-Cit moiety) was inserted into conventional PEG-modified TNF-α (PEG-TNF-α), facilitating its clinical use for anti-tumor therapy. Comparative pharmacokinetic and pharmacodynamic studies showed that the half-lives of both PEGylated forms of TNF-α were ~60-fold greater than that of unmodified TNF-α. In addition, the *in vitro* bioactivity of rPEG-TNF-α was greater than that of PEG-TNF-α with the same degree of PEG modification. Release of TNF-α from rPEG-TNF-α *in vitro* was dependent on the presence of cathepsin B and was inhibited by a cathepsin B inhibitor. Despite the potent cytotoxicity of unmodified TNF-α against normal cells, its PEGylated forms at higher TNF-α concentrations showed low cytotoxic activity against these cells. In contrast, both forms of PEGylated TNF-α showed potent cytotoxic activity against the B16 and L929 cell lines, with rPEG-TNF-α being 5- and 9-fold more potent, respectively, than PEG-TNF-α. Moreover, rPEG-TNF-α was a more potent *in vivo* antitumor agent than PEG-TNF-α.

tumor necrosis factor-α, releasable PEGylation, dipeptide, anti-tumor effect

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TNF-α, a cytokine secreted by activated macrophages, has potent and specific activity against tumor cells, and has been assessed as a novel anti-tumor agent [1,2]. Because of its very low *in vivo* stability and pleiotropic activity, TNF-α requires continuous infusion or frequent administration at high doses to show anti-tumor effects. This, however, results in the augmentation of its side effects, including fever, hypotension, and endotoxin-like shock. When TNF-α is used clinically systemically, its dose must be limited to 1/5–1/25 of the dose required to show anti-tumor activities [3].

To overcome these shortcomings, several strategies have been investigated, including new macromolecular carrier systems. One of such strategies is PEGylation with a permanent linkage, resulting in an effective drug delivery platform [4]. Conjugation of cytokines such as TNF-α with PEG has been shown to improve their *in vivo* stability and their therapeutic effects [5–7]. However, the target molecules conjugate randomly with PEG, and several active cysteine residues are unavoidably modified, reducing the biological activity of these polypeptides. For example, randomly mono-PEGylated interferon alpha 2a (IFN-α2a), in which IFN-α2a conjugated with a 40 kD branched PEG, has only about 7% of the bioactivity of unmodified IFN-α2a [8]. Recently, some PEGylation technologies involving rational design,

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such as site-specific linkage, have been demonstrated, using protein engineering and improved chemical linkages [9,10]. The site-specific linkages of PEG may enhance the retention of bioactivity, as well as reducing the heterogeneity of the PEGylated protein.

The *in vivo* bioactivity of protein PEGylated with a permanent linkage depends on the slow release of the parent drug from the bioconjugate. A new approach to PEGylation, in which the release of active drug is controlled, called releasable PEGylation (rPEGylation) [11], employs chemically designed linkers that are sensitive to proteases specific in tumor tissue, allows the release of active drug in tumor tissue. Thus rPEGylated compounds are actually prodrugs, biologically inactive in the circulation but active at their site of action.

Cathepsin B is a ubiquitous cysteine protease with properties conserved in multiple species [12]. It is never found extracellularly except in pathological conditions such as tumors or in areas of tissue destruction such as in rheumatoid arthritis [13]. The general substrates of cathepsin B and L include dipeptide and tetrapeptide [14]. Tetrapeptide such as Gly-Phe-Leu-Gly and Ala-Leu-Ala-Leu were regarded as having distinct disadvantages, because of their relatively slow drug release and hydrophobic nature, which could cause these tetrapeptides to precipitate or aggregate [15–17]. Consequently, a dipeptide linker sensitive to cathepsin B such as the Val-Cit moiety has been widely utilized in releasable conjugates [18–20]. Those conjugates are highly stable in plasma and efficiently release drugs in tumor tissues.

We have described the synthesis of a releasable PEGylated-TNF-α, using the Val-Cit moiety to conjugate PEG and TNF-α. This compound showed enhanced stability in human and mouse plasma and sensitivity to cathepsin B and rat liver lysosomes. Moreover, the conjugate showed potent anticancer activity against Meth-A solid tumors and was toxic *in vitro* to L929 cells [21]. Further studies are necessary to determine the anticancer activity of this conjugate. We have assessed the sensitivity to cathepsin B of the Val-Cit linker containing a *p*-aminobenzylcarbonyl (PABC) spacer (Figure 1), the pharmacokinetics (PK) and pharmacodynamics (PD) of this compound, its *in vitro* cytotoxicity various normal and cancer cell lines, and its activity again S-180 solid tumors. We also compared the activities of rPEG-TNF-α with those of conventional PEGylated-TNF-α (PEG-TNF-α) and unconjugated TNF-α. These results may provide fundamental information for designing more potent bioactive protein derivatives against tumors and various cardiovascular diseases, and for developing better drug delivery systems to further enhance the therapeutic activity and safety of conjugated bioactive proteins.

1 Materials and methods

1.1 Materials

Recombinant human TNF-α (17000 kD, 1×10^7 IU mg^{-1} in 0.2 mol L^{-1} phosphate buffer, pH 7.2) was obtained from Biosynthesis Biotechnology Co., Ltd (Beijing, China). The 40 kD branched N-hydroxylsuccinimide functionalized polyethylene glycol (Y-NHS-40k, Polydispersity, 1.05) and 40 kD branched N-hydroxylsuccinimide functionalized PEG-valine-citrulline containing a *p*-aminobenzylcarbonyl spacer (Y-40k-Val-Cit-PABC-NHS, Polydispersity, 1.05) were obtained from Jenkem Technology Co., Ltd. (Beijing, China). Cathepsin B from human liver (2759 U mg^{-1}) was purchased from Sigma Corporation. Vascular endothelial cells (VEC), melanoma cells (B16) and fibrosarcoma cells (L929) were grown in RPMI 1640 medium (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum. Fluorescein isothiocyanate (FITC) was obtained

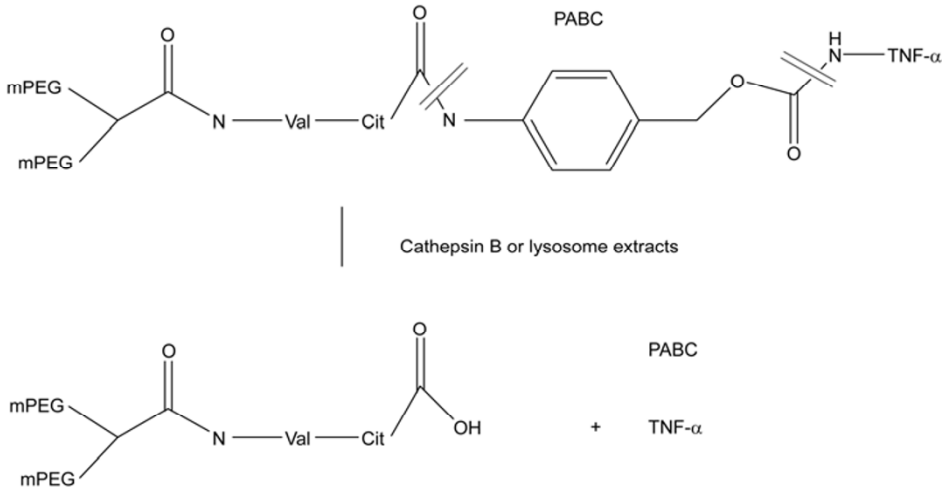

Figure 1 PEG conjugate and cathepsin B reaction products. Enzyme-mediated hydrolysis of the protease-sensitive Val-Cit dipeptide linker followed by PABC generates free TNF-α.

from Biosea Biotechnology Co., Ltd. (Beijing, China) and cell counting kits-8 (CCK-8) were from Dojindo Laboratories (Kumamoto, Japan). Other reagents and solvents used were of analytical grade. Animal care and handling were approved by the Institutional Authority for Laboratory Animal Care.

1.2 Preparation and characterization of TNF-α conjugates

The method of preparation of TNF-α conjugates has been describe [21]. Synthesized rPEG-TNF-α and PEG-TNF-α conjugates were each purified and separated into three fractions of various molecular weights by gel filtration chromatography (GFC) using a Sephacryl S-200HR column on a fast protein liquid chromatography system eluted with 0.1 mol L^{-1} phosphate buffer, pH 7.0. The molecular sizes of the PEG-TNF-α and rPEG-TNF-α conjugates were estimated by GFC analysis, and the degree of PEG-attachment to TNF-α was assayed by the fluorescamine method [22]. The specific *in vitro* bioactivity of the PEG-TNF-α and rPEG-TNF-α conjugates was measured using L-M cytotoxicity assay as described [23].

1.3 Pharmacokinetics studies

The pharmacokinetic profiles of TNF-α and TNF-α conjugates in the blood were measured as described [24]. Briefly, native TNF-α, PEG-TNF-α, and rPEG-TNF-α were radiolabeled with ^{125}I by the lactoperoxidase method, with each having a specific activity of 4.50 mCi mg^{-1} protein. The biological activities of ^{125}I radiolabeled TNF-α and its derivatives were indistinguishable from those of nonradiolabeled TNF-α and its derivatives (data not shown). Their pharmacokinetic profiles in the blood after intravenous (i.v.) injection into normal male BALB/c mice (5 weeks of age) were assessed at a dose of 31.6 ng of protein/mouse. Blood was collected from the tail vein at various time points, and radioactivity was measured in each sample.

1.4 *In vitro* evaluation of drug release

Lysosomes were collected from liver homogenates by centrifugation using the metrizamide discontinuous gradient method with slight modifications [21]. All drug release studies were performed at 37°C. The concentration of each conjugate was 20 μg mL^{-1} (17 μg TNF-α). TNF-α and its conjugates were labeled with FITC (data not shown). The stability of the TNF-α conjugates to hydrolysis by rat liver lysosomes, papain and cathepsin B was determined by incubating these TNF-α conjugates in 50 mmol L^{-1} sodium acetate (pH 5.0), 2 mmol L^{-1} dithiothreitol (DTT), and 25% (v/v) glycerol at 37°C for 15 min, followed by the addition of 20% (v/v) glycerol. The final concentration of lysosome was 1 mg mL^{-1} and the final concentrations of papain and cathepsin B were 8 μmol L^{-1} each. The effects of pH on drug release were evaluated by incubating the TNF-α conjugates in 40 mmol L^{-1} acetate or phosphate buffer at pH ranging from 3 to 7. The effects of the enzyme inhibitors aprotinin, bestatin, pepstatin, phosphoramidon, E-64, Z-Phe-Phe-CHN_2, and CA-074 were determined by adding each to the conjugate-lysosome mixtures till a final concentration of 10 μmol L^{-1}. During the process, 100 μL aliquots were periodically withdrawn. All the samples were lyophilized immediately by adding 1 mL chloroform, vortexing for 10 min, and centrifuged at 5000×*g* for 5 min. The upper layer in each tube was collected, and the chloroform was removed by lyophilization. The extracted PEG mixture was dissolved in 1 mL phosphate buffered saline (PBS) and analyzed by fluorescence spectrophotometry (λ_{ex}=492 nm, λ_{em}=519 nm). The results were expressed as concentrations of TNF-α.

1.5 *In vitro* cytotoxicity

Cytotoxicity under starved conditions, was assessed using the CCK-8 assay, which is based on the conversion of a water-soluble tetrazolium salt, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8), to a water-soluble formazan dye upon reduction by dehydrogenases in the presence of an electron carrier [25]. Normal VECs and the tumor cell lines B16 and L929 were grown in 96-well plates for 48 h and treated with TNF-α, PEG-TNF-α, or rPEG-TNF-α over a range of concentrations, with the volume in each well adjusted to 200 μL by adding culture medium. Following incubation for 24 h, the cells were washed, and cell viability was assessed using a CCK-8 assay (Dojindo, Kumamoto, Japan). CCK-8 solution (10 μL) was added to each well, followed by incubation for 2 h at 37°C. The absorbance at 450 nm was determined using a multiplate reader (Lambda Bio-20; Beckman). Cell viability was expressed relative to that of control (untreated) cells, and cytotoxicity (C %) was calculated using the equation $C=(A-B)/A\times100\%$, where A is the absorption in a control well and, B is the absorption in a test well.

1.6 Screening and evaluation of *in vivo* anti-tumor effects

The *in vivo* anti-tumor effects of native and conjugated TNF-α were assessed in mice bearing S-180 solid tumors. S-l80 cells were implanted intradermally (4×10^5 cells/site) in male C57BL/6 mice. The mice were assigned to three groups of six mice each, and seven days later (tumor size, 7–8 mm in diameter), each mouse received an intravenous injection of native TNF-α, PEG-TNF-α, or rPEG-TNF-α. The antitumor potency of TNF-α, PEG-TNF-α and rPEG-

TNF-α was evaluated by measuring tumor volume, tumor hemorrhagic necrosis scores, and survival time. Tumor volume [26] and tumor hemorrhagic necrosis scores [27] were determined as described. Sudden death was defined as death occurring within 24 h after injection of TNF-α, PEG-TNF-α, or rPEG-TNF-α. Complete regression was defined as tumors that no longer regrew after more than 150 d. The side effects of TNF-α, PEG-TNF-α and rPEG-TNF-α were assessed by measuring changes in body weight over a 25-day period.

1.7 Statistical analysis

All data are shown as mean±SD, unless otherwise mentioned. Groups were compared using Student's unpaired *t*-test and one-way analysis of variance.

2 Results and discussion

PEGylation is defined as the modification of a protein, peptide or non-peptide molecule by linking of one or more PEG chains. These chains are non-toxic, non-immunogenic, non-antigenic, highly soluble in water and FDA approved. PEGylated drugs have several advantages, including a prolonged *in vivo* half-life, decreased degradation by metabolic enzymes and reduced or absent protein immunogenicity. PEGylation now plays an important role in drug delivery, enhancing the therapeutic potentials of peptides and proteins. However, random conjugation of a polypeptide with PEG results in the unavoidable modification of some active cysteine residues, reducing the biological activity of these drugs. To overcome these limitations, we designed a novel rPEG-TNF-α, with a cleavable Val-Cit moiety. To assess its therapeutic effects, we compared the activities of rPEG-TNF-α, PEG-TNF-α and native TNF-α.

2.1 Preparation and characterization of TNF-α conjugates

Recombinant human TNF-α was chemically conjugated with activated PEG or PEG-Val-Cit containing a PABC spacer (average Mw 40000; Mw/average Mw, 1.05) via amide bonds between the amino groups of TNF-α and the N-hydroxysuccinimide groups of PEG at the end of the main chain. PEG attachment to TNF-α increased over time (data not shown). The resulting PEGylated TNF-α conjugates were purified from native TNF-α and separated into three fractions of various molecular sizes by gel filtration-high performance liquid chromatography (protein standard). Table 1 shows the average Mw, PK, and PD of PEG-TNF-α and rPEG-TNF-α. The dipeptide Val-Cit linker did not alter the PK of rPEG-TNF-α. The *in vivo* half-life ($t_{1/2}$) of rPEG-TNF-α was 60- to 110-fold higher than that of native TNF-α, depending on the degree of PEG modification. The activities of PEG-TNF-α, decreased as the molecular weight and degree of PEG modification increased, with the *in vitro* bioactivities of Fractions 2 and 3 being less than 10% that of native TNF-α. These changes in PK and PD profiles were similar to those previously reported for PEG of TNF-α, and suggest that PEG chains sterically inhibit PEF-TNF-α binding to TNF receptors. Insertion of the dipeptide Val-Cit into PEGylated TNF-α resulted in a markedly improved PD profile. Although changes in rPEG-TNF-α activity were similar to those of PEG-TNF-α, with activity associated with increased molecular weight and degree of PEG modification, the bioactivity of rPEG-TNF-α was noticeably higher than that of PEG-TNF-α with the same degree of PEG modification. The bioactivity of rPEG-TNF-α Fraction 1 (Mw 58000) was 77.2% relative to that of native TNF-α, whereas the bioactivity of PEG-TNF-α Fraction 1 (Mw 58700), with the same degree of modification as rPEG-TNF-α Fraction 1, was only 18.4%. These findings suggest that the dipeptide residue in rPEG-TNF-α plays an important role in increasing the bioactivity of rPEG-TNF-α. All further experiments, except for those indicated, used Fraction 1 of rPEG-TNF-α.

The Val-Cit moiety of rPEG-TNF-α is a substrate of cathepsin B. When administrated *in vivo*, rPEG-TNF-α was cleaved by cathepsin B, releasing-TNF-α.

Table 1 Comparative pharmacokinetic (PK) and pharmacodynamic (PD) parameters of rPEG-TNF-α, PEG-TNF-α and native TNF-α (n=6 mice each)

	Fraction No.	Average molecular weight[a)]	PK		PD
			$t_{1/2}$(h)[b)]	Fold difference[c)]	*In vitro* activity retained (%)[d)]
rPEG-TNF-α	1	58000	18.86±2.33$^{**\#}$	↑ ×60.8	77.2±6.97$^{*\#\#}$
	2	101000	21.22±2.34**	↑ ×68.5	48.6±5.71$^{**\#}$
	3	138000	34.09±3.25**	↑ ×110	28.9±4.22$^{**\#}$
PEG-TNF-α	1	58700	25.10±1.23**	↑ ×81	18.4±1.73**
	2	99000	27.23±1.76**	↑ ×87.8	9.1±0.12**
	3	137000	35.74±1.07**	↑ ×115.3	6.8±0.28**
Native TNF-α		17000	0.31±0.2	×1	100.0

a) Molecular size determined by GFC (protein standard). b) Half time in plasma *in vivo* after intravenous administration to mice the three TNF-α formulations. c) Compared with native TNF-α. d) The specific activities of native TNF-α, PEG-TNF-α and rPEG-TNF-α were measured by growth inhibition L-M cytotoxic assay. (*P<0.05; **P<0.01 vs. native TNF-α; #P<0.05 vs. PEG-TNF-α with the same degree of modification). rPEG-TNF-α, releasable PEGylated-TNF-α; PEG-TNF-α, PEGylated-TNF-α.

2.2 *In vitro* evaluation of drug release

To confirm whether TNF-α can be released by rPEG-TNF-α, we tested the enzyme and pH specificity of the linker. After incubation for 24 h, we found that these TNF-α conjugates were resistant to rat liver lysosomes and pure enzymes, although they were less resistant to rat liver lysosomes and cathepsin B than to papain (Figure 2A). Each enzyme has an optimal pH, at which the enzymatic activity is maximal. To examine the optimal pH for drug release, rPEG-TNF-α was incubated with rat liver lysosomes or pure enzymes in buffers of pH ranging from neutral (pH 7) to acidic (pH 3) for 24 h. We found that the optimal pH for TNF-α release was approximately pH 4, identical to that of cathepsin B, suggesting a crucial role for this enzyme in drug release. To confirm these results, we investigated the effects of proteinase inhibitors on TNF-α release. We found that inhibitors of amino-(bestatin), aspartyl-(pepstatin), and metallo-(phosphoramidon) proteinases were all ineffective at concentrations of 10 μmol L^{-1} (Figure 2B). However, E-64, a general inhibitor of cysteine proteases, completely inhibited TNF-α release from rPEG-TNF-α. The serine proteinase inhibitor aprotinin partially inhibited this release, probably due to its high concentration. These findings confirm that cysteine proteases are highly responsible for the cleavage of the Val-Cit linker. Cathepsins B and L are lysosomal cysteine proteases, which are involved in maintaining normal cellular metabolism [28]. To determine their contribution to rPEG-TNF-α cleavage, similar experiments were performed using specific inhibitors of cathepsin B, (CA-074) [29], and cathepsin L, (Z-Phe-Phe-CHN_2) [30]. We found that both inhibitors suppressed TNF-α release, even at 10 μmol L^{-1} (Figure 2B), confirming that lysosomal cathepsin B is responsible for the *in vivo* cleavage of rPEG-TNF-α.

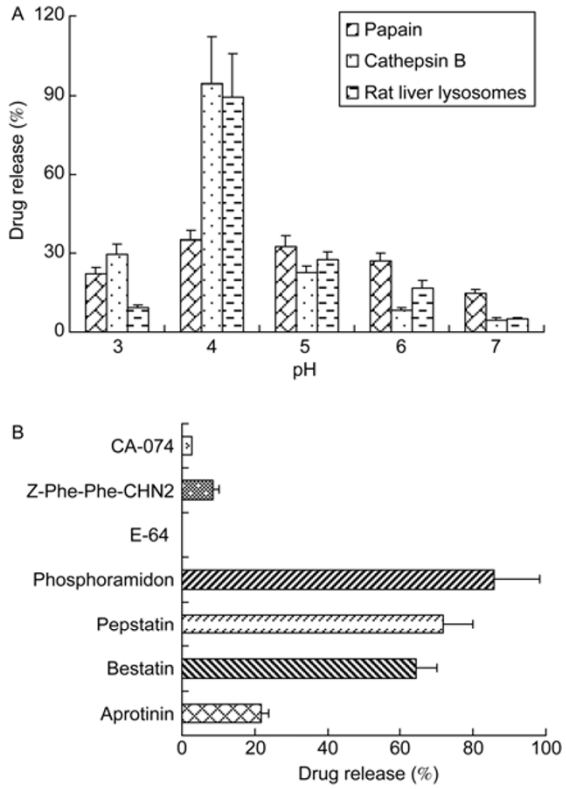

Figure 2 Effects of various enzymes at different pH (A) and enzyme inhibitors (B) on TNF-α release after 24 h incubation of PEG-TNF-α at 37°C. TNF-α release was expressed as a percentage relative to that of native TNF-α. FITC-labeled TNF-α conjugates, rat liver lysosome, and inhibitor concentrations were approximately 20 μg mL^{-1}, 0.5 mg mL^{-1} of protein, and 10 μmol L^{-1}, respectively. Conditions: 40 mmol L^{-1} acetate buffer (pH 4.0), 1 mmol L^{-1} EDTA, 5 mmol L^{-1} reduced glutathione, 0.1% Triton X-100, 37°C (n=4).

2.3 *In vitro* cytotoxicity

We evaluated the cytotoxicity of TNF-α and its conjugates using one normal cell line, VEC, and two cancer cell lines, B16 and L929. Both TNF-α conjugates showed slight cytotoxic activity against VECs, with 5000 U mL^{-1}, of each having less than 20% cytotoxicity (Figure 3). Moreover, the cytotoxicities of PEG-TNF-α and rPEG-TNF-α did not differ significantly. At higher concentrations (>5000 U mL^{-1}); however, these conjugates exhibited stronger cytotoxicity, up to 60%, against VECs (Figure 3A). At the same doses, both forms of PEGylated TNF-α were more cytotoxic to B16 and L929 cells, a difference likely due to the presence of cathepsin B in tumor tissue [16]. Moreover, the cytotoxicity of rPEG-TNF-α toward B16 (IC_{50} (5368.06±46.28) IU mL^{-1}; Figure 3B) and L929 (IC_{50} (3263.18±32.27) IU mL^{-1}; Figure 3C) cells, was about 5 and 9-fold higher than that of PEG-TNF-α against B16 (IC_{50} (30233.06±204.22) IU mL^{-1}) and L929 (IC_{50} (27356.81±274.08) IU mL^{-1}) cells, respectively. Additional modification of the dipeptide linkers or of PEGylation may enhance the *in vitro* cytotoxicity of rPEG-TNF-α. We also found that the cytotoxicity of rPEG-TNF-α was dose- and time-dependent. The low cytotoxicity of PEG-TNF-α may be due to the incomplete modification of PEG. PEGylation of proteins is accompanied by a significant loss of their activities.

2.4 Screening and evaluation of *in vivo* anti-tumor effects

We also compared the anti-tumor effects of single intravenous injections of rPEG-TNF-α, PEG-TNF-α and native TNF-α into S-180 solid tumors (Figure 4A). Although native TNF-α inhibited tumor growth in a dose-dependent manner, all mice receiving 10000 IU native TNF-α experienced side effects, including a transient decrease in body weight. Consequently, 10000 IU of native TNF-α was considered the maximum applicable dose. We found that PEG-TNF-α at doses of 200 and 1000 IU/mouse slightly inhibited S-180 tumor growth, whereas 50 IU rPEG-TNF-α

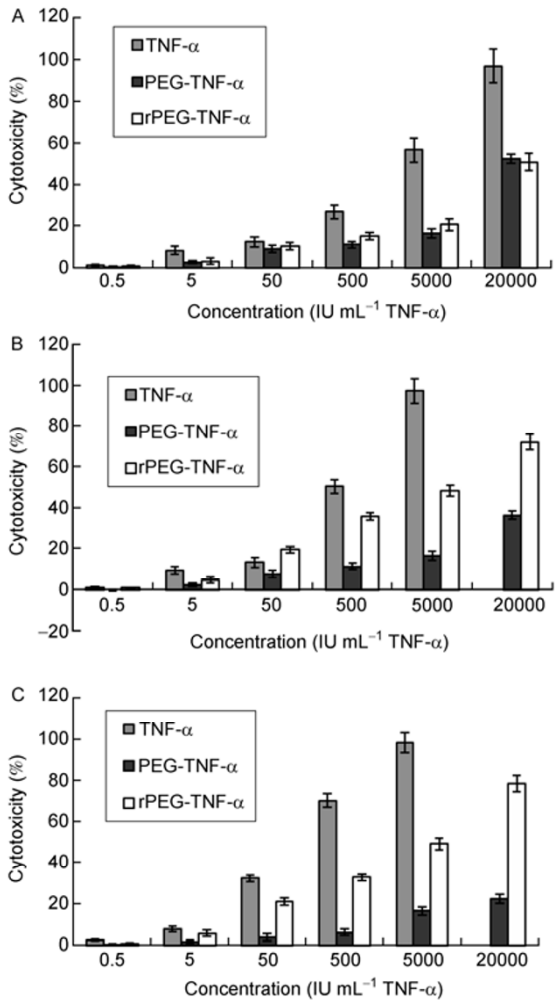

Figure 3 In *vitro* cytotoxicity of TNF-α and TNF-α conjugates on VEC (A), B16 (B), and L929 (C) cells. Cytotoxicity was measured using CCK-8 assays after incubation for 48 h (n=6). rPEG-TNF-α, releasable PEGylated-TNF-α; PEG-TNF-α, PEGylated-TNF-α.

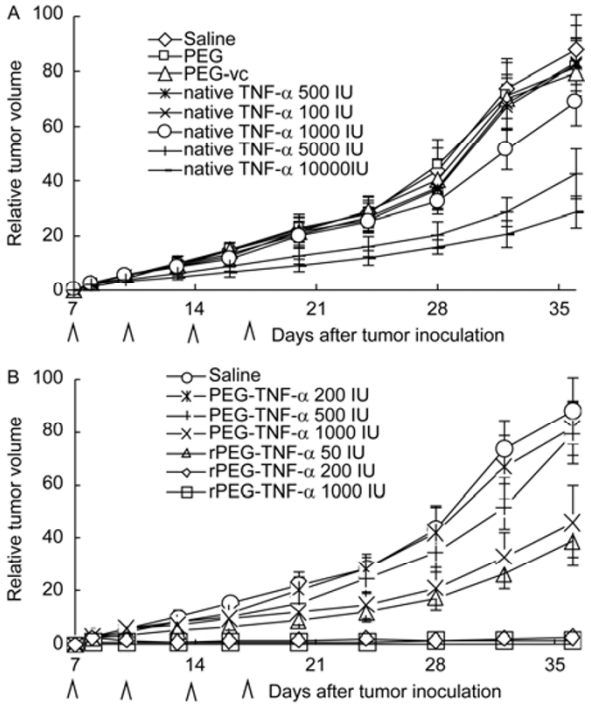

Figure 4 Anti-tumor effects of native TNF-α and TNF-α conjugates on S-180 solid tumors in mice. Eleven days after tumor inoculation, 6 mice each were intravenously injected with native TNF-α (A) or TNF-α conjugates (B) twice weekly for two weeks (arrows). Each value represents the mean±SD. *, P<0.01 compared with saline control. rPEG-TNF-α, releasable PEGylated-TNF-α; PEG-TNF-α, PEGylated-TNF-α.

and 1000 IU PEG-TNF-α showed anti-tumor effects comparable to those of native 10000 IU TNF-α (Figure 4B). Both TNF-α conjugates were well tolerated, with none of the mice showing body weight reductions. However, the anti-tumor effects of rPEG-TNF-α were superior to those of PEG-TNF-α, suggesting that the Val-Cit moiety contributed to the improvement in PD. PEG shielding of the proteolytic cleavage sites of TNF-α and the reduction in renal clearance due to increased molecular size increased the circulation time and plasma stability of both PEG-TNF-α and rPEG-TNF-α, as shown in our PK experiments. In addition, the accumulation and retention of macromolecules are greater in tumor than in normal tissues, a phenomenon called the "enhanced permeability and retention (EPR)" effect [31]. rPEG-TNF-α was cleaved by cathepsin B, which is abundant in tumor tissues, resulting in the efficient release of TNF-α and ultimately, a higher anti-tumor potency than observed for PEG-TNF-α.

To confirm that the Val-Cit moiety is a polymeric modifier and that rPEG-TNF-α is a systemic anti-tumor agent, we compared the anti-tumor potencies of rPEG-TNF-α, PEG-TNF-α and native TNF-α against S-180 solid tumors. Following a single intravenous administration, native TNF-α dose-dependently induced tumor hemorrhagic necrosis in S-180 solid tumors (Figure 5) and slightly inhibited tumor growth (data not shown). However, four of the six mice that received 10000 U native TNF-α died within 24 h after injection, and the remaining mice showed toxic side effects such as piloerection, tissue inflammation, and/or decreased body weight. In comparison, the anti-tumor effects of rPEG-TNF-α were markedly increased compared with native TNF-α, with 50 IU of rPEG-TNF-α, causing marked hemorrhagic necrosis, similar to that observed with 10000 IU of native TNF-α. In contrast to the latter, none of the mice administered rPEG-TNF-α experienced sudden death or toxic side effects. We found that the anti-tumor effects of 1000 IU PEG-TNF-α were equivalent to those of 50 IU rPEG-TNF-α. These results indicated that rPEG-TNF-α was approximately 200-fold more potent than native TNF-α and 20-fold more potent PEG-TNF-α.

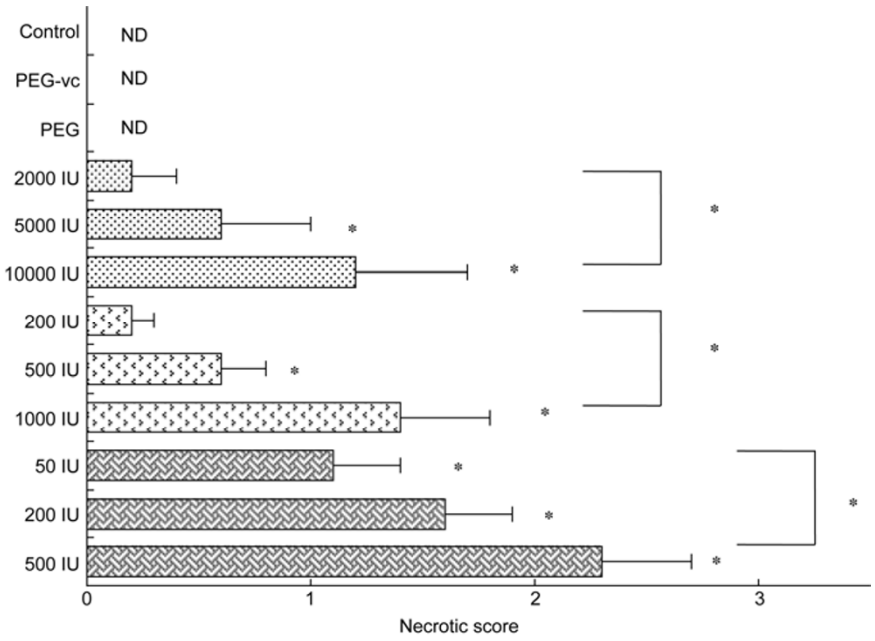

Figure 5 Tumor necrotic effects of native TNF-α, rPEG-TNF-α, and PEG-TNF-α on S-180 solid tumors. Mice were assigned into groups of six. Necrosis scores of 3, 2, 1, and 0 indicated that ⩾50%, 25%–50% necrotic, <25%, and 0%, respectively, of the tumor was. Each value represents the mean±SD. *, $P<0.01$ compared with saline control. ND, not detected. rPEG-TNF-α, releasable PEGylated-TNF-α; PEG-TNF-α, PEGylated-TNF-α.

3 Conclusion

We found that PEGylation of TNF-α using a Val-Cit linker containing a PABC spacer yielded a more potent product than conventional PEGylation. Although both types of PEGylated TNF-α had similar structure, rPEG-TNF-α showed more potent cytotoxic and anti-tumor activities, indicating that the conjugation of TNF-α with PEG and a Val-Cit moiety selectively increased its bioactivity *in vitro* and *in vivo*, rPEG-TNF-α may therefore be a more potent anti-tumor therapeutic agent than PEG-TNF-α.

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