Expression and identification of recombinant soluble single-chain variable fragment of monoclonal antibody MC3

Feng-Tian He, Yong-Zhan Nie, Bao-Jun Chen, Tai-Dong Qiao, Dai-Ming Fan, Rong-Fen Li, Yun-Sheng Kang, Yan Zhang

INTRODUCTION
Progress in the use of murine monoclonal antibodies (McAbs) for the in vivo study on diagnosis and treatment of human tumors is limited by a number of factors, including poor penetration of the intact antibody molecule into the tumors, their inability to reach the tumor in sufficient quantities without significant toxicity to normal tissue, and the development of a human anti-mouse antibody response to the injected McAb[19]. One possible way to alter the pharmacology of antibody is via the use of smaller molecular weight antibody fragment called scFv. ScFv molecules offer several advantages as carriers for the selective delivery of radionuclides and toxins to tumors, including rapid blood clearance, low kidney uptake, small size suitable for rapid penetration through tumor tissue and less possibility of developing antinouse antibody response[16-18]. Colorectal and gastric cancers are frequent causes of death of the cancers of digestive system. MC3 is a specific monoclonal antibody directed against colorectal and gastric carcinomas[19], which has a potential use for in vivo diagnosis and therapy of the corresponding carcinomas. In order to overcome the disadvantages of the intact McAb applied in vivo and to offer the antibody a stable genetic source, soluble ScFv of MC3 was generated by advanced recombinant phage antibody technique, which may provide a novel tumor-targeting vehicle for in vivo study on the diagnosis and treatment of colorectal and gastric carcinomas.

MATERIALS AND METHODS
Materials
The hybridoma cell line producing McAb MC3 (isotype IgG1,κ) was generated by the Institute of Digestive Disease, Xi‘an, China[19]. Mouse ScFv DNA construction kit, phage-displayed ScFv expression and detection kits, anti-E tag antibody and pCANTAB5 Sequencing Primer Set were purchased from Pharmacia, Sweden. mRNA isolation kit, Tag DNA polymerase, T4 DNA ligase, SfiIand NsiI Restriction enzymes were bought from Promega, USA. The gastric carcinoma cell line AGS highly expressing MC3-binding antigen was from ATCC, USA. Horseradish peroxidase (HRP)-labeled goat anti-mouse IgG was from Sino-American Biotechnology Company, China.

Preparation of the phage-displayed ScFv
Total DNA was extracted from guanidine thiocyanate homogenates from 5×10⁶ MC3-producing hybridoma cells[20], and the mRNA was isolated from the total RNA using mRNA isolation system according to the protocol supplied by the manufacturer. Subsequently the phage-displayed ScFvs were generated using the Mouse ScFv DNA construction kit and ScFv expression kit[21-27]. The purified mRNA was transcribed into cDNA using random primers, and the VH and VL DNAs were separately amplified through PCR program 1 (30 cycles: 94°C×1min, 55°C×2min, 72°C×2min). Gel-purified VH and VL DNAs were mixed with linker primers at an equimolar ratio and assembled in vivo ScFv DNA in fill-in reaction, designed
program 2 (7 cycles: 94°C×1min, 63°C×4min). In a second PCR reaction (same as program 1), the ScFv DNA was amplified and provided with a SfiI site at the 5’ end and a NotI site at the 3’ end. After digestion with restriction enzymes SfiI and NotI, the ScFv DNA was ligated into the phagemid vector pCANTABSE, and the ligated sample was transformed into competent E. coli TG1 cells to express phage-displayed ScFv. The transformants were grown in 2×YT medium containing ampicillin and glucose (2×YT-AG medium) up to an OD₆₀₀=0.5. Bacteria were infected with M13KO7 helper phage for 1 h at 37°C with shaking. The cells were sedimented by centrifugation, and the supernatant was carefully removed and discarded. The pellet was gently resuspended in 2×YT medium containing ampicillin and kanamycin (2×YT-AM medium) and incubated overnight at 37°C with shaking. The supernatant containing the recombinant phages was harvested by centrifugation and the phages were precipitated with PEG8000 and NaCl and resuspended in 2×YT medium, filtered through a 0.45μm filter and stored at 4°C.

Panning to select for antigen-positive phage-displayed ScFv
Recombinant phage expressing the MC3 ScFv were selected by panning[25-27]. The AGS cells highly expressing MC3-binding antigen were grown as an attached monolayer in 25-cm² flasks until almost confluent, washed with PBS, and fixed with 0.25% glutaraldehyde for 8min at room temperature(RT). The fixed cells were washed with PBS and blocked with 50g/L nonfat dry milk in PBS for 2h at RT. The cells were washed 3 times with PBS and the PEG-precipitated recombinant phages diluted 8:7 with 100g/L nonfat dry milk in PBS containing 0.1g/L sodium azide were added to the fixed cells. The culture flask was shaken gently for 2h at 37°C and the cells were washed 20 times with PBS and 20 times with PBS containing 0.1% Tween20 (PBST). Log phase E. coli TG1 cells were incubated with bound phages for 1h at 37°C with shaking. Ampicillin, glucose and M13KO7 were added to the bacterial suspension, and the culture was incubated for 1h at 37°C with shaking. Subsequently, preparation of PEG-precipitated recombinant phage was completed as above, and the second round of panning was performed as described for the first panning step. After the second round of panning, the reinfected TG1 bacteria were plated on SOBAG plates and grown overnight at 30°C, and single colonies were grown in 2×YT-AG medium overnight at 30°C with shaking. The ligated sample was transformed into competent HB2151 cells and stored at -20°C until use. One of the two cell pellets was resuspended in 0.5mL of ice-cold 1×TES (0.2mol/L Tris-HCl, pH8.0, 0.5mmol/L EDTA, 0.5mol/L sucrose) followed by adding 0.75mL of 1/5×TES. After incubation for 30min on ice, the supernatant containing the periplasmatic fraction was centrifuged and stored at -20°C. Whole-cell extract was prepared by resuspending the second pellet in 0.5mL PBS and boiling for 5min following centrifugation.

Detection of soluble MC3 ScFv by Dot blot
Extracellular fraction and its concentrate precipitated by ice-cold 200g/L trichloroacetic acid (TCA)(to 1/5 of the original volume), periplasmic extract diluted 1:4 with PBS, and whole-cell extracts were spotted onto the nitrocellulose membrane separately (2µL/well). The membrane was blocked for 2h at RT with PBS containing 100g/L nonfat dry milk(blocking buffer, BB)followed by incubation for 1h at RT with anti-E tag antibody (directed against the E tag-peptide fused to the VL region of the ScFv) diluted to a final concentration of 8mg/L with an equal volume mixture of PBS and BB containing 0.05% Tween20 (PBS/BBT). After being washed once with PBS containing 0.05% Tween20 and 4 times with PBS and followed by a 5min soak in PBS, the membrane was incubated for 1h at RT with HRP-labeled goat anti-mouse IgG diluted 1:150 with PBS/BBT and washed as above and developed using diaminobenzidine (DAB) substrate.

Detection of soluble ScFv by Western blot
Routine method was applied[28,33]. Peroxidase-labeled antibody diluted 1:4 with PBS and TCA-concentrated extracellular fraction were loaded into the wells (10µL/well) of 120g/L SDS polyacrylamide gels and followed by electrophoresis, and the fractionated proteins were then transferred onto a nitrocellulose membrane. After being blocked and developed as described in Dot blot.

ELISA for assay of the reactivity with antigen of soluble ScFv
The AGS cells were grown in 96-well plates, fixed and blocked as described in phage ELISA followed by incubation with 50µL of periplasmic and extracellular fraction for 1h at RT. After washed 6 times with PBST, anti-E tag antibody diluted 1:1000 with PBS/BBT was added (50µL/well) and incubated as above. The plate was washed 50µL of HRP-labeled goat anti-mouse IgG diluted 1:1000 with PBS/BBT added and incubated as above. After being washed, ABTS substrate was added and A₄₀₅ was measured. PBS was as a negative control, and the ScFv was considered as a binder when the ELISA response was at least 2×higher compared to the negative control.

ELISA for assay of the competition of soluble ScFv with MC3 in antigen-binding
It was done as described in references[36-38]. The AGS cells were

www.wjgnet.com

He FT, et al. Expression and identification of McAb MC3 259
grown in 96-well plates, fixed and blocked as described in phage ELISA followed by incubation with 50µL of periplasmic fraction for 1h at RT, with PBS as a negative control. After washed 6 times with PBST, MC3 was added to the plate (50µL/well) at a final concentration of 20µg/mL, and incubated for 1h at RT. After washed as above, HRP-labeled goat anti-mouse IgG diluted 1:1000 was added (50µL/well) and incubated and washed as above. Then TMB substrate was added and A450 was measured. Binding of MC3 with the cells was inhibited by the soluble ScFv, which was described as the inhibition rate: Inhibition rate (1-A450 of the tested sample/A450 of the control) ×100%.

DNA Sequencing
The phagemid derived from phage antibody clone 19 was used for DNA sequencing of VH and VL DNAs of the ScFv DNA, based on the dideoxy method, with the primers taken from the pCANTAB5 Sequencing Primer Set.

RESULTS
Cloning of MC3 ScFv DNA and Screening of phage-displayed ScFv
The amplified VH, VL, and ScFv DNAs were about 340 bp, 320 bp and 750 bp respectively. After two rounds of panning to the recombinant phages, 18 antigen-positive phage clones were selected from 30 preselected phage clones by ELISA. 4 out of the 18 clones (clones 12, 19, 23 and 30), which showed strong signal (clone 19 showed the strongest signal), were selected for expression of soluble MC3 ScFv in E. coli HB2151.

Identification of soluble MC3 ScFv
After expression of soluble ScFv in E. coli HB2151, extracellular, periplasmatic, and whole-cell fractions were checked for the presence and reactivity of ScFv by Dot blot, Western blot and ELISA. In a Dot blot, no visible signal was achieved for the whole-cell extracts, and only weak signal was shown for the extracellular extracts, while a strong signal for periplasmatic and TCA-precipitated extracellular extracts. Based on the results in the Dot blot, Western blot analysis was performed only with periplasmatic fraction, and the result showed that the VH and VL DNAs of MC3 ScFv DNA and the result showed that the VH and VL DNAs of MC3 ScFv DNA were 336 bp and 312 bp respectively, and they were identified as variable antibody genes belonging to the IgG1 subgroup, κ-type. The amplified VH, VL, and ScFv DNAs were about 340 bp, 320 bp and 750 bp respectively. After two rounds of panning to the recombinant phages, 18 antigen-positive phage clones were selected from 30 preselected phage clones by ELISA. 4 out of the 18 clones (clones 12, 19, 23 and 30), which showed strong signal (clone 19 showed the strongest signal), were selected for expression of soluble MC3 ScFv in E. coli HB2151.

Detection of soluble MC3 ScFv in periplasmatic extract by Western blot. Lanes 1-4: periplasmatic extracts derived from phage clones 12, 19, 23 and 30 respectively, diluted 1:4 with PBS; Lane 5: low molecular weight protein marker.

DNA Sequence analysis
The phagemid derived from clone 19 was used for DNA sequencing, and the result showed that the VH and VL DNAs of MC3 ScFv DNA were 336 bp and 312 bp respectively, and they were identified as variable antibody genes belonging to the IgG1 subgroup, κ-type. The detail sequence of the VH and VL DNAs were listed as follows.

**VH DNA of MC3 ScFv (336 bp)**
5'-ATGGCCCAAG TGAAAGTTCA AGCGGACTGA AGAAGGCTTG AGGAGACAGTC AGGATCTCCT GCAAGGCTTC TGCTATATCC TCCACAACCT CGTAAATGCA GTGGGTTCAA AAGATGCCAG GAAGGGGTTC GAAGTTTATG GTGCTTATTAC AGCCGAGGTT TGCCAGTTACG GCGAACTTT

**VL DNA of MC3 ScFv (312 bp)**
5'-GCTTCCTTAG TTGTTTCTGG TGGGCAATCT GCTGGCAAGG GCCAACCATCC CCGCGCGAGC CGACCCAAAGT TTGGATTAG TTTTGGCAC ACAGGCACAGG AAGAACACG AGCCCATC CTAATCCTGC AACCCCGGCT TCACCCGGCA GAGACGTGTCG GATGAGTCGG GACAGATTT GACCCCTAAC ATATATCGT TGGAGGAGGA TGATCCTGCA GTGATTTTCT GTCACCAAGT TAAGGAGGTT CTTACACGTC TCGGAAGGGG GACAAAGTGCT GAAATACAAAC GG-3'
DISCUSSION

Recent progress in antibody engineering have been directed toward the expression of antibody fragments in bacterial and phage display systems, leading to the creation of various applications in biology, clinical diagnosis and therapy. The power of the phage display system to express antibody fragments offers several advantages over hybridoma technology, allowing quick, economical production and generation of antibodies with increasing affinity and specificity by mimicking affinity maturation in the normal immune system, and offering the antibody a stable genetic source which can be easily manipulated[29-31]. Antibody fragments showed less possibility of developing anti-antibody response in comparison to the intact antibodies when applied in vivo. In this study, the ScFv of murine McAb MC3 directed against colorectal and gastric carcinomas was successfully produced by advanced phage antibody technology, which offered it the above advantages of the engineering antibody. MC3 ScFv was displayed on the surface of M13 phage as a fusion protein with gene g3p and additionally expressed in a soluble form secreted in the bacterial periplasm. Both expression forms retained their reactivity to the antigen. Because of the advantage of MC3 ScFv compared to the intact MC3 for in vivo diagnosis and therapy of the associated carcinomas, we have focused on the production of soluble ScFv. A critical step in cloning of the ScFv DNA was the assembly of VH and VL DNAs with linker DNA. The linker primers consist of 93 base oligonucleotides which are complementary to each other and have homology with the 3’ end of the VH gene and the 5’ end of the VL gene. 24 bases on either end of the linkers are complementary to the ends of the VH and VL. The central 45 bases of the linkers encode the flexible (Gly4-Ser)3 linker which joins the VH and the VL to form a ScFv fragment. In the assembly and fill-in reactions, an exact quantification of purified VH and VL products and linker DNA was very essential. Even slight deviations of the equimolar ratio VH:VL:linker lead to either no visible ScFv product or to the formation of VH-linker or VL-linker dimers, apparently 450bp in size.

McAb MC3 was prepared by immunizing mice with human colorectal carcinoma cells, but immunohistochemical detection indicated that the MC3-binding antigen was highly expressed in both colorectal and gastric carcinomas[32]. Because no purified antigen was available so far, the recombinant phages were selected in a panning reaction with AGS cells highly expressing MC3-binding antigen. After two rounds of panning, 18 antigen-positive phage clones were selected by ELISA, and 4 clones showing strong signal were used to express soluble ScFv. Clone 19, which showed the strongest signal in ELISA, was used for DNA sequencing and identified as variable antibody fragment. MC3-binding antigen was highly expressed in both colorectal and gastric carcinoma cell lines.

In this study, the ScFv of murine McAb MC3 directed against colorectal and gastric carcinomas, and also targeting vehicle for the preparation of soluble ScFv of MC3 may provide a promising therapeutic application to colorectal and gastric carcinomas, and also offer the antibody a stable genetic source.

REFERENCES

1. DeNardo SJ, Kramer EL, O’Donnell RT, Richman CM, Salaka QA, Shen S, Noe M, Glenn SD, Ceriani RL, DeNardo GL. Radioimmunotherapy for breast cancer using indium-111/yttrium-90 BrE-3: results of a phase I clinical trial. J Nucl Med 1997;38:1180-1185
2. Alvarez RD, Partridge EE, Khazaee MB, Pfett G, Austin M, Kilgore L, LaRagiole AE, MT, Gonzalez WE, Schijon J, LuBragio AF, Meredith FT. Intraportal radioimmunotherapy of ovarian cancer with 177Lu-Cc49: a phase I/II study. Gynecol Oncol 1997;65:94-101
3. Tempeiro M, Leichner, Dalrymple G, Harrison K, Augustine S, Schlam J, Anderson J, Wiseacre J, Colcher D. High dose therapy with iodine-131-labeled monoclonal antibody CC49 in patients with gastrointestinal cancers: a phase I trial. J Clin Oncol 1997;15:1518-1528
4. Buchsbaum DA, Khazaee MB, Liu T, Bright S, Richardon K, Jones M, Meredith R. Fractionated radioimmunotherapy of human colon carcinoma xenografts with 131I-labeled monoclonal antibody CC49. Cancer Res 1995;55(Suppl):5883-5887
5. Liu HF, Liu WW, Fang DC, Men RP. Expression and significant of preapoptotic gene Bax in gastric carcinoma. World J Gastroenterol 1999; 5:15-17
6. Goel A, Augustine S, Baranowska-Kortlewicz J, Colcher D, Booth BJ, Pavlinskova G, Tempero M, Batra SK. Single-Dose versus fractionated radioimmunotherapy of human colon carcinoma xenografts using 131I-labeled multivalent CC49-single chain fvs. Clin Cancer Res 2001;7:175-184
7. Goel A, Baranowska-Kortlewicz J, Hinrichs SH, Wiseacre J, Pavlinskova G, Augustine S, Colcher D, Booth BJ, Batra SK. 131I-Tc-labeled divalent and tetravalent cc49-single chain fvs: novel imaging agents for rapid in vivo localization of human colon carcinoma. J Nucl Med 2001;42:1519-1527
8. Khare PD, Shao XL, Kuroki M, Hirose Y, Arakawa F, Nakamura K, Tomity Y, Kuroki M. Specifically targeted killing of carcinomaobryonic antigen (CEA)-expressing cells by a retroviral vector displaying single chain variable fragment antibody to CEA and carrying the gene for inducible nitric oxide synthase. Cancer Res 2001;61:370-375
9. Schmidt M, MccWatters A, White RA, Groner B, Wels W, Fan Z, Bast RC Jr. Synergistic interaction between an anti-p185 HER-2 pseudomonoecytic xenotoxin fusion protein [scFv(FRP5)-ETA] and ionizing radiation for inhibiting growth of ovarian cancer cells overexpressing HER-2. Gynecol Oncol 2001;80:145-155
10. Mayer A, Tsiofanou E, O’Malley D, Boxer GM, Bhatia J, Flynn AA, Chester KA, Davidson BR, Lewis AA, Winslet MC, Haffil AP, Hilton AJ, Begent RH. Radioimmunoguided surgery in colorectal cancer using a genetically engineered anti-CEA single-chain Fv antibody. Clin Cancer Res 2000;6:1711-1719
11. Barth S, Huhn M, Matthey B, Tawadros S, Schnell R, Schinkothe T, Diehl V, Engert A. Ki-4(scFv)-ETA’, a new recombinant anti-CD30 immunotoxin against a highly-specific cytotoxic activity against disseminated Hodgkin tumors in SCID mice. Blood 2000/95:3909-3914
12. Barth S, Huhn M, Matthey B, Schnell R, Tawadros S, Schinkothe T, Lorenzen J, Diehl V, Engert A. Recombinant anti-CD25 immunotoxin RFTS(scFv)-ETA demonstrates successful elimination of disseminated human SCID mice with the CD25+ cell line SCID 2000/36:718-724
13. Onda M, Otafsen T, Tsutsumi Y, Bruland OS, Pantan J. Cytotoxicity of antioest zeroes recombinant immunotoxins composed of TP3-Fv fragments and a truncated Pseudomonas exotoxin A. J Immunother 2001; 24:144-150
14. Hoffmann P, Mueller N, Shively JE, Fleisher B, Neuhauser M. Fusion proteins of B7.1 and a carcinomaobryonic antigen (CEA)-specific antibody fragment opsonize CEA-expressing tumor cells and coactivate T-cell immunity. Int J Cancer 2001;92:725-732
15. Roovers RC, van der Linden E, de Bruijne AP, Arenda JW, Hoogenboom HR. In vitro characterization of a monovalent and bivalent form of a fully human anti-ep-CAM phage antibody. Cancer Immunol Immunother 2001;50:51-59
16. Mersmann M, Schmidt A, Rippmann JF, Waest T, Brocks B, Retting WJ, Garin-Chesa P, Pfitzner M, Moosmayer D. Human antibody derivatives directed against the fibrolast activation protein for tumor stroma targeting of carcinomas. Int J Cancer 2001;92:240-248
17. Kashentseva EA, Seki T, Curiel DT, Dmitriev IP. Adenovirus targetting to c-erbB-2 oncoprotein by single-chain antibody fused to trimeric form of adenovirus receptor ectodomain. Cancer Res 2002;62:609-616
18. Tag MK, Sasse S, Stocker M, Djakhelkur K, Huhn M, Matthey B, Gottstein C, Pfitzner T, Engert A, Barth S. An anti-GD2 single chain Fv selected by phage display and fused to Pseudomonas exotoxin A develops specific cytotoxic activity against neuroblastoma derived cell lines. Int J Mol Med 2000;5:579-584
19. Fan DM, Zhang YX, Chen XT, Mou ZX, Chen BJ, Qiao TD, Yang HB, Fang ZD. Establishment of four monoclonal antibodies to colonic cancer cells and immunohistochemical study on their corresponding antibodies. Disi

www.wjgnet.com
