^{99m}Tc labeled HAb18 McAb Fab fragment for radioimmunoimaging in nude mice bearing human hepatocellular carcinoma *

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Abstract

AIM To establish a method of labeling anti-hepatoma McAb (HAb18) Fab fragment modifier with ^{99m}Tc.

METHODS HAb18 Fab was modified with 2-iminotholane and labeled with ^{99m}Tc by transchelation from ^{99m}Tc GH. Labeling yield, radiochemical purity and immunoreactivity were determined by thin layer chromatography (TLC-SG), paper chromatography (PC), gel chromatography (GC) and cell binding assay, respectively. The nude mice bearing human hepatoma were used for radioimmunoimaging (RII).

RESULTS A radiolabeling yield of 50%-80% was obtained, and immunoreactivity (IR) was 30%-40%. Radioimaging results showed that ^{99m}Tc-HAb18 McAb Fab fragment was concentrated in the tumor 4-8 hours after injection, and the maximum concentration was seen in 12-24 hours, and the T/NT value was 5.18 and 7.48 at 6h and 8h after the injection.

CONCLUSION ^{99m}Tc-HAb18 McAb Fab fragment could be specifically localized in the tumor of nude mice bearing human hepatocellular carcinoma within 24 hours and this method might be effectively used for labeling McAb Fab fragment with 99mTc.

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INTRODUCTION

Many methods have been reported to radiolabel intact MAbs with ^{99m}Tc[1]. Since the long imaging time of intact McAb Hab18 (> 48 hours) is not compatible with the 6h physical $T^{1/2}$ of ^{99m}Tc, it seems preferable to use antibody fragments for ^{99m}Tc labeling, as the fragments have smaller molecular mass, can be cleared more rapidly from the blood, and potentially increase the tumor/non-tumor ratio (T/NT). The method of labeling fragment with ^{99m} Tc is therefore one exploring aspect. In this paper, 2-iminotholane (IT) was used to modify anti-hepatoma McAb HAb18 Fab fragment, and then IT-Fab fragment modifier was labeled with ^{99m}Tc by transchelation from ^{99m}Tc-glucoheptonate (GH). Successful results were obtained when ^{99m}Tc-IT-Fab was injected into the nude mice bearing human hepatoma for radioimmunoimaging (RII).

MATERIALS AND METHODS

Reagents

The anti-hepatoma McAb HAb18 (10g/L, purity >95%) was prepared and supplied by the Hepatoma Targetting Drug Research Laboratory, Department of Pathology, the Fourth Military Medical University. McAb HAb18 belongs to IgG_1 subclass and its affinity coefficient (ka) is 8.17×10.9L/mol. The antigen is about 61kd and has no crossreaction with AFP, CEA, and ferritin. IT is a product of Sigma Chemical Co.; 5.5'-dithiobis (2-nitrobenzoic acid) (DTNB), Fluka Co; papain, Merck Co.; and GH and MDP kit was supplied by the Institute of Jiangsu Nuclear Medicine. TEA-B-1 solution: a mixture of 1.0mol/L TEA and TEA HCl solution (1:1 v/v), pH 8.0; TEA-B2 solution: a mixture of KCl (50mmol/L) and $MgCl_2$ (1mmol/L) with the TEA-B-1 solution; TEA-B-SH solution: 1% 2-mercaptoethanol of TEA-B-2 solution; IT solution: IT (0.25mol/L) dissolved in TEA-B-1 solution.

Human hepatoma nude mice model

Human hepatoma was subcutaneously implanted in the neck or the upper lumber side of NC or BALB/c nude mice. The model was named SMMC-LTNM[2]. The mice were maintained in an aseptic condition and used for experiment when the tumor increased to 0.5 cm-1.4 cm in diameter.

Instrument

Packard A5301D γ-counter produced by Packard Instrument Co.; Diacam/Maxdelta 3000 SPECT system by Siemens Co.; fast protein liquid chromatograpy system (FPLC): Waters 650 with DEAE-sepharose fast flow column; and HPLC: Bio-Rad 5000T.

HAb18 Fab fragment

Prepared by papain digestion and purified by FPLC using DEAE-sepharose fast flow column[3].

Fab fragment modification

Jue's method[4] was used with some modification. Briefly, HAb18 Fab fragment (4g/L) was dialyzed overnight against TEA-B-SH solution, then mixed with IT solution (6mmol/L in concentration) at 0℃ reaction for 20min. The mixture was dialyzed against the TEA-B-2 buffer at room temperature (20℃) for 3h with a buffer change each hour. After that, the mixture was run through sephadex G50 column which was absorbed with bovine serum albumin (BSA), and eluted with 0.05M PBS at a speed of 1 ml/min. The protein fraction was collected under UV-280nm monitoring, concentrated to 4.4g/L, divided into 220μg/vial, and lyophilized for us.

Determination of sulfhydryl groups with DTNB

According to Ellman's method[5], protein samples (0.5ml) were mixed with 2.5ml 0.05mol/L Tris-Hcl buffer (pH 8.4), and 0.025ml 0.01mol/L DTNB solution. Half a mililiter of distilled water instead of protein sample solution was used as blank control. The increase in absorbance at 412nm was followed using a spectrophotometer (Lambda 2S). By means of the formula sulfhydryl group (μmol)= A412/0.5 × 13.6, the number of sulfhydryl group was calculated according to protein concentration (μmol).

^{99m}Tc labeling of IT-Fab fragment

The labeling procedure was as follows: ① 370-555MBq (1ml-1.5ml) of freshly eluted pertechnetate from a ^{99}Mo/^{99m}Tc generator was added into an GH kit (containing 10mg sodium glucoheptonate and 1mg stannous chloride), and was placed 10min, ^{99m}Tc-GH was prepared. ② Lyophilized IT-Fab (220 μg/vial) was dissolved in 100μl 0.1mol/L citrate buffer, pH 6.5, bubbled with N_2. ③ 0.1ml-0.5ml ^{99m}Tc-GH solution was added to the IT-Fab vial and incubated at 37℃ for over 1 hour.

Quality control of ^{99m}Tc-IT-Fab

Radiolabeling yield and radiochemical purity were determined by paper chromatography (PC) using No.1 Xinhua filter paper strips (saline as a solvent, ^{99m}Tc-IT-Fab and $^{99m}TcO_2$ Rf=0-0.1, $^{99m}TcO_4$ Rf= 0.7-0.8. Acetone as a solvent, ^{99m}Tc-GH Rf=0, $^{99m}TcO_4$ Rf = 1); or instant thin layer chromatography (ITLC) using ITLC-SG paper (Gelman Co), (1% BSA pretreated, alcohol: NH_3H_2O:H_2O = 2:1: 5 as a solvent, ^{99m}Tc-IT-Fab and $^{99m}TcO_4$ Rf = 1.0 $^{99m}TcO_2$ Rf = 0) and gel column chromatography (GCC) with sephadex G50 (0.9 cm × 15 cm diameter), 0.05 mol/L PBS, pH 7.4, as an eluent. The protein labeling yield was calculated by routine method. Immunoreactivity assessment: in vitro immunoreactivity of ^{99m}Tc-IT-Fab was evaluated by a conventional live cell assay. Briefly, hepatoma cell suspension was prepared by mincing SMMC-LTNM tumor tissues from the mice model. It was centrifuged and washed with phosphate-buffered saline (0.1 mol/L- PBS); then 10μl diluted ^{99m}Tc-IT-Fab (10×10^3 cpm) and 0.5ml PBS was added, and the mixture was incubated at 37℃ for 2 hours. After incubation, the samples were then centrifuged and the supernatants were removed. The cells were further washed with 1ml PBS solution by centrifugation. Before and after the removal of supernatant, the activity of samples was counted in a γ-counter, and the percentage of binding was calculated.

Animal biodistribution study

^{99m}Tc-IT-Fab 18.5 MBq/0.2ml was intravenously injected into each nude mouse bearing human hepatoma xenografted model by the tail vein, and the mice were sacrificed 5 hours after the injection. The blood and major organs were removed, weighed and counted in the γ-counter for radioactivity, the tumor/blood ratio and the tumor/organ ratios were calculated.

Animal imaging study

^{99m}Tc-IT-Fab 18.5-74 MBq/0.2ml was injected intravenously or intraperitonealy into the tumor-bearing nude mice (n = 6 each in half), and the same dose of ^{99m}Tc-GH was intravenously injected into the nude mice (n = 2) as control. The mice were imaged with a SPECT at 30min, 2 h, 4 h, 6 h, 12 and 22 h after administration. The T/NT ratios were calculated by ROI technique.

RESULTS

Fab fragmant yield was 30%-40%, and single peak was found by HPLC. The molecular mass weight was identified as 45kd by SDS-PAGE. The number of sulfhydryl group per molecule of IT-Fab and Fab fragment was 0.3 and 0, respectively. $^{99m}TcO_2$ was less than 0.5%. The immunoreactivity was 30%-40% by cell binding assay. The biodistribution study showed that the T/NT ratios of tumor to the blood,

the heart, liver, lung, speen, kidney, stomach, intestine, muscle, and brain were 0.89, 4.1, 1.1, 8.0, 5.2, 0.1, 1.6, 4.8, 4.8 and 33.1, respectively. Animal imaging showed that ^{99m}Tc-IT-Fab was rapidly distributed to the whole body when injected i.v. and the radioactivity rapidly appeared in the bladder. The body distribution via intraperitoneal was 10min-30min later than that of the i.v. injection. The tumor was visible with high background at 1h-2h; the accumulation of the tumor was evident with clear kidney imaging 4h-8h after the injection, and a relatively increased tumor localization was seen with low background and decreased kidney outline 12h-22h after injection (Figure 1). The T/NT (on the back muscles) ratios by ROI were 5.18 and 7.48 at 4h and 8h. In control group, ^{99m}Tc-GH rapidly appeared in the blood pool and the whole body, and accumulated rapidly in the bladder. The two kidney imagings were clear, but no tumor accumulation was seen 6h after the injection with two dim kidney outlines and low background. Radioactivity was removed in urine and the bladder image disappeared gradually.

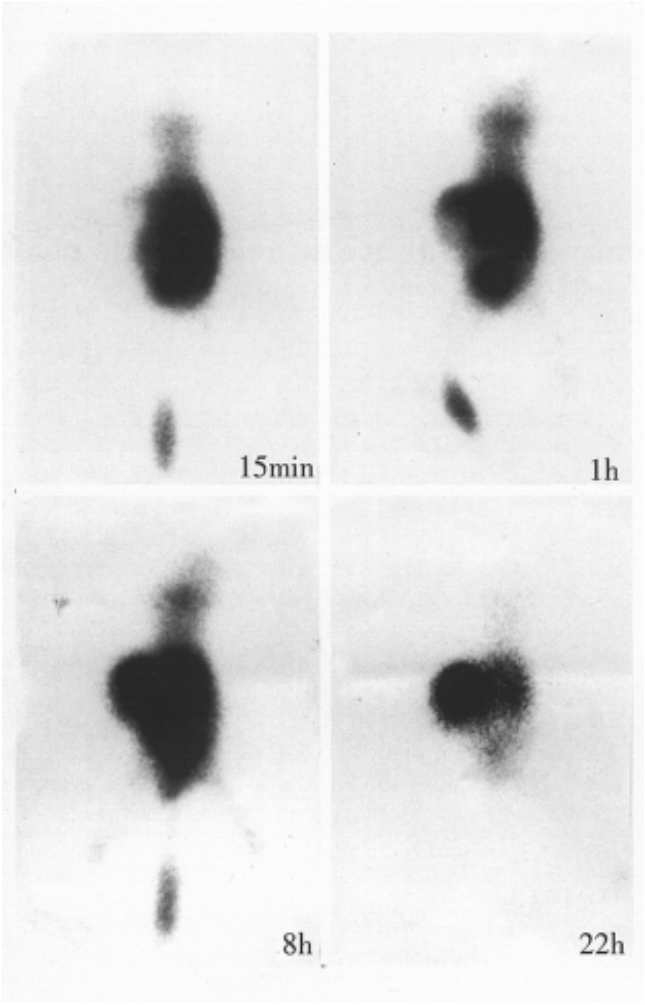

Figure 1 Dynamic imaging of ^{99m}Tc-IT-Fab in nude mice bearing human hepatoma model (posterianterior position).

DISCUSSION

^{99m}Tc labeling McAb F(ab')$_2$ and Fab' fragments have been reported by some authors[1], but Fab fragment was rarely seen. In terms of the clearance, intact IgG was the slowest, F(ab')$_2$ fragment in the middle, and Fab' or Fab fragment the fastest. As for preparation, Fab' fragment was prepared from the reduction of F(ab')$_2$ fragment and then was followed by purifying procedure. The key step was the difficult control of reduction condition. F(ab')$_2$ fragment was prepared and purified from pepsin or papain digestion. Fab fragment was also prepared by papain digestion and then purification, but because of the small number of sulfhydryl group per molecule, Fab fragment was difficult to label with ^{99m}Tc. We used 2-iminothiolane (a chemical compound, MW: 137) to modify Fab fragment, so the number of sulfhydryl group per molecule of IT-Fab was increased from 0 to 0.3. Park *et al*[6] reported that the number of sulfhydryl group per molecular of F(ab')$_2$ was 4.2 and Fab 0.9. The difference might have resulted from the treatment with $SnCl_2$ before the measurement. Rhodes[7] reviewed that the protein to be labeled need to contain free sulfhydryl (-SH) group or reactive monosulfides (-S or -S-metal), and the labeling yield was significantly and positively related to the number of sulfhydryl group. We have modified F(ab')$_2$ with 2-iminothone in the same way (with the number of SH per molecule of 0.7) and labeled with ^{99m}Tc. The labeling yield was 92% by PC, which was higher than that of ^{99m}Tc-IT-Fab (50%-80%). This result proved what was mentioned above. Paik[6] proposed that there were two sites on the IgG molecule, one with a high-affinity and low-capacity and the other with a low-affinity and high-capacity. In our study, 50%-80% of labeling yield was obtained by PC, and 20%-30% by GCC, which suggested that the same phenomenon existed on the McAb fragment. Because of the low-affinity binding, ^{99m}Tc was cleared on the column by Sephadex G_{50}. GH, as a renal function imaging agent, was rapidly excreted from the kidney, and was reported to have some tumor-affinity characteristics[8]. But the present resultsdemonstrated that ^{99m}Tc-GH had no affinity to hepatoma. This proved the specificity of ^{99m}Tc-IT-Fab localized on the tumor. The best tumor imaging was at 12 h-22 h after the administration of ^{99m}Tc-IT-Fab. The background excretion was mainly by kidney route. This was similar to the reported imaging time and excretion route of ^{99m}Tc-Fab'[9]. The biodistribution was mainly in the kidney (32.6% ID/g) and next in the blood, the tumor, liver, and stomach. Our results demontrated that ^{99m}Tc-IT-Fab could bespecifically localized in the tumor of nude

mice bearing human heptocellular carcinoma within 24 hours, and this method could be effectively used for labeling McAb Fab fragment with ^{99m}Tc.

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