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• COLORECTAL CANCER •

Construction, expression and tumor targeting of a single-chain Fv against human colorectal carcinoma

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Abstract

AIM: A single-chain antibody fragment, ND-1scFv, against human colorectal carcinoma was constructed and expressed in *E.coli*, and its biodistribution and pharmacokinetic properties were studied in mice bearing tumor.

METHODS: V_H and V_L genes were amplified from hybridoma cell IC-2, secreting monoclonal antibody ND-1, by RT-PCR, and connected by linker $(Gly_4Ser)_3$ to form scFv gene, which was cloned into expression vector pET 28a(+) and finally expressed in *E.coli*. The expressed product ND-1scFv was purified by metal affinity chromatography using Ni-NTA, its purity and biological activity were determined using SDS-PAGE and ELISA. ND-1scFv was labeled with ^{99m}Tc, and then injected into mice bearing colorectal carcinoma xenograft for phamacokinetic study *in vivo*.

RESULTS: SDS-PAGE analysis showed that the relative molecular weight of recombinant protein was 30kDa with purity of 94 %. ELIAS assay revealed that ND-1scFv retained the immunoactivity of parent mAb, being capable of binding specifically to human colorectal carcinoma cell line expressing associated antigen. Radiolabeled ND-1scFv exhibited rapid tumor targeting, with specific distribution in mice bearing colorectal carcinoma xenograft observed as early as 1 h following injection. *In vivo* pharmacokinetic studies also demonstrated that ND-1scFv had very rapid plasma clearance ($T_{1/2}\alpha$ of 5.7 min, $T_{1/2}\beta$ of 2.6 h).

CONCLUSION: ND-1scFv shows significant immunoactivity, and better pharmacokinetic and biodistribution characteristics compared with intact mAbs, demonstrating the possibility as a carrier for tumor-imaging.

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INTRODUCTION

Colorectal carcinoma is one of the common malignant tumors with relatively high incidence, occupying the fourth rate of mortality in China. Therefore, efficient diagnosis and therapeutic approaches are important for colorectal carcinoma research. Although in recent years some progress has been made in respect to application of monoclonal antibodies for the therapy and diagnosis of colorectal carcinoma, most mAbs are of murine origin, so that repeated administration can induce human anti- mouse antibodies (HAMA), moreover, intact mAbs are generally too large (Mt 150 000) to penetrate tumor masses, which can severely limit the efficacy of antibody in clinical utilization[1]. To overcome such deficiencies, gene engineering antibody, including human origin antibodies, single chain Fv (scFv), human-murine chimed antibodies are developed to improve murine origin mAbs[2-9]. ScFv, which is comprised of immunoglobulin heavy- and light-chain variable regions that are connected by a short peptide linker, is the gene engineered antibody employed most widely at present. The main advantages of scFv over intact mAbs and Fab fragment are their small size (M_r 30 000, amounting to one sixth of intact mAbs), making them penetrate a solid tumor mass rapidly and evenly. In addition, the lack of Fc domains makes them less immunogenic responsive and less capable of binding to Fc receptors distributed on normal cells. These characteristics make scFv potentially useful in tumor diagnosis and therapy as a carrier[10,11].

ND-1 is a murine monoclonal antibody against tumor-associated antigen LEA , mainly expressed in human colorectal carcinoma, developed by Jindan Song in 1986, which was obtained by immunizing Balb/c mouse with CCL-187 human colorectal carcinoma cell line. The histochemical determination of one thousand pathologic samples showed that ND-1 binded specifically to well differentiated and moderately differentiated colorectal carcinoma tissues and its specificity is superior to mAbs against CEA. ^{131}I labeled ND-1 also exhibited excellent imaging of tumor tissue in mice bearing colorectal carcinoma xenograft. We constructed a scFv by gene engineering technology from the V_L and V_H of ND-1, a monoclonal antibody against human colorectal carcinoma, and determined the biological properties of ND-1 scFv *in vivo* and *in vitro*.

MATERIALS AND METHODS

Materials

IC-2 is murine hybridoma cell that secrets monoclonal antibody ND-1against human colorectal carcinoma. Both IC-2 and HeLa human cervical carcinoma cell line were from our group. pET28a(+) expression vector and *E. coli* BL21 were kindly provided by Dr. YH. Chen. CCL-187 human colorectal carcinoma cell line was kindly provided by Tumor Research Institution of Medical College of Harvard University. pMD18-T vector, *E.coli* JM109 component cell, DNA polymerase, restriction enzyme, and DNA recovery kit were purchased from TarkaRa Biotechnology (Dalian, China). mRNA purification kit and T4 DNA ligase were bought from Pharmacia Biotech. Anti-His6 tag antibody was from Invitrogen. Ni-NTA resin was provided by Qaigen company. MDP and ^{99m}Tc were kindly provided by Department of Nuclear Medicine at China Medical University. Heavy chain primer 1 and 2, light chain primer mix, linker primer mix, and RS primer mix was purchased from Pharmacia Biotech.

Genetic construction of ND-1scFv

ND-1scFv gene was constructed as previously described. Briefly, mRNA was extracted from 5×10^6 hybridoma cells IC-2 and cDNA was synthesized by reverse transcription using random primer. V_H and V_L gene were separately amplified from the cDNA by PCR using heavy chain primer and light primer mix. The V_H and V_L gene fragments were recovered and mixed in equimolar ratios for two PCR reactions, the first one using linker primer mix for 7 cycles, followed by the second one using RS primer mix for 30 cycles. As a result, V_H and V_L gene fragments were connected to form scFv gene by extension overlap splicing PCR, and then, obtained ND-1 scFv gene was cloned into pMD18-T, and transformed into *E. coli* JM109, positive clones were identified by colony PCR and DNA sequencing.

Oligonucleotide primers S1 and S2 were designed to add *Eco*RI site at the 5' end of ND-1scFv, and *Hind* III site, *Sal*Isite at the 3' end. S1: 5' ACTGAATTCATGGCCCAGGT GCAGCTGCAGC 3' , S2: 5' CGCAAGCTTCTAGTCGAC TTTCCAGCTTGGTC 3' . pMD18-T-ND-1scFv was used as template for a PCR by primer S1 and S2, and the product was cloned into the vector pET28a(+) after digestion with *Eco*RI and *Hind*III, and then transformed into competent *E.coli* BL21cells for protein expression.

DNA sequencing

ND-1scFv genes cloned into pMD18T and pET28a(+) were sequenced by the dideoxy chain termination method with M13 primer, T7 promoter primer and T7 terminator primer.

Expression and purification of ND-1scFv

E.coli BL21 cells containing pET28a(+)-ND-1scFv plasmid were grown in 100 ml LB broth with 50 μg/ml kanamycin at 37 ℃, when $O.D_{600}$ of the culture attained about 0.6, IPTG was added in a final concentration of 1 mmol, and cells were shaken at 37 ℃, after 3.5 h, the culture was centrifuged at 5 000 rpm for 10 min, the cell pellet was treated with lyses solution. After sonication and centrifugation, inclusion body containing scFv protein was solubilized and denatured in the presence of 6 mol/L Guanidine hydrochloride. Affinity chromatography on Ni-NTA resin was performed to purify scFv, the column was eluted with 8 mol /L urea at pH8.0, pH6.5 and pH4.2, and the component of pH4.2, containing scFv, was collected, following renaturing by dialysis. Purity and concentration of protein were determined with Bradford assay.

ELISA assay for activity of ND-1scFv

CCL-187 cells and HeLa cells (5×10^4) were grown in 96- well microtiter plates at 37 ℃ for 24 h, then fixed with 2.5 % glutaradehyde and blocked with 1 % BSA, followed by incubation with ND-1IgG or ND-1scFv at 37 ℃ for 2 h; after washing 3 times with PBS, anti-His6 antibody was added into wells with ND-1scFv and incubated as above, the plate was washed and HRP-labeled goat anti-mouse IgG was added into both ND-IgG and ND-1scFv wells, incubating at 37 ℃ for 2 h, substrate TMB was added, incubated in darkness for 30 min, the reaction was terminated with1N H_2SO_4; PBS was used as a negative control.

Tumor model

Human colorectal carcinoma cells (1×10^6) were injected s.c. into the back of athymic mice (nu/nu)(4-6 weeks old). When a tumor developed at 0.5-1.5 cm in diameter, biodistribution and pharmacokinetics were studied.

Biodistribution and pharmacokinetics studies

ND-1scFv and ND-1IgG were labeled with ^{99m}Tc using MDP. Excess β-mercaptoethanol was added to the solution containing ND-1scFv and ND-1IgG, reduced product (1 mg) was mixed with 40 μl MDP (2.5 mg/ml) and 370MBq ^{99m}Tc. Biodistribution study was performed using tumor-bearing mice injected i.p. with 0.2 ml ^{99m}Tc-ND-1scFv, the mice were killed at different periods. Blood, tumor and all the main organs were collected and weighed. The radioactivity was counted in a gamma scintillation counter. The T/NT value for each organ was calculated.

Pharmacokinetic study was performed by the tumor- bearing mice injected via the tail vein with 0.1 ml ^{99m}Tc-ND-1scFv and ^{99m}Tc-ND-1 IgG. Blood samples were obtained via tail bleeds at 0, 5, 10, 15, 30, 60, 120, 180 min and 24 h after injection, the radioactivity was counted in a gamma scintillation counter, and pharmacokinetic parameters were calculated.

RESULTS

Clone of ND-1scFv gene

V_H and V_L gene were amplified from hybridoma cell IC-2 that secreted monoclonal antibody against human colorectal carcinoma, and then were connected by a linker $(Gly_3Ser)_4$ using extension overlap splicing PCR to construct scFv gene, which had *Eco*RI site at 5' end and *Hind*III site at 3' end. scFv gene was cloned into the vector pET28a(+) and expressed in *E.coli* BL21. Restriction enzyme digestion analysis showed scFv gene had been accurately inserted into vector pET28a (+). Sequence analysis revealed that scFv gene consisted of 732bp, encoding 243 amino acids. Of which, 354bp for heavy chain gene, was located upstream of scFv gene, and 330bp for the light chain gene, was located downstream. They were connected by a 45bp linker sequence. The deduced protein sequence of ND-1scFv was showed in Figure 1.

M A Q V Q L Q Q S G P G L V A P S Q S L
S I T CT V S G F S L T T D V H W V R Q P
P R K G L E W L G L V W A N G R T N C T
S A L M S R I S I T R D T S K N Q V F L T
M N S L Q T D D T A M Y Y C A R G S Y
G A V D F W G Q G T T V T V S S G G G G
S G G G G S G G G G S D I E L T Q S P A
linker
S L A V S L G Q R A T I S Y R A S K S V S
T S G Y S Y M H W N Q Q K P G Q P P R L
L I Y L V S N L E S G V P A R F S G S G S
G T D F TL N I H P V E E E DA A T Y Y C
Q H I R E L T R S E G G P S W K

Figure 1 Amino acid sequences of ND-1scFv deduced from nucleotide sequences.

Expression and purification of ND-1scFv

Plasmid ND-1scFv-pET28a(+) was transformed into *E.coli* BL21, the protein was expressed with induction of IPTG. SDS-PAGE analysis showed that the lysates of BL21 cell expressing scFv protein exhibited a new protein band with molecular weight at 30kDa (Figure 2). Because a sequence encoding a short peptide His-tag exists at the upstream of multi-clone site (MCS) of vector pET28a(+), ND-1scFv was expressed as a recombinant fusion protein with His tag, consisting of 26kDa for scFv and 4kDa for His-tag and its upstream sequence, which was consistent with the theoretically predicted value. SDS-PAGE analysis also showed that no new protein component was found in the supernatant of cell lysate of *E.coli* BL21induced by IPTG, which indicated scFv protein was expressed in the form of inclusion body. Inclusion body protein was purified by metal affinity chromatography using Ni-NTA resin which could bind to the His-tag protein marker located on the N terminal end of scFv specifically, purity of purified scFv was 94 % purity.

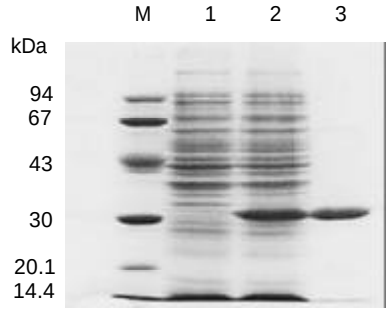

Figure 2 Expressed ND-1scFv. M: Protein marker; 1: Expression of pET28a(+)-ND-1scFv without induction; 2: Expression of pET28a(+)-ND-1scFv with induction of IPTG; 3: purified ND-1scFv protein.

Determination of immunoactivity of ND-1scFv

The immunoreactivity of purified ND-1scFv was determined by ELISA, the result revealed that scFv exhibited an immunoreactivity similar to the parent ND-1 antibody, and showed strong binding to CCL-187 cells expressing colorectal carcinoma associated antigen LEA, and weak binding to LEA-negative HeLa cells. This suggested that scFv had excellent specificity and still retained higher activity after undergoing refolding and purifying procedures.

Table 1 Immune activity of ND-1scFv determined by ELISA

Sample	OD_{450nm} ($\bar{x}\pm s$)	
	CCL-187	HeLa
ND-1IgG	1.92±0.28	0.20±0.06
ND-1scFv	0.87±0.17	0.19±0.03
PBS	0.14±0.03	0.13±0.01

In vivo distribution studies

ND-1scFv was labeled with ^{99m}Tc. ^{99m}Tc-ND-1scFv was injected into mice bearing the CCL-187 xenograft for biodistribution studies. Radioactivity in blood, tumor and normal tissue was determined at 1 and 3 h following injection, and the ratios of radioactivity between tumor tissue and normal tissue (T/NT) were evaluated. The result showed that labeled scFv displayed rapid localization in tumors, accumulation was found in tumors in high concentrations 1 hour after injection, and scFv uptake in tumor was significantly higher than that in other normal tissues (Table 2).

Table 2 Distribution of ^{99m}Tc- labeled ND-1scFv in mice-bearing tumor ($\bar{x}\pm s$)

Tissue	T/NT value	
	1 h	3 h
Blood	2.61±0.97	2.16±1.05
Liver	1.20±0.40	1.75±1.10
Spleen	2.72±0.10	1.23±0.65
Kidney	0.07±0.05	0.26±0.01
Heart	1.75±0.51	1.90±0.60
Lung	0.83±0.31	0.62±0.16

Pharmocokinetic studies

Studies were conducted to define the pharmocokinetic properties of plasma clearance of ^{99m}Tc labeled ND-1scFv in mice bearing tumor (Table 3). Compared with intact ND-1 IgG, ^{99m}Tc-ND-1scFv exhibited an extremely rapid clearance from the plasma, 80 % of the scFv was cleared out of the plasma pool at 15 min following injection, $T_{1/2}\alpha$ phase for the scFv was 5.7 min, $T_{1/2}\beta$ phase was 2.6 h, while $T_{1/2}\alpha$ and $T_{1/2}\beta$ for the ND-1 were 60 min and 18 h, respectively.

Table 3 Pharmacokinetic parameter of ^{99m}Tc-labeled ND-1scFv in mice-bearing tumor

	ND-1IgG	ND-1scFv
Alpha half-life (min)	5.7	60
Beta half-life (h)	2.6	18

DISCUSSION

The critical issue in application of mAbs is its high specificity and good *in vivo* biological features. In this study, single chain Fv ND-1scFv against human colorectal carcinoma was constructed by fusing gene of variable region of heavy chain with gene of variable region of light chain, and the ND-1scFv protein was functionally expressed in *E.coli*. ELISA analysis showed that ND-1scFv had an immunoactivity similar to the parent ND-1 mAbs, and binded specifically to the CCL-187 human colorectal carcinoma cell that expressed associated antigen LEA. This suggests that ND-1 mAb with only a Fv segment still retained its immunoactivity of binding to corresponding antigens, which is consistent to the previous reports. In addition, ND-1scFv also exhibited excellent specific distribution and pharmacokinetic characteristic in tumor-bearing mice.

A linker sequence was required to connect V_H and V_L for the construction of scFv, the linker widely used at present was a 15-amino acid sequence consisting of repetition of four Gly and one Ser (GGGGS)[12-15]. In this study, the $(Gly_4Ser)_3$ sequence was used, and the fusion molecule was constructed in V_H-linker-V_L order, the expressed ND-1scFv protein retained favorable stability during the renaturing and dialyzing, and retained biological activity similar to the parent antibody. In addition, a *Sal*I site was provided at scFv 3' end except for adding a *Hind*III site for ligasing the vector. In another experiment, we have already constructed a fusion protein of ND-1scFv and yeast cytosine deaminase using the same restriction site.

E.coli gene expression system is known as the earliest developed and most widely applied system for gene engineering. Although expressed proteins are usually lack of the effective modification such as glycosylation, there are some evidences suggesting that a variety of antibody fragments expressed in *E.coli* were able to fold and assemble correctly into bioactive products without the processing[16-20], which was also confirmed in own studies. pET vector which belongs to the T7 expression system propagating in *E.coli* was used to express of ND-1scFv. This vector contains T7 promoter, which can achieve high level controlled gene transcription in the presence of T7 RNA polymerase[19, 21-24]. ND-1scFv protein was intensively expressed to 17 % of the total bacterial protein. In addition, a 6×His tag sequence exists at the upstream of clone site in the pET vector, which was expressed in the form of fusion protein with the downstream scFv. Since it did not influence the bioactivity of expressed products, no enzyme hydrolysis process was required to remove it from the final products. This simplified the whole expression procedure. It was even more worth noticing that this sequence could be used as a protein marker for the determination and purification of expressed proteins[25-27]. ND-1scFv protein was purified by metal affinity chromatography using Ni-NTA resin which can bind to His tag specifically located on -NH_3 of scFv. SDS-PAGE analysis showed that the purity of ND-1scFv was as

high as 94 %, and the concentration was 1.5 mg/ml, demonstrating its potential usefulness in clinical application.

After being reconstructed into small molecules, the molecular weight of mAbs usually reduced to as 1/3 or 1/6 of intact mAbs, which significantly increased their penetrability to tumor tissue. Related experimental observation revealed that intact mAbs mainly concentrated nearby the blood vessel, while scFv seemed to be distributed uniformly within the tumor tissue and performing targeting function with high efficiency[28,29]. Furthermore, scFv exhibited two- phase pharmacokinetic characteristic *in vivo*, its $T_{1/2}\alpha$ (equilibrium phase) is much shorter than that of intact mAbs, implying that the *in vivo* equilibrated distribution of scFv may be reached rapidly, and its penetration into the interior of solid tumor could be achieved in a short time. In our experiment, ^{99m}Tc labeled ND-1scFv accumulated in tumor tissue in high concentration rapidly only 1 h after being injected into mice bearing xenograft. The radioactivity was significantly higher than that in most of normal tissues, while intact ND-1 required 20-24 h to obtain similar accumulation. Plasma pharmacokinetic studies in mice bearing tumor also showed a rapid plasma clearance of ND-1scFv superior to intact ND-1. Strong penetrability, rapid localization and elimination are the main biological behavior of scFv *in vivo*, making it an ideal localizing diagnostic agent for clinical applications. These were further validated by the immunoimaging experiments in mice bearing tumor using a various of scFvs against different tumor antigen[30-32]. Hitherto, the superiority of ND-1 developed by our group over the commercial product, mAb *vs* CEA, both in specificity and affinity, has been demonstrated in a number *in vivo* and *in vitro* experiments. Thus ND-1scFv, constructed from V_H and V_L of ND-1, may provide a new approach for clinical diagnosis and treatment of human colorectal carcinoma.

In this study, we observed that labeled scFv simultaneously accumulated intensively in kidney and in tumors of mice bearing xenograft, which also has been reported by other researchers[30]. On one hand, relative small size of scFv promotes its rapid uptake by kidney, so that the accumulation in kidney occurs shortly after injection, on the other hand, the half life of ^{99m}Tc is shorter, which, although beneficial for *in vivo* fast imaging, also increases the uptake of labeled scFv by kidney[28]. Recently, Goel *et al*[33,34] constructed divalent $[sc(Fv)_2]$ and tetravalent $\{[sc(Fv)_2]_2\}$ by covalent interaction, which increased the valence of scFvs and improved their affinity. Compared to the monovalent scFv, the divalent scFvs showed approximately 20-fold higher affinities. Furthermore, the molecular weight of multivalent scFvs was larger than scFv, but still smaller than intact IgG, so the in vivo pharmacokinetic behavior would be more promising[35-40]. Some researchers suggested that this uptake also may be related to the IP of the scFv, thus, there exists the possibility of directly modifying the isoelectric point of the scFv by introducing mutation in framework regions. A lower IP may reduce non-specific uptake into tissues such as the kidney[31]. The ND-1scFv constructed in this study retained the immunoactivity of parent mAbs and the clinical application are demonstrated preliminarily in radiolabling experiment with mice bearing tumor. With further development, it may become a promising targeting carrier for clinical diagnosis.

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