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Linkage with cathepsin B-sensitive dipeptide promotes the *in vitro* and *in vivo* anticancer activity of PEGylated tumor necrosis factor-alpha (TNF-α) against murine fibrosarcoma

DAI ChuanYun[1,2], FU Ya [2], LI Biao[3], WANG YiGuang[1], ZHANG Xuan[1], WANG JianCheng[1] & ZHANG Qiang[1*]

[1]*State Key Laboratory of Natural and Biomimetic Drugs, School of Pharmaceutical Sciences, Peking University, Beijing 100191, China;*
[2]*Department of Biology, Chongqing University of Science and Technology, Chongqing 401331, China;*
[3]*Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100193, China*

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To improve the pharmacological profile of tumor necrosis factor alpha (TNF-α), we have synthesized a new PEGylated prodrug, PEG-vcTNF-α, using a cathepsin B-sensitive dipeptide (valine-citrulline, vc) to link branched PEG and TNF-α. PEG-modified TNF-α without the dipeptide linker (PEG-TNF-α) and unconjugated TNF-α were also tested as controls. It was found for the first time that TNF-α released from PEG-vcTNF-α was specifically dependent on the presence of cathepsin B. PEG-vcTNF-α induced higher cytotoxicity and greater apoptosis against L929 murine fibrosarcoma cells than PEG-TNF-α. Reversal of these effects by a cathepsin-B inhibitor confirmed that these effects were mediated by cathepsin B-specific release of TNF-α. *In vivo* pharmacokinetics studies demonstrated that the plasma stability of PEG-vcTNF-α was significantly increased compared to TNF-α. Finally, the improved anticancer efficacy of PEG-vcTNF-α and the distinct activities among the three formulations confirmed the positive contribution of both PEGylation and the dipeptide linkage to the improved drug-like properties of PEG-vcTNF-α. The results here indicate that linking proteins and PEG via the cathepsin B-sensitive dipeptide may be a promising strategy for developing protein therapeutics.

tumor necrosis factor alpha, PEGylation, cathepsin B-sensitive dipeptide, antitumor effect

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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 Math-A fibrosarcoma [1]. TNF-α has a direct anti-tumor effect, and stimulates host anti-tumor immune responses via specific interactions with tumor vascular endothelial cells [2]. TNF-α can bind to TNF-α receptors (the 55 kD TNF-α R1 and the 75 kD TNF-α R2), which are located on the plasma membrane of most cells throughout the body except red blood cells. Acting via these receptors, TNF-α induces two main forms of "extrinsic" (or death receptor-mediated) programmed cell death (PCD): (i) classical apoptosis, characterized by caspase-dependent chromatin condensation and fragmentation, membrane blebbing and generation of apoptotic bodies; and (ii) necrosis-like caspase-independent PCD, characterized by absent or marginal chromatin condensation, lack of nuclear fragmentation and disruption of membrane integrity [3]. Thus TNF-α has been considered as a promising new therapeutic agent [4,5]. However, it is cleared rapidly from the circulation and widely distributed to various tissues after intravenous administration, resulting in limited

*Corresponding author (email: zqdodo@bjmu.edu.cn)

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bioavailability. Although systemic administration of TNF-α causes the regression of various transplanted solid tumors [6], the high doses required for significant clinical anti-tumor effects often also elicit unexpected side-effects, such as tissue inflammation and injury, as well as the lethal endotoxic shock-like syndrome [7,8]. Similar *in vivo* drawbacks to those seen with TNF-α are also found in clinical applications of other bioactive proteins [9]. Thus, the development of a drug delivery system (DDS) seems necessary for the promotion of protein therapies, following recent marked advances in biotechnology-based pharmaceuticals.

In recent years, PEGylation has been identified as one of the most promising strategies for optimizing protein therapeutics [10]. The attachment of polyethylene glycol (PEG) to bioactive proteins (PEGylation) increases their molecular size and steric hindrance, leading to increased plasma half-life and *in vivo* stability. However, protein PEGylation is mostly non-specific and occurs at all the lysine residues of the protein, some of which may be in or near an active site. Furthermore, most PEG conjugates may not be cleavable *in vivo*. As a result, protein PEGylation is usually accompanied by a significant loss of bioactivity because of the loss of a binding site and/or steric hindrance. For instance, $BPEG_{40k}$-IFN-α2a, a mixture of various positional isomers of interferon-α2a randomly conjugated with 40 kD branched PEG, lost approximately 90% of its bioactivity compared to unmodified IFN-α2a [11,12]. Thus, the clinical application of PEGylated proteins has been limited.

To overcome the problem of PEGylation, some investigators have attempted replacing all lysine residues in the protein with other amino acid residues. This lysine-deficient protein was then site-specifically mono-PEGylated at its N-terminus. For instance, site-specific PEGylation of TNF-α [13,14] and human basic fibroblast growth factor [15] has been reported. Nevertheless, the slow rate of free drug release from the conjugate strongly influenced the *in vivo* antitumor activity of these PEGylated proteins. However, it is logical to hypothesize that i.v. administration of a TNF-α contained system in which the PEG and TNF-α are linked by an oligopeptide that is the substrate of a tumor tissue-specific protease may lead to high anticancer activity, even at low doses, because of the combined effect of extended circulation time and tumor site-specific drug release.

Cathepsin B is a ubiquitous cysteine protease whose properties differ very little between species [16,17]. It is never found in extracellular areas, except in pathological conditions such as tumors or in areas of tissue destruction in rheumatoid arthritis [18]. Therefore, the cathepsin B-sensitive peptide is likely to be stable in circulation. The physiological substrates of cathepsin B and L include valine-citrulline and phenylalanine-arginine [19]. Previous reports have demonstrated the success of conjugating a cytotoxic agent to a tumor-selective monoclonal antibody (mAb) using a protease-sensitive dipeptide linker. These antibody-drug conjugates include cBR96-valine-citrulline-MMAE and BR96-valine-citrulline-DOX (specific to Lewis Y antigen on carcinomas), cAC10-valine-citrulline-MMAE (specific to CD30 on hematological malignancies), and huPr1-valine-citrulline-MMAE (specific to hormone-resistant prostate cancer), and have high stability in plasma and efficient release of drug in tumor tissues [20–25].

In the present study, we have designed a novel PEGylated protein using a cathepsin B-cleavable dipeptide linker, valine-citrulline, to link the PEG and TNF-α, to overcome the short half-life and poor tissue selectivity of unconjugated TNF-α and the low therapeutic efficacy of standard PEGylated TNF-α (PEG-TNF-α). This validation study of this novel antitumor prodrug, PEG-valine-citrulline-TNF-α (PEG-vcTNF-α), includes assessment of the specific bioactivities, stability, sensitivity to cathepsin B, *in vitro* cytotoxicity and apoptosis, pharmacokinetics, and antitumor activity, compared with the standard PEGylated TNF-α or unconjugated TNF-α, in the hope that we can prove the hypothesis mentioned above.

1 Materials and methods

1.1 Materials

Recombinant human tumor necrosis factor-α (rhTNF-α, 17000 kD, 1×10^7 IU mg^{-1} TNF-α in 0.2 mol L^{-1} phosphate buffer, pH 7.2) was obtained from Biosynthesis Biotechnology Co., Ltd. (Beijing, China); TNF-α ELISA Detection Kit was from Senxiong Technical Industry Co, Ltd. (Shanghai, China); 40 kD branched PEG-NHS (Polydispersity, 1.05) and 40 kD branched PEG-val-cit-NHS (Polydispersity, 1.05) were supplied from Jenkem Technology Co., Ltd. (Beijing, China); Cathepsin B from human liver (2759 units mg^{-1} protein) was purchased from Sigma Corporation; L929 murine fibrosarcoma cells lines were from Life Technologies (Gaithersburg, MD) and were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum. Annexin V-FITC Apoptosis Detection Kits and FITC were obtained from Biosea Biotechnology Co., Ltd. (Beijing, China) and Cell Counting Kits (CCK-8) were obtained from Dojindo Laboratories (Kumamoto, Japan).

1.2 Preparation and characterization of TNF-α conjugates

Branched 40 kD PEG-NHS or PEG-vc-NHS moiety was added at room temperature and under rapid stirring in 0.1 mol L^{-1} phosphate buffer, pH 8.5. For these experiments, recombinant human TNF-α was reacted with a 30-fold molar excess of activated branched PEG moiety. The conjugation reaction was carried out for 30 min, while the pH was maintained with 0.05 mol L^{-1} NaOH. Subsequently, 6-ami-

nocaproic acid (5-fold molar excess against activated branched PEG moiety) was added to stop the reaction and the pH was adjusted to 6.0 with 0.1 mol L^{-1} HCl. The synthesized PEG-TNF-α or PEG-vcTNF-α conjugates were purified from the unreacted polymer and the side reaction products by gel filtration chromatography using a Sephacryl S-200HR column on a FPLC system eluted with 0.1 mol L^{-1} phosphate buffer/0.3 mol L^{-1} NaCl, pH 7.0. The molecular size of PEG-TNF-α or PEG-vcTNF-α conjugates was estimated by analytical gel filtration chromatography using a Sephacryl S-300HR column, eluted with 10 mol L^{-1} phosphate buffer/0.3 mol L^{-1} NaCl, pH 7.0. and the degree of modification by PEG attachment to TNF-α was assayed by fluorescamine methods as described by Stocks *et al*. [26]. The *in vitro* specific bioactivity of PEG-TNF-α or PEG-vcTNF-α conjugates was measured by L-M cytotoxicity assay, according to the method described by Yamazaki *et al*. [27].

1.3 Stability of TNF-α conjugates in plasma

To evaluate stability of PEG-TNF-α and PEG-vcTNF-α in plasma, amounts of PEG-TNF-α or PEG-vcTNF-α conjugates were labeled with FITC yielding PEG-TNF-α-FITC or PEG-vcTNF-α-FITC conjugates [28]. Conjugates containing 17 μg of protein in each sample were incubated in 1 mL of freshly drawn mouse or human plasma pre-incubated at 37°C. Aliquots of 100 μL were sampled periodically. All samples were lyophilized immediately, then dissolved in 1 mL chloroform, vortexed for 10 min, and centrifuged at 5000×*g* for 5 min. The upper layer was collected and chloroform was removed by lyophilization. The resulting extracted PEG mixture was renewably dissolved in 1 mL phosphate-buffered saline (PBS) and analyzed by a fluorescence spectrophotometer (λ_{ex}=492 nm, λ_{em}=519 nm). This result was then converted into the TNF-α concentration (validation data of assay not shown).

1.4 Purification of lysosomes

Sprague-Dawley rats (200–300 g) were sacrificed to harvest and purify their liver lysosomes. Lysosomes were collected from liver homogenate by centrifugation using the metrizamide discontinuous gradient method with a slight modification [29]. Briefly, non-fasted rats were anesthetized with ether and blooded. The liver was removed and homogenized using a Polytron mixer (Kinematica, Switzerland). The homogenate (4 mL of chilled 0.25 mol L^{-1} sucrose/g of liver) was centrifuged in succession at 4800×*g* for 5 min, and 17000×*g* for 10 min. The sediment of the second centrifugation was washed at 17000×*g* for 10 min, resuspended in 57% metrizamide, and 10 mL thereof was placed on the bottom of an Ultraclear tube (Beckman, CA). On top, a discontinuous gradient of metrizamide was constructed (layers from bottom to top were: 57%, 32.8%, 26.3%, and 19.8% metrizamide). Centrifugation was for 1 h in a 70.1 Ti rotor (Beckman) at 141000×*g*. Lysosomes were collected from the top layer and from the 26.3/19.8 interface. Using this procedure, the degree of purification of lysosomes relative to the original homogenate (as measured by acid phosphatase activity) was 20–30-fold and the yield 3%–5%. The protein concentrations in lysosomal pellets were determined by the BCA assay (Pierce, Rockford, IL). Acid phosphatase activity was determined using β-glycerophosphate or ρ-nitrowith phenyl phosphate as the substrate. The activities of cathepsin B and lysosomes were determined by using Z-Arg-Arg-AMC as the substrate.

1.5 *In vitro* evaluation of drug release

All release studies were carried out at 37°C. The concentrations of conjugates labeled with FITC in the incubation mixture were approximately 20 μg mL^{-1}, corresponding to around 17 μg mL^{-1} of TNF-α. Four *in vitro* testing protocols were performed. First, the hydrolysis stability of TNF-α conjugates to the liver lysosomes or homogenates was assessed. TNF-α conjugates were incubated in 50 mmol L^{-1} sodium acetate (pH 5.0), 2 mmol L^{-1} dithiothreitol (DTT), and 25% (v/v) glycerol for 15 min at 37°C and then added as a 20% (v/v) addition to initiate each reaction [26]. The final concentrations of lysosomes and homogenates were 1 mg of protein mL^{-1} and 20% w/w, respectively. Second, we studied the hydrolysis stability of TNF-α conjugates in the presence of various types of pure enzymes at concentrations of approximately 8 μmol L^{-1}. This panel of enzymes included include serine proteases (trypsin and chymotrypsin), cysteine proteases (papain and cathepsin B), aspartic acid proteases (cathepsin D and pepsin A), and metalloproteases (thermolysin and collagenase). Also, to examine the effect of pH on drug release, acetate or phosphate buffers (40 mmol L^{-1}), with a pH range from 3 to 7, were used. In the case of serine or metalloproteases, a final concentration of 10 mmol L^{-1} $CaCl_2$ was added, while in the case of cysteine proteases, final concentrations of 1 mmol L^{-1} EDTA and 5 mmol L^{-1} reduced glutathione were added. Third, the effect of several enzyme inhibitors was evaluated. They include aprotinin, bestatin, pepstatin, phosphoramidon, E-64, Z-Phe-Phe-CHN_2, and CA-074. Conjugate-lysosome mixtures were as described above, but contained a final inhibitor concentration of 10 μmol L^{-1}. Aliquots of 100 μL were sampled periodically, and all samples were treated with the same methods described above (in section "Stability of TNF-α conjugates in plasma").

1.6 *In vitro* cytotoxicity

Cells (2×10^4 cells $well^{-1}$ in 100 μL medium) were placed into wells of a 96-well microtiter plate. After 24 h incuba-

tion, TNF-α, PEG-TNF-α, PEG-vcTNF-α, cathepsin B (final concentration, 10 units mL^{-1}), CA-074 (final concentration, 10 μmol L^{-1}) or PBS was added to the wells according to experiment design. The volume was adjusted by culture medium to 200 μL, and the incubation was carried out. Cell viability assessment was performed by using Cell Counting Kits (CCK-8), following the manufacturer's instructions. Cytotoxicity (C%) was calculated from the equation $C=(A-B)/A\times100\%$, where A is the absorption in a control well and B is the absorption in the test well. L929 cells were placed in wells (1×10^6 cells $well^{-1}$) and incubated for 24 h prior to adding TNF-α (10 IU mL^{-1}), PEG-TNF-α or PEG-vcTNF-α. In the latter two conditions (PEG-TNF-α or PEG-vcTNF-α), 10 IU mL^{-1} TNF-α was also included. Cells were incubated for a further 12 h. The culture medium was discarded and replaced with 100 μL of cold binding buffer. Cell staining was performed using Annexin V-FITC according to the instructions of the manufacturer. Samples were analyzed using a FACS Canto (BD, USA) flow cytometer.

1.7 *In vivo* study on antitumor effects

All of the animal experiments adhered to the principles of care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee of Peking University. The antitumor effects of TNF-α were assessed in mice bearing Meth-A fibrosarcoma. The Meth-A cells were implanted intradermally (2×10^5 cells per site) in 5-week-old female BALB/c mice. Seven days later (when tumor diameter was ~7–8 mm), native TNF-α, PEG-TNF-α, and PEG-vcTNF-α were administered by a single intravenous injection. The antitumor potency was estimated from the tumor volume and tumor hemorrhagic necrosis 24 h after the injection. Antitumor effects of TNF-α *in vivo* were screened by determining mean tumor volume (V) as calculated using the formula: $V=(\text{length}\times(\text{width})^2)/2$.

1.8 Pharmacokinetics studies in mice

Native TNF-α, PEG-TNF-α, and PEG-vcTNF-α were radiolabeled with ^{125}I by the lactoperoxidase method, yielding ^{125}I-TNF-α, ^{125}I-PEG-TNF-α, and ^{125}I-PEG-vcTNF-α with specific activities of 4.50 mCi mg^{-1} protein. The biological activities of ^{125}I-radiolabeled TNF-α and its derivatives were indistinguishable from those of non-radiolabeled TNF-α and its derivatives (data not shown). Their pharmacokinetic profiles in blood circulation after i.v. injection into normal male BALB/c mice (5 weeks of age) were studied at a dose of 31.6 ng of protein per mouse. Blood was collected from the tail vein at various time points, and radioactivity was measured in each sample.

1.9 Statistical analysis

All data are shown as mean±SD, unless specified otherwise. Statistical evaluation was performed with Student's unpaired *t*-test and one-way analysis of variance.

2 Results

2.1 Preparation of conjugates

Recombinant human TNF-α was conjugated with activated PEG (average molecular weight (MW) 40000; MW/average MW, 1.05) via amide bonds between amino groups of TNF-α and *N*-hydroxysuccinimide groups of PEG at the end of the main chain. The rate of PEG attachment to TNF-α increased with increasing reaction 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, degree of PEG modification, and the activities of native TNF-α, PEG-TNF-α and PEG-vcTNF-α. It was demonstrated that activity decreased with

Table 1 Comparison of molecular properties of TNF-α, PEG-TNF-α and PEG-vcTNF-α (*n*=5, mean±SD)

	Fraction no.	Average molecular weight[a)]	Degree of modification (%)[b)]	Specific activity ($\times10^5$ IU mg^{-1} TNF-α)[c)]	Remaining activity (%)	Yield (%)
PEG-TNF-α	1	137000	40.46	6.23±0.56**#	6.2	31.04
	2	99000	27.95	18.38±0.87**#	9.4	24.55
	3	58000	8.21	27.40±0.39**#	18.6	9.38
PEG-vcTNF-α	1	138000	38.34	28.74±0.72**	28.7	30.67
	2	101000	27.48	46.66±0.43*	46.7	23.73
	3	58000	9.03	74.19±0.76*	74.2	8.38
TNF-α		17000	0	100.0±0.21	100.0	

a) The molecular size was determined by gel filtration chromatography (protein standard). b) Degree of modification was calculated by fluorescamine methods. c) The specific activities of native TNF-α, PEG-TNF-α and PEG-vcTNF-α were measured by growth inhibition L-M cytotoxic assay. *, $P<0.05$; **, $P<0.01$ vs. native TNF-α. #<0.05 vs. PEG-vcTNF-α under the same degree of modification.

increasing molecular weight and extensive PEG modification. The specific activity of PEG-vcTNF-α was noticeably higher than that of PEG-TNF-α with the same degree of PEG modification. Fraction 2 was used for further experiments because it combined a moderate degree of modification with relatively high specific activity.

2.2 Stability of TNF-α conjugates in plasma

To further evaluate the stability of the PEG-TNF-α or PEG-vcTNF-α, conjugates (containing 2 μg TNF-α) were incubated in human or mouse plasma at 37°C for a period of 120 h. Endogenous TNF-α was detected in freshly drawn mouse or human plasma but only in trace amounts (data no shown). The stability characteristics of PEG conjugates in human or mouse plasma at 37°C are shown in Figure 1, and it can be seen that the two TNF-α conjugates were very stable in plasma. The half-life ($t_{1/2}$) of PEG-vcTNF-α *in vitro* is 129.63 h in human plasma and 48.73 h in mouse plasma, respectively, while that of PEG-TNF-α is 170.04 h in human plasma and 60.18 h in mouse plasma, respectively. Although the $t_{1/2}$ of PEG-TNF-α is longer than that of PEG-vcTNF-α, there is no significant difference between these two formulations. We also found that release of drugs from the two TNF-α conjugates was faster in human plasma than in mouse plasma.

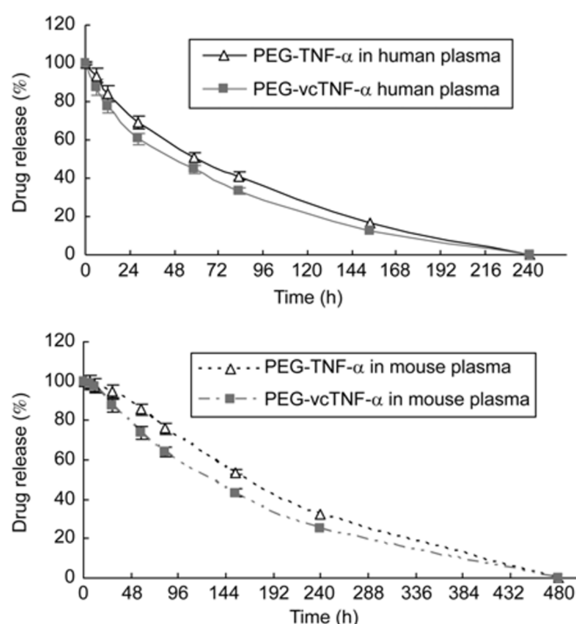

Figure 1 Plasma stability (at 37°C) of conjugated TNF-α constructs (PEG-TNF-α and PEG-vcTNF-α). PEG-TNF-α and PEG-vcTNF-α conjugates labeled with FITC and containing 17 μg of protein in each sample were incubated in 1 mL of freshly drawn mouse or human plasma pre-incubated at 37°C. Aliquots of 100 μL were sampled at various time points. PEG or PEG conjugates were extracted with chloroform. Fluorescence intensity was analyzed by a fluorescent spectrophotometer (n=4).

2.3 *In vitro* evaluation of drug release

Our focus was on the enzyme specificity of the linker cleavage. First, the excellent stability of TNF-α conjugates in human plasma as well as mouse plasma led us to the hypothesis that lysosomal enzymes are an effective means of TNF-α release. To test this hypothesis, we compared the kinetics of release when TNF-α conjugates were incubated with rat liver homogenates and with lysosomes, prepared using a well-established method for lysosome purification from rat liver. Results showed that the release rate of PEG-vcTNF-α in the liver homogenate was very slow, but was markedly accelerated in the presence of lysosomes, supporting our hypothesis. The 20% w/w homogenate, corresponding to approximately 40 mg mL^{-1} of protein, was virtually unable to release TNF-α, whereas the lysosomes at 1 mg mL^{-1} of protein liberated the drug significantly. Second, to elucidate the enzymes responsible, we measured TNF-α released after 24 h treatment of TNF-α conjugates with different types of pure enzymes. The results are shown in Table 2, indicating that TNF-α conjugates were very resistant to various kinds of enzymes. However, it was found that cysteine protease was the only enzyme effective in cleaving PEG-vcTNF-α, although the release rate varied between papain and bovine spleen cathepsin B (which was also sensitive to pH). PEG-TNF-α was not cleaved at all by any of the enzyme classes (including cysteine protease). Third, to further explore the optimum pH conditions for drug release, PEG-vcTNF-α was incubated with rat liver lysosomes in buffers covering a pH range from neutral (pH 7) to acidic (pH 3) for 24 h. Figure 2 shows that the pH optimum was approximately 4, identical to that in the case of cathepsin B (Table 2), suggesting that this may be the crucial enzyme in mediating drug release from PEG-vcTNF-α.

2.4 *In vitro* inhibition studies

To consolidate these results, we investigated the effect of protease inhibitors. Figure 3 compares the percentages of TNF-α released due to lysosomes in the presence of an inhibitor relative to the control. Inhibitors of amino protease (bestatin), asparatate protease (pepstatin), and metalloprotease (phosphoramidon) were all ineffective at 10 μmol L^{-1}. A general inhibitor for cysteine protease (E-64) abolished the release completely, although serine protease inhibitor (aprotinin) also arrested the release to some extent, probably because of its high concentration. This confirms that cysteine proteases are largely responsible for the cleavage of the valine-citrulline linker. Cathepsin B and L are lysosomal cysteine proteases, which play a variety of digestive and processing roles to maintain the normal cellular metabolism [29]. To determine their contributions to the cleavage, similar studies were performed using a specific inhibitor for cathepsin B, CA-074 [30], and for cathepsin L, Z-Phe-Phe-

Table 2 Effects of enzyme specificity and pH on drug release after 24 h incubation of TNF-α conjugates at 37°C

	pH	Serine proteases		Cysteine proteases		Aspartate proteases		Metalloproteases	
		Trypsin	Chymotrypsin	Papain	Cathepsin B	Cathepsin D	Pepsin A	Thermolysin	Collagenase
PEG-vcTNF-α	3	0	0	22.4	10.8	0	0	0	0
	4	0	0	27.0	79.6	0.4	0	0	0
	5	0	0	32.6	6.7	0	0.1	1.7	0
	6	0	0	27.3	1.5	0	0	1.4	0.2
	7	0	0	20.8	1.9	0	0	0	0
PEG-TNF-α	4	0	0	0	0	0	0	0	0

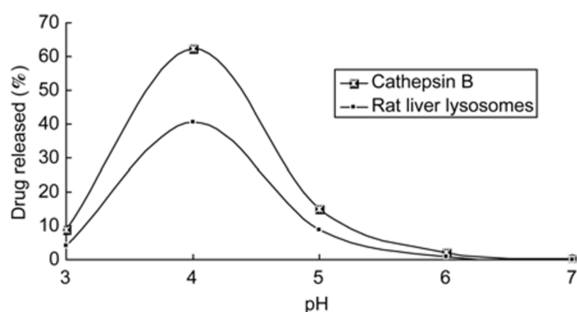

Figure 2 Optimum pH of enzyme activity for releasing TNF-α from PEG-vcTNF-α labeled with FITC after 24 h incubation with either 10 units mL^{-1} cathepsin B or 0.5 mg protein mL^{-1} of a rat liver lysosome preparation. Conditions: 40 mmol L^{-1} acetate buffer (pH 3–7), 1 mmol L^{-1} EDTA, 5 mmol L^{-1} reduced glutathione, 0.1% Triton X-100; 37°C.

CHN_2 [31]. As shown in Figure 3, both CA-074 and Z-Phe-Phe-CHN_2 were able to dramatically suppress TNF-α release even at 10 μmol L^{-1}. This suggests that lysosomal cathepsin B and cathepsin L are responsible for the linker cleavage.

2.5 *In vitro* cytotoxicity

As shown in Figure 4, cytotoxicity was reduced in the PEGylated TNF-α constructs, although PEG-vcTNF-α had slightly more toxicity than PEG-TNF-α. The IC_{50} values for cytotoxicity of and PEG-TNF-α were (5246±85) and (22310±295) IU mL^{-1}, respectively, and both were considerably less cytotoxic than native TNF-α (IC_{50}: (122.45±3.84) IU mL^{-1}) (Figure 4A). After pulsed (2 h) and long-term (24 h) drug exposure, it was shown that cytotoxic effect of PEG-vcTNF-α increased with the length of exposure time, and the growth inhibition rate of long-term PEG-vcTNF-α exposure was higher than that of pulsed term PEG-vcTNF-α exposure, and almost as high (at high concentrations) as that induced by 2 h treatment with TNF-α (Figure 4B). Cytotoxic effects of PEG-vcTNF-α decreased with CA-074 (10 μmol L^{-1}) and increased with cathepsin B (10 units mL^{-1}), compared with the same dose of PEG-vcTNF-α alone. Furthermore, the cytotoxic effects of PEG-vcTNF-α with 10

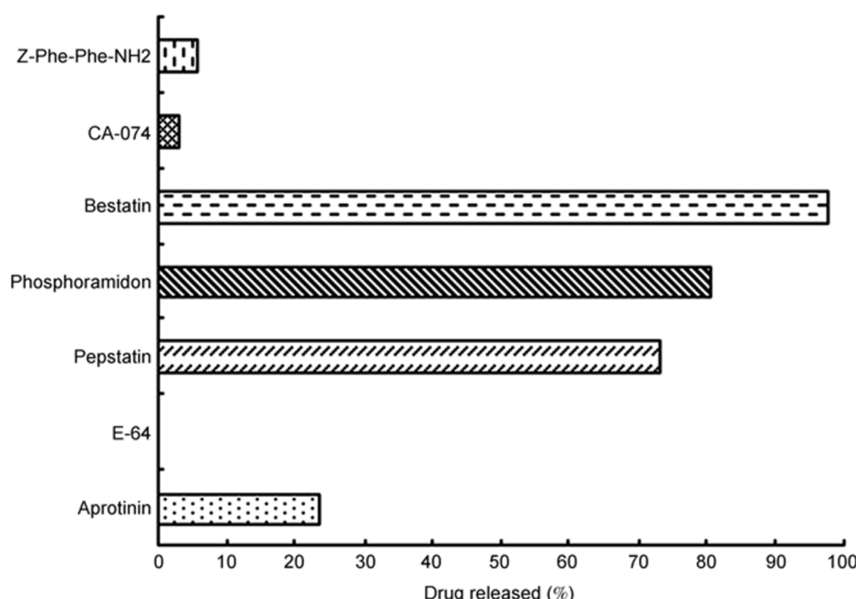

Figure 3 Effect of various protease inhibitors on TNF-α release by incubation with rat liver lysosome extracts. TNF-α release for 24 h is expressed as percentage relative to the control. TNF-α conjugates labeled with FITC, rat liver lysosome, and inhibitor concentrations were approximately 20 μg mL^{-1}, 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.

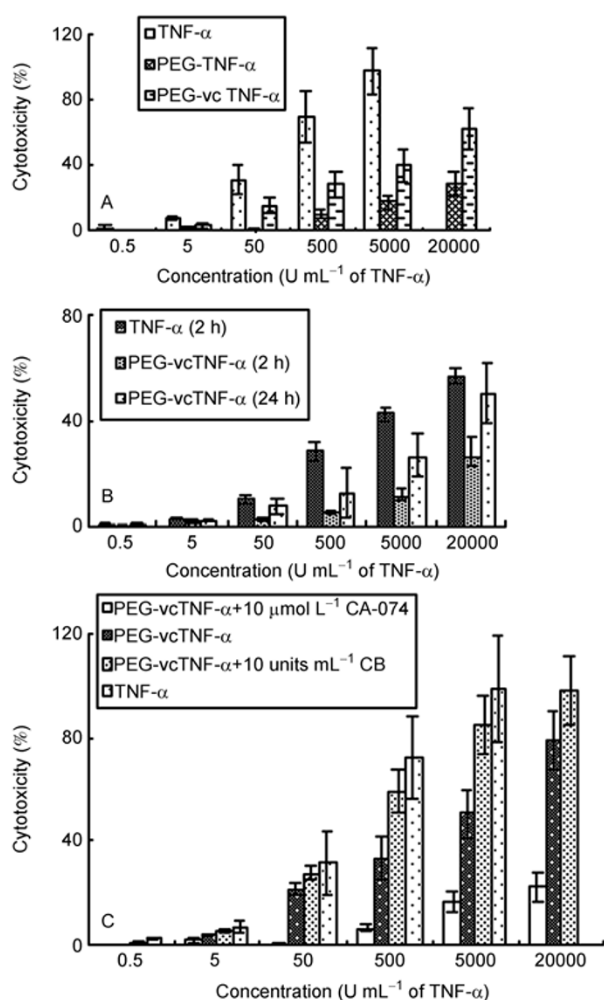

Figure 4 *In vitro* cytotoxicity of PEG-vcTNF-α and its effect on L929 cell line. CCK-8 assay was used to measure cytotoxicity. Effects of TNF-α, PEG-TNF-α or PEG-vcTNF-α were determined after L929 cells were incubated for 48 h (A). Shorter incubations (2 h and 24 h) were also tested (B). The effects of cathepsin B (10 units mL^{-1}) and CA-074 (10 μmol L^{-1}) on PEG-vcTNF-α cytotoxicity after a 48 h incubation were also examined (C). Cytotoxicity (%) was calculated relative to control culture incubated without TNF-α, PEG-TNF-α or PEG-vcTNF-α. *n*=4.

units mL^{-1} cathepsin B were almost identical to that induced by the same dose of native TNF-α (Figure 4C), suggesting that under these conditions, the entirety of the conjugated drug is being released to act in the same way as native TNF-α. To characterize the killing mechanism of L929 cells induced by TNF-α, PEG-TNF-α and PEG-vcTNF-α, we compared the content of viable, necrotic and apoptotic cells in cell cultures treated for 12 h with these formulations. As seen in Figure 5A, approximately 83% of L929 cells in control cultures were viable (bottom left quadrant). Incubation with TNF-α resulted in a large increase in apoptotic cell number (Figure 5B), which was less apparent after incubation with PEG-TNF-α (Figure 5C) and PEG-vcTNF-α (Figure 5D). The number of apoptotic cells decreased when cells were cultured with PEG-vcTNF-α in the presence of CA-074 (Figure 5E), and increased following combined treatment with PEG-vcTNF-α and cathepsin B (Figure 5F). Neither cathepsin B nor CA-074 alone resulted in cell necrosis or apoptosis (data not shown).

2.6 *In vivo* study of antitumor effects

We compared the antitumor potency of PEG-vcTNF-α to those of native TNF-α and PEG-TNF-α with scheduled i.v. injections on Meth-A solid tumors. Control mice (saline or PEG alone) showed no antitumor effect (data not shown), whereas native TNF-α showed suppression of tumor growth (Figure 6). However, three of eight mice administered native TNF-α at a dose of 10000 IU died within 24 h, and the remaining mice developed piloerection, tissue inflammation and a loss in body weight (data not shown). Whereas the minimal dose of native TNF-α (200 IU) was not effective for tumor growth inhibition. PEG-vcTNF-α and PEG-TNF-α had markedly increased antitumor potencies compared with native TNF-α. As shown in Figure 6, PEG-TNF-α at a dose of 2000 IU showed the maximal antitumor effects without any toxic side effects and had antitumor effects superior to that of native TNF-α at a dose of 10000 IU. On the other hand, only 200 IU of PEG-vcTNF-α was needed to exhibit a marked antitumor potency, and tumor growth was completely inhibited during the observation period. This reversal of tumor growth was only seen at 2000 IU of PEG-TNF-α and at 10000 IU of native TNF-α, indicating that PEG-vcTNF-α was a more potent antitumor agent by approximately 10- and 50-fold, respectively, compared to unlinked PEG-TNF-α and native TNF-α.

2.7 Pharmacokinetics studies in mice

The plasma concentrations of TNF-α after i.v. administration of native TNF-α, PEG-TNF-α or PEG-vcTNF-α are shown in Figure 7. Native TNF-α was rapidly eliminated from the circulation, and its plasma half-life was only 0.31 h (Table 3) which corresponds to that reported previously [32]. In contrast, the plasma clearance of both PEG-TNF-α and PEG-vcTNF-α was markedly decreased relative to that of native TNF-α and their plasma half-lives were 27.23 and 21.22 h, respectively, approximately 85- and 65-fold longer than that of native TNF-α.

3 Discussion

The peptide-linked PEGylated protein reported here is distinguished from the general PEGylated proteins described elsewhere in three important aspects: stability in plasma, sensitivity to cathepsin B, and specific activity. Generally,

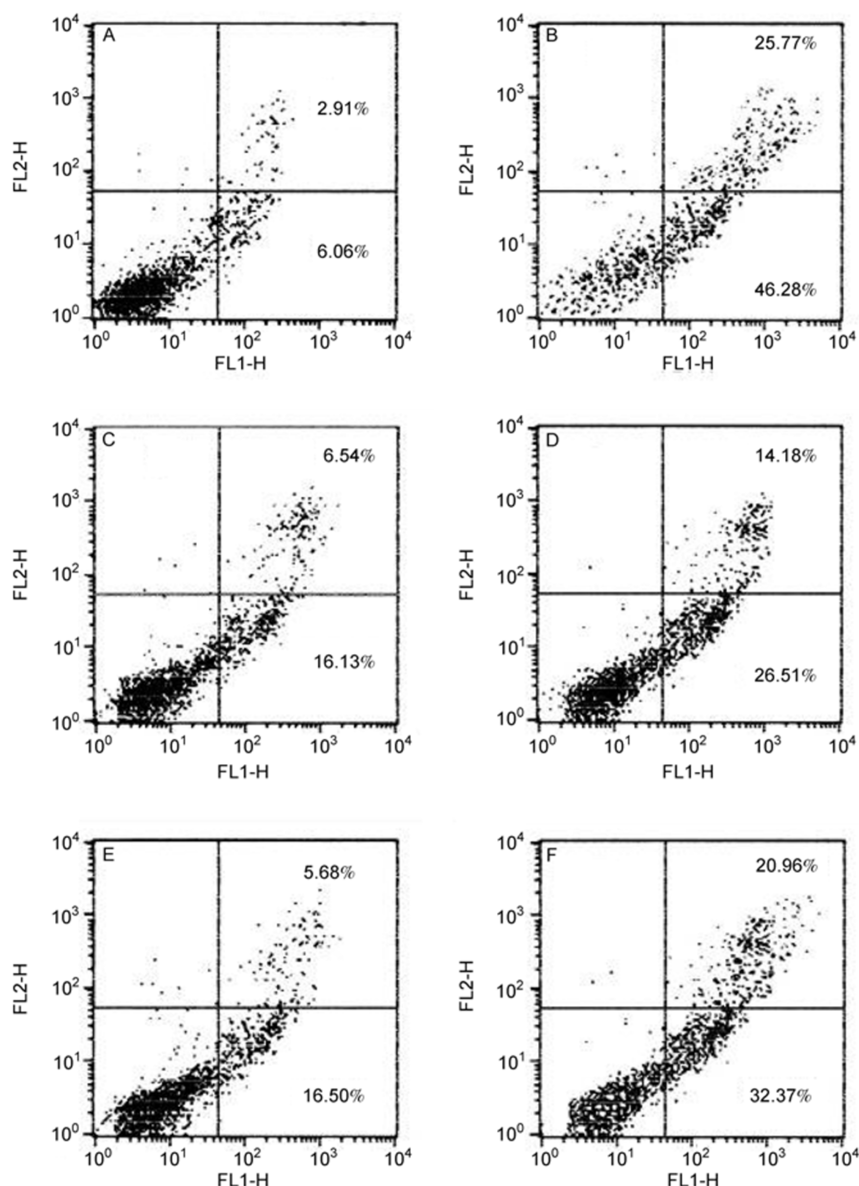

Figure 5 The effect of PEG conjugates on L929 cell apoptosis. L929 cells (1×10^6 cells well^{-1}) were plated in multiwell plates. After 24 h, buffer (A), TNF-α (10 IU mL^{-1}) (B), PEG-TNF-α (C), PEG-vcTNF-α (D), PEG-vcTNF-α with CA-074 (10 μmol L^{-1}) (e) or PEG-vcTNF-α with cathepsin B (10 units mL^{-1}) (F) was added. Cells were incubated for a further 12 h. Culture medium was then replaced with 100 μL cold binding buffer. Cell staining was performed using Annexin V-FITC according to the manufacturer's instructions. Samples were analyzed using a FACSCanto (BD, USA) flow cytometer.

PEGylation of a protein drug enhances its plasma half-life and *in vivo* stability because of its increased molecular size and steric hindrance, but these improvements are accompanied by a significant loss of their specific activity. In this paper, we describe the use of a cathepsin B-cleavable dipeptide linker in novel PEGylated conjugates to overcome the contradiction between long plasma half-life but low therapeutic efficacy for PEGylated proteins and short half-life/poor tissue selectivity for native TNF-α.

PEG polymer is nontoxic and is approved by the FDA for use in drugs, so is used widely to modify therapeutic proteins. Literally thousands of research articles have described almost every aspect of attachment of PEG to a therapeutic protein. In this work, an N-hydroxysuccinimide derivative of the activated polymer (PEG-NHS or PEG-vc-NHS) was used to attach PEG to TNF-α. The preparation process of conjugates was according to previously described methods. For PEG-vcTNF-α, two amino acids were added to the N terminal of TNF-α prior to attachment to PEG through the N-hydroxysuccinimide activated ester. We showed that PEG modification had negative effects on the bioactivity of TNF-α, which caused reduction in specific activity to between 6.23% to 74.19% depending on the degree of modification. For general PEGylation, the remaining

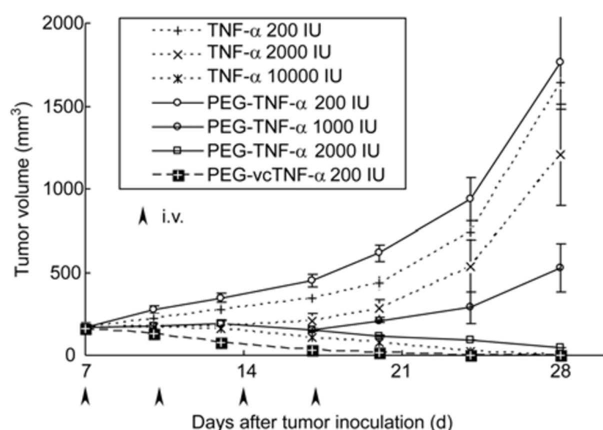

Figure 6 Antitumor effect on Meth-A solid tumors of native TNF-α, PEG-TNF-α and PEG-vcTNF-α (at the concentrations shown, per mouse per injection) administered by i.v. injection (at time points indicated by arrows). PEG and saline were used as controls. n=6.

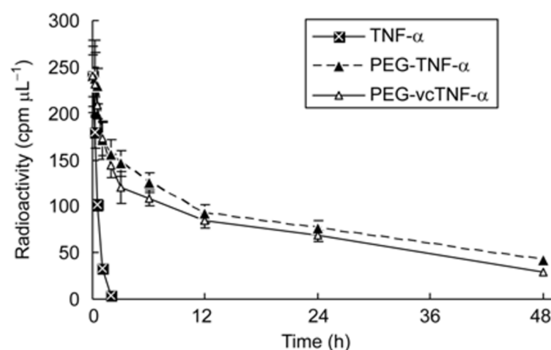

Figure 7 Changes in TNF-α concentration in serum after administration of native TNF-α, PEG-TNF-α and PEG-vcTNF-α. After i.v. administration of [^{125}I]-native TNF-α or [^{125}I]-bioconjugated TNF-α to normal male BALB/c mice, blood was collected from tail vein at various time points, and radioactivity was measured. Each bar represents the mean±SD (n=6).

Table 3 Pharmacokinetic parameters of TNF-α in plasma after i.v. administration of different formulations to rats. Data are mean±SD, n=6

Formulation	$t_{1/2}$ (h)	AUC (0-INF) (cpm μL^{-1}) h	MRT (h)
TNF-α	0.31±0.03	133±17	0.44±0.04
PEG-TNF-α	27.23±5.72	3472±898	39.29±8.25
PEG-vcTNF-α	21.22±3.1	3554±549	30.62±4.47

bioactivity of TNF-α decreased as modification degree increased, because of the loss of binding site(s) and/or steric hindrance. This finding suggested that PEG chains sterically hindered the TNF-receptor binding site for PEG-TNF-α, consistent with many literature reports. However, when a valine-citrulline moiety was added between PEG and TNF-α, we found, unexpectedly, that the loss of bioactivity was much less than without the valine-citrulline linker, indicating that this moiety plays an important role in protecting TNF-α bioactivity.

We hypothesized that TNF-α could be liberated from their conjugates by incubation with proteases. However, released TNF-α will theoretically be degraded by components of the plasma or by the proteases used in these experiments. This prediction is supported by the absence TNF-α in our test conditions (data not shown). Evaluation of PEG conjugate stability in samples taken from plasma or other solutions presents a challenge for drug quantitation, because TNF-α is easily degraded by unspecific proteases in plasma or by lysosomal proteases such as cathepsin B. When TNF-α is released from PEG conjugates into plasma, it is cleared very quickly, so the free drug was undetectable (data not shown). Accordingly, it is very difficult to directly quantify released TNF-α in the presence of plasma or proteases. In this study, PEG is used as an amphichroic molecule, such that when PEG attaches to a protein, that protein becomes similarly amphichroic. Making use of the amphichroic features of PEG or PEG conjugates, TNF-α conjugates labeled with FITC or PEG in plasma samples were extracted with chloroform [26]. Fluorescence intensity was then analyzed with a fluorescence spectrophotometer. TNF-α concentration (including conjugates) can be validated by to this process, which was highly reproducible (data not shown).

As shown in Figure 1, significant drug loss occurs in plasma, possibly because of the presence of unspecific proteases in mouse and human plasma [33]. However, the stability of TNF-α in both PEG conjugates in plasma was similar, indicating that the degradation mostly results from the direct effect of these proteases on the TNF-α rather than on the cathepsin B-sensitive peptide linker (valine-citrulline). The cathepsin B-sensitive peptide linker therefore seems resistant to protease actions while in the circulation. It was found that the half-life of drug loss for PEG-vcTNF-α is more than five days when incubated in human plasma, confirming that the dipeptide linkers used in PEG-vcTNF-α offer a significant stability advantage in human plasma, which is crucial for the *in vivo* efficacy of the conjugate. Drug loss from both conjugates was faster when incubated in human plasma, suggesting that more protease activity is present in human plasma than in mouse plasma. This finding was consistent with the report that human plasma has much higher levels of protease activity than mouse plasma [33].

Knowledge of enzyme specificity permits the design of drug-polymer conjugates that can be activated specifically at the tumor site. Cathepsin B is an attractive target for the release of drugs, because increases in the levels of mRNA, protein, enzyme activity, membrane association, and secretion of cathepsin B have all been observed in tumors. Therefore, cathepsin B-specific drug release observed in the case of PEGylated protein is thought to be advantageous from the viewpoint of presenting the cytotoxic agent selectively at the tumor site. We showed that PEG-vcTNF-α was cleaved by cysteine proteases, but not other proteases, to release TNF-α, and this release is dependent upon pH. Our finding that the valine-citrulline linker peptide is specifically targeted by cysteine proteases to release the peptide drug from the PEGylated conjugate is in agreement with previous reports [20–24].

Here the question arises of whether cathepsin B liberates TNF-α within the lysosomes or outside the cell. The answer is to be found in our observations that the release of TNF-α relies on pH, with the optimum condition being pH 4. With regard to pH, tumors were thought for many years to have a more acidic pH than most normal tissues. The major difference lies in the extracellular pH. In normal tissue extracellular pH is at about 7.4, whereas in many tumors it is about 0.3–0.5 pH units more acidic in comparison to intracellular pH (pH 7.1–7.2) [34]. This extracellular acidification in tumors is insufficient for the release of TNF-α, indicating that drug release occurs in the lysosomes.

To further prove that it is cathepsin B that acts on the peptide linker, the effect of several enzyme inhibitors was evaluated, including aprotinin, bestatin, pepstatin, phosphoramidon, E-64, Z-Phe-Phe-CHN_2, and CA-074. In Figure 3, E-64, CA-074 and Z-Phe-Phe-CHN_2 were all able to dramatically suppress the release. E-64 is a non-specific inhibitor of cysteine proteases, while CA-074 and Z-Phe-Phe-CHN_2 are specific inhibitors of cathepsin B and cathepsin L, respectively. This suggests that lysosomal cathepsins B and L are responsible for the cleavage and further support our earlier results.

The L929 cell was used in these tests because this cell line is particularly sensitive to TNF-α. It was proven again at the cellular level that PEGylation of proteins is accompanied by a significant loss of their activities. However, we show in Figures 4 and 5 that PEG-vcTNF-α induced higher cytotoxicity and more apoptosis in these cells than PEG-TNF-α, through the mechanism of cathepsin-specific release of TNF-α, as demonstrated by the enhancement and inhibition of this response by cathepsin B and the cathepsin B inhibitor, CA-074, respectively. Thus, the dipeptide linkers used appear to play an important role on the improvement of *in vivo* antitumor efficacy of PEG-vcTNF-α. Cytotoxicity of PEG-vcTNF-α was observed to be both dose- and time-dependent. Although very low, PEG-TNF-α showed some cytotoxicity, possibly because of incomplete modification by PEG.

The antitumor effect of PEG-vcTNF-α compared with PEG-TNF-α and native TNF-α was improved greatly, which might reflect their improved plasma stability, circulation time, tumor selectivity etc. The poor antitumor effect of native TNF-α indicates its fast elimination from the plasma, while the improved efficacy of PEG-vcTNF-α suggests that more drug is released in the tumor tissues. In comparison with native TNF-α, the antitumor effect of PEG conjugates increased significantly, about 50-fold for PEG-vcTNF-α and 5-fold for PEG-TNF-α, respectively. As shown in Figure 6, PEG has no toxicity when administered intravenously at this dose and has not shown antitumor effects in tumor-bearing mice. Thus, it is the PEGylation of TNF-α, not the PEG itself, which contributes to the improvements in pharmacodynamics. The shielding of proteolytic cleavage sites of TNF-α by the PEG chain and reduction of the renal clearance due to increased molecular size lead to the increase in circulation time and plasma stability for both PEG-TNF-α and PEG-vcTNF-α, as demonstrated in the our pharmacokinetic and *in vitro* stability studies. Additionally, it is well known that macromolecules are accumulated and retained in the tumor tissue much more than in normal tissues, a phenomenon termed the "enhanced permeability and retention (EPR)" effect [35,36]. Many macromolecular anticancer agents, such as synthetic polymer-conjugating drugs, polymeric micelle-containing drugs and others, have been reported accordingly [32,37]. The increased tumor growth inhibition seen here with the TNF-α conjugates might also result from the EPR effect of PEGylation.

However, with the same degree of PEG modification, PEG-vcTNF-α was approximately 10-fold more potent than PEG-TNF-α, suggesting that the linkage with the cathepsin B-sensitive valine-citrulline dipeptide as a cleavable moiety further promotes the anticancer activity of the PEGylated TNF-α against murine fibrosarcoma. Although the inserted dipeptide is important for the selective release of drugs from the conjugate in tumor tissue, it is necessary to recognize the contribution of PEGylation *per se* in conferring increases in circulation time, plasma stability, and the EPR effect. Thus, it is crucial to combine both PEG and a protease-sensitive linker to achieve better anticancer activity. We have demonstrated here the *in vivo* mechanism of action of PEG-vcTNF-α after administration by i.v. injection. PEG-vcTNF-α has a longer circulation time than TNF-α, and accumulates and is retained in the tumor tissue because of the EPR effect. Once sequestered in this way, PEG-vcTNF-α is cleaved and TNF-α is released from the dipeptide PEG conjugates through enzyme hydrolysis in the tumor tissue, where cathepsin B was expressed extracellularly. This leads to high local concentrations of TNF-α in the tumor tissue, which greatly enhances it anticancer efficacy. We believe that this technology is a major advance in the delivery of

protein anti-cancer agents to their intended site of action, and will hopefully lead to improved therapies in the fight against cancer.

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1 Carswell E A, Old L J, Kassel R L, *et al*. An endotoxin-induced serum factor that causes necrosis of tumors. Proc Natl Acad Sci USA, 1975, 72: 3666–3670
2 Debs R J, Fuchs H J, Philip R, *et al*. Immunomodulatory and toxic effects of free and liposome-encapsulated tumor necrosis factor alpha in rats. Cancer Res, 1990, 50: 375–380
3 Leist M, Jaattela M. Four deaths and a funeral: From caspases to alternative mechanisms. Nat Rev Mol Cell Biol, 2001, 2: 589–598
4 Blick M, Sherwin S A, Rosenblum M, *et al*. Phase I study of recombinant tumor necrosis factor in cancer patients. Cancer Res, 1987, 47: 2986–2989
5 Creaven P J, Plager J E, Dupere S, *et al*. Phase I clinical trial of recombinant human tumor necrosis factor. Cancer Chemoth Pharm, 1987, 20: 137–144
6 Kimura K, Taguchi T, Urushizaki I, *et al*. Phase I study of recombinant human tumor necrosis factor. Cancer Chemoth Pharm, 1987, 20: 223–229
7 Sherman M L, Spriggs D R, Arthur K A, *et al*. Recombinant human tumor necrosis factor administered as a five-day continuous infusion in cancer patients: phase I toxicity and effects on lipid metabolism. J Clin Oncol, 1988, 6: 344–350
8 Chapman P B, Lester T J, Casper E S, *et al*. Clinical pharmacology of recombinant human tumor necrosis factor in patients with advanced cancer. J Clin Oncol, 1987, 5: 1942–1951
9 Rosenberg S A, Lotze M T, Muul L M, *et al*. A progress report on the treatment of 157 patients with advanced cancer using lymphokine-activated killer cells and interleukin-2 or high-dose interleukin-2 alone. New Engl J Med, 1987, 316: 889–897
10 Caliceti P, Veronese F M. Pharmacokinetic and biodistribution properties of poly(ethylene glycol)-protein conjugates. Adv Drug Deliv Rev, 2003, 55: 1261–1277
11 Wang Y S, Youngster S, Grace M, *et al*. Structural and biological characterization of PEGylated recombinant interferon alpha-2b and its therapeutic implications. Adv Drug Deliv Rev, 2002, 54: 547–570
12 Kozlowski A, Harris J M. Improvements in protein PEGylation: PEGylated interferons for treatment of hepatitis C. J Control Release, 2001, 72: 217–224
13 Yoshioka Y, Tsutsumi Y, Ikemizu S, *et al*. Optimal site-specific PEGylation of mutant TNF-α improves its antitumor potency. Biochem Bioph Res Co, 2004, 315: 808–814
14 Yamamoto Y, Tsutsumi Y, Yoshioka Y, *et al*. Site-specific PEGylation of a lysine-deficient TNF-alpha with full bioactivity. Nat Biotech, 2003, 21: 546–552
15 Wu X, Li X, Zeng Y, *et al*. Site-directed PEGylation of human basic fibroblast growth factor. Protein Expres Purif, 2006, 48: 24–27
16 Otto H H, Schirmeister T. Cysteine proteases and their inhibitors. Chem Rev 1997, 97: 133–172
17 Devetzi M, Scorilas A, Tsiambas E, *et al*. Cathepsin B protein levels in endometrial cancer: Potential value as a tumour biomarker. Gynecol Oncol, 2009, 112: 531–536
18 Hashimoto Y, Kakegawa H, Narita Y, *et al*. Significance of cathepsin B accumulation in synovial fluid of rheumatoid arthritis. Biochem Bioph Res Co, 2001, 283: 334–339
19 Barrett A J, Kembhavi A A, Brown M A, *et al*. L-trans-Epoxysuccinyl-leucylamido (4-guanidino) butane (E-64) and its analogues as inhibitors of cysteine proteases including cathepsins B, H and L. Biochem J, 1982, 201: 189–198
20 Francisco J A, Cerveny C G, Meyer D L, *et al*. cAC10-vcMMAE, an anti-CD30-monomethyl auristatin E conjugate with potent and selective antitumor activity. Blood, 2003, 102: 1458–1465
21 Afar D E H, Bhaskar V, Ibsen E, *et al*. Preclinical validation of anti-TMEFF2-auristatin E-conjugated antibodies in the treatment of prostate cancer. Mol Cancer Ther, 2004, 3: 921–932
22 Dubowchik G M, Firestone R A, Padilla L, *et al*. Cathepsin B-labile dipeptide linkers for lysosomal release of doxorubicin from internalizing immunoconjugates: model studies of enzymatic drug release and antigen-specific *in vitro* anticancer activity. Bioconjug Chem, 2002, 13: 855–869
23 Sanderson R J, Hering M A, James S F, *et al*. *In vivo* drug-linker stability of an anti-CD30 dipeptide-linked auristatin immunoconjugate. Clin Cancer Res, 2005, 11: 843–852
24 Doronina S O, Toki B E, Torgov M Y, *et al*. Development of potent monoclonal antibody auristatin conjugates for cancer therapy. Nat Biotech, 2003, 21: 778–784
25 Ajaj K A, Biniossek M L, Kratz F. Development of protein-binding bifunctional linkers for a new generation of dual-acting prodrugs. Bioconjug Chem, 2009, 20: 390–396
26 Stocks S J, Jones A J M, Ramey C W, *et al*. A fluorometric assay of the degree of modification of protein primary amines with polyethylene glycol. Anal Biochem, 1986, 154: 232–234
27 Yamazaki S, Onishi E, Enami K, *et al*. Proposal of standardized methods and reference for assaying recombinant human tumor necrosis factor. Jpn J Med Sci Biol, 1986, 39: 105–118
28 Faure M P, Gaudreau P, Shaw I, *et al*. Synthesis of a biologically active fluorescent probe for labeling neurotensin receptors. J Biol Chem, 1994, 42: 755–763
29 Aniento F, Roche E, Cuervo A M, *et al*. Uptake and degradation of glyceraldehyde-3-phosphate dehydrogenase by rat liver lysosomes. J Biol Chem, 1993, 268: 10463–10470
30 Murata M, Miyashita S, Yokoo C, *et al*. Novel epoxysuccinyl peptides. Selective inhibitors of cathepsin B, *in vitro*. FEBS Lett, 1991, 280: 307–310
31 Barrett A J, Kirschke H. Cathepsin B, Cathepsin H, and Cathepsin L. Methods Enzymol, 1981, 80: 535–561
32 Tsutsumi Y, Kihira T, Tsunoda S, *et al*. Molecular design of hybrid tumor necrosis factor-alpha III: Polyethylene glycol-modified tumor necrosis factor-alpha has markedly enhanced antitumor potency due to longer plasma half-life and higher tumor accumulation. J Pharmacol Exp Ther, 1996, 278: 1006–1011
33 Satoh T, Hosokawa M. The mammalian carboxylesterases: From molecules to functions. Annu Rev Pharmacol Toxicol, 1998, 38: 257–288
34 Vaupel P, Kallinowski F, Okunieff P. Blood flow, oxygen consumption and tissue oxygenation of human tumors. Adv Exp Med Biol, 1990, 277: 895–905
35 Mahajan S S, Hou L, Doneanu C, *et al*. Optimization of bivalent glutathione S-transferase inhibitors by combinatorial linker design. J Am Chem Soc, 2006, 128, 8615–8625
36 Vasey P A, Kaye S B, Morrison R, *et al*. Phase I clinical and pharmacokinetic study of PK1 (HPMA copolymer doxorubicin): First member of a new class of chemotherapeutic agents-drug-polymer conjugates. Clin Cancer Res, 1999, 5: 83–94
37 Yokoyama M, Okano T, Sakurai Y, *et al*. Toxicity and antitumor activity against solid tumors of micelle-forming polymeric anticancer drug and its extremely long circulation in blood. Cancer Res, 1991, 51: 3229–3236

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