Stealth Polycyanoacrylate Nanoparticles as Tumor Necrosis Factor-α Carriers: 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] Jian-Jun ZHOU,[b] Xiu-Jian GAO,[a] and Jian-Hua ZHU[a]

Department of Pharmaceutics, School of Pharmacy, Fudan University,[a] 138 Yi Xue 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 4, 2000; accepted February 26, 2001

The objective of this study was to investigate the pharmacokinetics and *in vivo* anti-tumor effect of recombinant human tumor necrosis factor-α (rHuTNF-α) encapsulated in poly(methoxypolyethyleneglycol cyanoacrylate-co-*n*-hexadecyl cyanoacrylate) (PEG-PHDCA) nanoparticles. Our experimental results showed that PEG-PHDCA nanoparticles could extend the half-life of rHuTNF-α to 7.42 h and obviously change the protein biodistribution in tissues, and in particular, increase accumulation of rHuTNF-α in tumor. Compared with PHDCA nanoparticles and free rHuTNF-α, PEG-PHDCA nanoparticles loaded with rHuTNF-α showed higher anti-tumor potency at the same dose, which might be related to its higher accumulation in tumor tissues and longer plasma circulation time. Therefore, PEG-PHDCA nanoparticles could be an effective carrier for rHuTNF-α.

Key words poly(ethylene glycol); poly(alkyl cyanoacrylate); nanoparticle; stealth; poly(methoxypolyethyleneglycol cyanoacrylate-co-*n*-hexadecyl cyanoacrylate); tumor necrosis factor-α

Tumor necrosis factor-α (TNF-α) exhibits striking biological effects, such as direct cytotoxicity against various kinds of tumor cells, activation of immune anti-tumor response and inducement of hemorrhagic necrosis of certain transplanted solid tumors with selective impairment of tumor vascular endothelial cells.[1—3] However, excessively frequent and high doses of TNF-α are required for significant anti-tumor effects because of its short plasma half-life, and it was found to have severe toxic side effects in phase I-II studies.[4—6] These drawbacks make effective systemic treatment with free TNF-α difficult.

In order to overcome these problems, liposomes were tested as carriers of TNF-α.[7—11] Unfortunately, the conventional liposomes were rapidly recognized by cells of the mononuclear phagocyte system (MPS) and showed a short circulation time and a disappointing localization in treating non-reticuloendothelial system (RES) tumors. Recently, stealth liposomes of TNF-α were studied by several work groups.[12—16] The long circulating liposomes showed low toxicity and increased bioavailability, but rapid drug leakage and poor storage stability limits their human use.[17]

In past few years, there has been an increasing interest to develop stealth nanoparticles or long-circulating nanoparticles as drug carriers, whose striking advantages would be less uptake by MPS, extended half-life and a better role in controlled delivery of the pharmacological agent to its target.[18] The stealth nanoparticles could be prepared by modification of their surface with hydrophilic, flexible and non-ionic polymers, such as poly(ethylene glycol) (PEG).[19—21] More recently, a more rapidly biodegradable copolymer, poly(methoxypolyethyleneglycol cyanoacrylate-co-*n*-hexadecyl cyanoacrylate) (PEG-PHDCA), has been developed for the preparation of stealth nanoparticles,[22—25] but there have not been any reports on PEG-PHDCA nanoparticles as protein or peptide carriers, and PEG-PHDCA was synthesized using only a small molecular weight PEG (MW=2000), which might not be ideal in extending half-life.

The purpose of the present work was to further investigate and assess the merits of other PEG-PHDCA nanoparticles as recombinant human tumor necrosis factor-α (rHuTNF-α) carriers following our previous work.[26] The pharmacokinetics and *in vivo* anti-tumor effect of rHuTNF-α encapsulated in the stealth nanoparticles were investigated. The results of this study will provide fundamental information enabling us to design a useful delivery system with long circulating half-life and selective localization in the vascular space against tumors, so further enhancing the therapeutic activity and safety of rHuTNF-α.

MATERIALS AND METHODS

Materials Recombinant human tumor necrosis factor-α (rHuTNF-α, MW=17000) was kindly supplied by Shanghai Research Center of Biotechnology, Chinese Academy of Sciences (Shanghai, China). Cyanoacetic acid (purity >99%), poly(vinylalcohol) (PVA) (MW=16000, 98% hydrolyzed), monomethoxy polyethylene glycol (MePEG, MW=5000, purity >95%), human serum albumin (HSA) and carrier-free Na[^{125}I] were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Dichloromethane (DCM) of HPLC grade was used in this study. All other reagents and solvents were of analytical grade.

Poly(methoxypolyethyleneglycol cyanoacrylate-co-*n*-hexadecyl cyanoacrylate) (PEG-PHDCA) was synthesized by polymerization of MePEG (MW=5000) cyanoacetate and *n*-hexadecyl cyanoacetate, and purifed by GPC. MePEG cyanoacetate and *n*-hexadecyl cyanoacetate were obtained by esterification of the cyanoacetic acid and MePEG or *n*-hexadecanol. Details are given elsewhere.[27]

Animals Female Kunming strain mice (20±2 g) were supplied by the 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.

* To whom correspondence should be addressed. e-mail: ypli@mail.shcnc.ac.cn

Radioiodination of rHuTNF-α rHuTNF-α was labeled with ^{125}I using the IODO-GEN procedure. Briefly, 60 μg of protein in 50 μl of 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 mCi 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 Cobra II-Autogamma (Packard Instruments, CT, U.S.A.).

Preparation of Nanoparticles Nanoparticles loaded with rHuTNF-α were prepared by the two-step emulsification procedure as described elsewhere.[26] Briefly, the 0.5 ml solution of rHuTNF-α in 0.1 M PBS (pH 7.2, 1%, w/v) containing HSA (2%, w/v) was emulsified in 5 ml of DCM containing PEG-PHDCA copolymer (75 mg) or PHDCA polymer (75 mg) by homogenization at 10000 rpm in an ice bath for 3×15 s (Bailing, Model DS-200, China) to form a primary emulsion (w/o). Thereafter, the first emulsion was poured into 50 ml of PVA solution (0.4%, w/v) and homogenized at 10000 rpm in an ice bath for 3×15 s (Bailing, Model DS-200, China). The double emulsion (w/o/w) was diluted in 150 ml PVA solution (0.1%, w/v) and the organic solvent was rapidly eliminated by evaporation under reduced pressure. Finally, the nanoparticles were collected by centrifugation at 25000×***g*** for 20 min and washed three times with PBS. The physicochemical properties and encapsulation efficiency of nanoparticles were measured as described elsewhere.[26] Nanoparticles loaded with ^{125}I-iodinated rHuTNF-α were used for study of pharmacokinetics described as follows.

Pharmacokinetics of Nanoparticles Three groups of female Kunming strain mice (n=8) were used in this experiment, group 1 being treated with free rHuTNF-α, group 2 with PHDCA nanoparticles and group 3 with PEG-PHDCA nanoparticles. For administration, nanoparticles were suspended in a certain volume of PBS (PH 7.4) in order to obtain the required concentration. Simultaneously, a solution of free rHuTNF-α in the same vehicle and of the same concentration was prepared. Each animal was dosed intravenously with 0.5 μg protein with a trace of ^{125}I (1 μCi).

After intravenous administration, blood was collected at 0.25, 0.5, 1, 2, 4, 6, 8, 12, and 24 h from the tail vein and the radioactivity levels were measured. The pharmacokinetic parameters of free rHuTNF-α and nanoparticles were calculated using the Practical Pharmacokinetic Program–Version 87.

In order to study the tissue distribution, six groups of female Kunming strain mice with S-180 tumor nodules of 9—10 mm in diameter were used. The groups 1, 2 were treated with free rHuTNF-α, groups 3, 4 with PHDCA nanoparticles and groups 5, 6 with PEG-PHDCA nanoparticles. Each animal was dosed intravenously with 0.5 μg protein with a trace of ^{125}I (1 μCi). The mice were dehematized at the abdominal aorta at 1 and 6 h after intravenous injection. Tissues were collected and weighed and the radioactivities were measured.

***In Vivo* Anti-tumor Activity** Six 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. Simultaneously, rHuTNF-α and nanoparticles suspensions were given by intravenous injection once every two days for ten days. Group 1 was treated with saline, groups 2—4 with free rHuTNF-α at the doses of 0.1, 0.5, and 2.5 μg per mouse, respectively, and group 5 with PHDCA nanoparticles and group 6 with PEG-PHDCA nanoparticles at the dose of 0.5 μg per mouse, respectively. Anti-tumor effects against S-180 solid tumor were expressed according to mean tumor weight.

Statistical Analysis Statistical evaluation of tumor weight was analyzed by using Student's t-test.

Table 1. Physicochemical Characterization and Encapsulation Efficiency of rHuTNF-α Loaded in PHDCA and PEG-PHDCA Nanoparticles

Polymer	Size (nm)	Zeta-potential (mV)	Entrapment efficiency (%)[a]
PHDCA	161.7±8.9	−21.9±0.8	75.1±2.4
PEG-PHDCA	153.6±10.1	−9.1±0.5	58.4±3.1

a) Mean±S.D. (n=3).

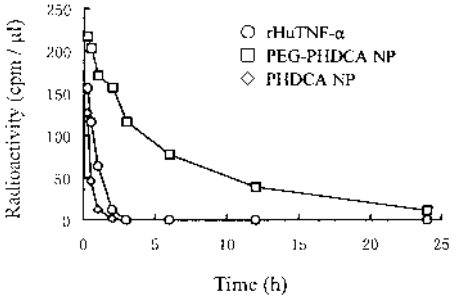

Fig. 1. Blood Clearance Curves of Free rHuTNF-α (○), PHDCA (◇) and PEG-PHDCA (□) Nanoparticles

RESULTS

The physicochemical properties and encapsulation efficiency of PHDCA and PEG-PHDCA nanoparticles loaded with rHuTNF-α are shown in Table 1. Both nanoparticle sizes were about 150 nm. The encapsulation efficiency values achieved for rHuTNF-α were obviously influenced by the presence of PEG in the PHDCA chain. Compared with PHDCA nanoparticles (75.1%), PEG-PHDCA nanoparticles showed lower encapsulation efficiency (58.4%). The values of the zeta potential also were obviously affected by the presence of PEG. Higher negative values were obtained for PHDCA nanoparticles (−21.9 mV), and a marked decrease in the surface charge for PEG-PHDCA nanoparticles occurred (−9.1 mV).

The blood clearance curves for rHuTNF-α loaded in nanoparticles after intravenous injection are shown in Fig. 1. The PEG-PHDCA nanoparticles showed initial high blood circulating levels compared with PHDCA nanoparticles and free rHuTNF-α. In fact, the radioactivity of PEG-PHDCA nanoparticles in blood at 1 h after intravenous administration was about 12 fold that observed for the PHDCA nanoparticles and 3 fold for free rHuTNF-α. Free rHuTNF-α and PHDCA nanoparticles were quickly removed from the circulating system. On the contrary, PEG-PHDCA nanoparticles exhibited a markedly delayed blood clearance. It could be seen that the blood-associated radioactivity remained higher after 24 h compared with those of PHDCA nanoparticles and

free rHuTNF-α. The radioactivity-time curves for PEG-PHDCA nanoparticles in mice are fitted by the two-compartment model and its pharmacokinetic parameters are shown in Table 2. But the radioactivity-time curve of PHDCA nanoparticles and free rHuTNF-α are fitted with one-compartment model and their pharmacokinetic parameters are shown in Table 3.

The distribution profiles of free rHuTNF-α and nanoparticles loaded with rHuTNF-α in S-180 solid tumor-bearing mice after intravenous administration are shown in Fig. 2. After 1 h, free rHuTNF-α and rHuTNF-α loaded in PHDCA nanoparticles were mainly distributed to the liver, spleen, lung and kidney. The tumor accumulation of free rHuTNF-α and rHuTNF-α loaded in PHDCA nanoparticles was very low. At 6 h after intravenous injection, free rHuTNF-α and PHDCA nanoparticles were gradually eliminated from all tissues. PEG-PHDCA nanoparticles were transported from the blood to normal tissues to a different extent and profile than free rHuTNF-α or PHDCA nanoparticles. The plasma levels of rHuTNF-α loaded in PEG-PHDCA nanoparticles were markedly higher than in PHDCA nanoparticles and free rHuTNF-α at the same time points and their transport to the liver and kidney were found to be markedly limited.

The inhibitory actions of nanoparticles loaded with rHuTNF-α in S-180 solid tumor-bearing mice after intravenous administration are shown in Table 4. The rHuTNF-α at the dose of 2.5 μg per mouse induced an obvious anti-tumor response, but four of the mice died during the experimental period, and the other surviving mice developed piloerection. PHDCA nanoparticles (0.5 μg per mouse) had an obvious inhibitory tumor growth action compared with rHuTNF-α of the same dose ($p<0.05$), and PHDCA nanoparticles were well tolerated. PEG-PHDCA nanoparticles had higher anti-tumor potency than free rHuTNF-α and PHDCA nanoparticles at the same doses. During the experimental period, PEG-PHDCA nanoparticles were well tolerated and body weight reduction was not observed. Saline and rHuTNF-α (0.1 μg, 0.5 μg per mouse) did not inhibit tumor growth.

Table 2. Pharmacokinetic Parameters of PEG-PHDCA Nanoparticles in Mice

Parameters	Values of parameters (X±S.D.)
$T_{1/2\alpha}$ [a] (h)	1.29±0.26
$T_{1/2\beta}$ [b] (h)	7.42±0.95
K_{21} [c] (h^{-1})	0.35±0.07
K_{10} [d] (h^{-1})	0.14±0.01
K_{12} [e] (h^{-1})	0.19±0.03
Vc [f] (ml)	5.56±0.30
$AUC_{0—24\,h}$ [g] (h·cpm/μl)	1570.56±56.85

a) $T_{1/2\alpha}$, plasma half-life in the alpha phase. *b*) $T_{1/2\beta}$, plasma half-life in the beta phase. *c*) K_{21}, transport rate constant from peripheral compartment to central compartment. *d*) K_{10}, elimination rate constant from central compartment. *e*) K_{12}, transport rate constant from central compartment to peripheral compartment. *f*) *Vc*, steady-state volume of central compartment. *g*) *AUC*, area under the plasma concentration curve.

Table 3. Pharmacokinetic Parameters of Free rHuTNF-α and PHDCA Nanoparticles in Mice

	$T_{1/2}$ [a] (h)	V [b] (ml)	$AUC_{0—24\,h}$ [c] (h·cpm/μl)
rHuTNF-α	0.48±0.09	1.80±0.29	510.78±48.67
PHDCA-NP	0.29±0.03	0.08±0.17	85.24±4.61

a) $T_{1/2}$, plasma elimination half-life. *b*) *V*, steady-state volume of distribution. *c*) *AUC*, area under the plasma concentration curve.

DISCUSSION

The encapsulation efficiency values achieved for rHuTNF-α were different between PHDCA nanoparticles and PEG-PHDCA nanoparticles. The difference might come from the presence of PEG in the PHDCA chains, but its mechanism was indistinct. Compared with PHDCA nanoparticles, PEG-

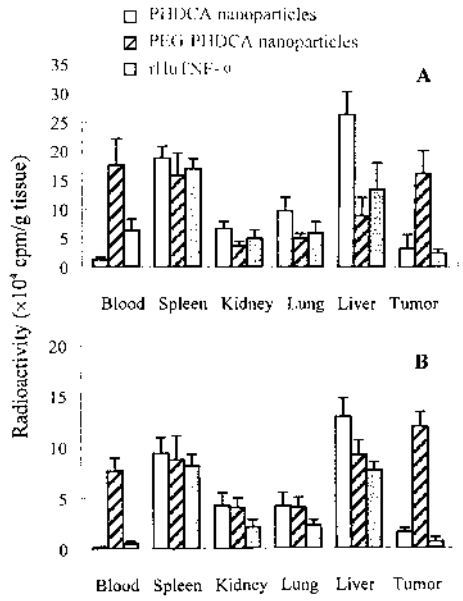

Fig. 2. Tissue Distribution of rHuTNF-α at 1 h (A) and 6 h (B) in PHDCA and PEG-PHDCA Nanoparticles

Table 4. *In Vivo* Anti-tumor Effects of rHuTNF-α and rHuTNF-α Loaded in Nanoparticles against Murine S-180 Sarcoma

Drug	Dose (μg/mouse)	Route	Mice In.[a]/Fi.[b]	Body wt. (g) In.[a]/Fi.[b]	Tumor wt. (g) X±S.D.	Inhibition (%)	p
NS	0.2[c]	i.v.	10/10	21.2/26.1	2.21±0.31		
rHuTNF-α	0.1	i.v.	10/10	21.4/25.2	2.12±0.19	4.1	>0.05
rHuTNF-α	0.5	i.v.	10/9	20.6/20.3	1.87±0.12	15.4	>0.05
rHuTNF-α	2.5	i.v.	10/6	21.9/21.2	1.14±0.18	48.4	<0.01
PHDCA NP	0.5	i.v.	10/9	20.8/22.5	1.46±0.22	33.9	<0.05
PEG-PHDCA NP	0.5	i.v.	10/10	21.3/25.8	0.48±0.10	78.3	<0.01

a) 0 d of experiment, *b*) 11 d of experiment, *c*) ml/mouse.

PHDCA nanoparticles showed a marked decrease in the surface charge. This might be related to a shift of the hydrodynamic phase of shear to greater distances from the nanoparticles surface. The same observations have been reported for PLGA-PLA : PEG coated nanoparticles.[28)]

Our experimental results showed that PEG-PHDCA nanoparticles could extend the half-life of rHuTNF-α to 7.42 h and obviously change the protein biodistribution in tissues, in particular, increase accumulation of rHuTNF-α in tumor. This result confirmed the idea of forming nanoparticles with a steric PEG barrier that would prevent their rapid uptake by MPS, improve circulatory half-life of rHuTNF-α and also contribute to the enhanced vascular permeability of tumors compared with normal tissues. It has been reported that relatively small size stealth liposomes (100—200 nm) effectively accumulate in many tumors *via* the 'impaired filtration' mechanism.[29,30)] The same might also occur with the nanoparticles.[31)]

Compared with PHDCA nanoparticles and free rHuTNF-α, PEG-PHDCA nanoparticles loaded with rHuTNF-α showed higher anti-tumor potency at the same doses. This result might be related to its higher accumulation in tumor tissues and longer plasma circulation time. It is well known that TNF-α can induce the hemorrhagic necrosis of tumor *via* specific interactions with tumor-vascular endothelial cells and it also has direct cytotoxicity against various tumor cells and stimulates the host immune anti-tumor response. Therefore, an increase in tumor accumulation and prolongation of the plasma half-life must produce higher anti-tumor potency.

In conclusion, PEG-PHDCA nanoparticles could obviously alter the *in vivo* behavioral characteristics of rHuTNF-α. PEG-PHDCA nanoparticles extended the half-life of rHuTNF-α to 7.42 h and obviously increased accumulation of rHuTNF-α in tumor. Compared with PHDCA nanoparticles and free rHuTNF-α, PEG-PHDCA nanoparticles loaded with rHuTNF-α showed the higher anti-tumor potency at the same doses. These results demonstrated that PEG-PHDCA was a useful carrier with selective localization in the vascular space against tumors and further enhances the therapeutic activity of rHuTNF-α.

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