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Studies on the Distribution and Radioimmunoimaging of ^{99m}Tc-Labeled 5-Fluorouracil Loaded Immunological Nanoparticles in Tissues and Human Gastric Carcinoma Xenografts

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OBJECTIVE To explore the method of preparation of ^{99m}Tc labeled Anti-VEGF McAb 5-FU loaded polylactic acid nanoparticles (^{99m}Tc-5-FU-Ab-NPs), and investigate the biological distribution of the nanoparticles in human gastric carcinoma xenografts.

METHODS Anti-VEGF monoclonal antibodyes (MCAB) in 5-FU-Ab-NPs were labeled with ^{99m}Tc using a modified Schwarz method. After isolation of the ^{99m}Tc-5-FU-Ab-NPs using a Sephadex G-250 column, the labeling percentage and radiochemical purity were determined using paper chromatography. The immunocompetence of the ^{99m}Tc-5-FU-Ab-NPs as tumor markers was determined using ELISA and immunohistochemistry. ^{99m}Tc-5-FU-Ab-NPs (experimental group), ^{99m}Tc-labelled murine multiclonal IgG loaded polylactic acid and nanoparticles (control group) were injected via the tail vein into SCID mice bearing human gastric carcinoma. A radio-immunity ECT image was developed at 2 and 6 h after the injection. Following the ECT imaging, the mice were sacrificed, their tissue and tumor radioactivity distribution determined, and percentage of the injected-dose per gram (%ID/g) and tumor/nontumor (T/NT) ratio calculated. High performance liquid chromatography (HPLC) was used to determine the 5-FU concentration in the tumor tissue and blood in the mice of both groups.

RESULTS The percentage of ^{99m}Tc-5-FU-Ab-NPs labeling was 90%~95%. There was no obvious decrease in the antibody activity before and after labeling. The radio-immuno-imaging (RII) showed that the tumor image had developed 2 h after injection of the ^{99m}Tc-5-FU-Ab-NPs, and with time it was clearer at the 6th hour following the injection. The %ID/g of the tumor tissue at both 2 h and 6 h after the injection was significantly higher compared to the control group. The tumor %ID/g and the tumor to blood activity ratio (TB) of the experimental group at 6 h following the injection increased compared to that at 2 h, and at the same time, 5-FU concentration in the tumor of the experimental group continuously increased over time, and showed a significant difference compared to the 5-FU concentration in the tumor of the control group.

CONCLUSION The ^{99m}Tc-5-FU-Ab-NPs prepared in this study are adequate to meet the demands of the RII, and the immune targeting ability of the anti-VEGF MCAB is reliable. Six hours after injection, the ^{99m}Tc-5-FU-Ab-NPs showed a relatively high specific concentration shadow in the human gastric carcinoma xenografts.

KEYWORDS: radionuclide imaging, gastric carcinoma, monoclonal antibody, nanoparticles.

INTRODUCTION

VEGF monoclonal antibodies (MCAB) for human gastric carcinoma can combine with the related antigen of human gastric carcinomas. These antibodies bound to nanometer particles can be used

to diagnose and treat a tumor by forming a conjugate with the tumor. Although ^{131}I labeled MCAB can be used for radio immuno-imaging (RII) and radioimmunotherapy, its nuclear characteristics are not ideal. Since the physical characteristics of ^{99m}Tc are better[1], we have studied ^{99m}Tc-labeled[2] anti-VEGF MCAB and conjugate-loaded 5-FU- NPs[3]. In our study we conducted an empirical examination of the antibodies in the tissues and RII in the SCID mouse bearing a human gastric carcinoma. In addition we conducted a preliminary investigation of the feasibility of anti-VEGF MCAB guided 5-FU-NPs targeted therapy for gastric carcinoma, to provide a basis for clinical applications.

MATERIALS AND METHODS

Materials

The human gastric MGC803 carcinoma cell line, with over expression of VEGF, was supplied by the Shanghai Cytology Institute, the Chinese Academy of Science. The cells were cultured in RPMI 1640 nutrient solution with 10% calf serum, 100 μg/ml penicillin and 100 μg/ml streptomycin, to lepidic growth at 37.5 ℃ under 5% CO_2 followed by 0.5% trypsinization passage. The ^{99m}Tc was provided by the Chinese Institute of Atomic Energy. The 5-FU NPs were prepared with polylactic acid using a supersonic emulsifying solvent volatilization method[4], producing particles with an orbicular contour and mean diameter of 191 nm. Besides a sustained-release ability in vitro, the drug-loading rate and envelope rate attained 15.2% and 45%.

Establishment of a SCID mouse model bearing human gastric carcinoma

Thirty SCID mice (bought from the Animal Breeding Institute, China Academy of Military Medical Science), were 4 to 6 weeks of age and weighed 18 to 22 g. The male to female ratio was 1:1 and all had a national animal inspection certificate. Initially, 6 mice (3 males and 3 females) were used to grow the MGC803 carcinoma cell suspensions into a solid tumor. Each mouse was injected subcutaneously into the lower left quadrant with 2×10^6 cells in 100 μl of the cell suspension. When the transplanted cells had grown to 1~1.5 cm, approximately 150 mg of the tumor was removed using a strict aseptic technique, and the tumor tissue was tailored to a size of 2~3 mm. Two or 3 pieces of the fresh gastric carcinoma tissue were quickly transplanted subcutaneously into the ventral part of the left hind limb of each the remaining 24 mice which had been divided into four groups of 6 mice each (3 males and 3 females). Two of these groups served as control groups, and two as experimental groups (to be sacrificed at 2 and 6 h after administration of their respective nanoparticles). Two weeks after successful tumor transplantation, a piece of the transplanted tumor tissue was taken out for a histopathological assessment, thereby confirming the conformity between the features of the transplantated tumor with that of the cancer cell line. This transplanted tumor model could be used for our study as the diameter of the tumor reached a size of 1 to 1.5 cm.

Anti-VEGF MCAB and nanoparticle conjugate preparation

The 5-FU-loaded anti-VEGF MCAB polylactic acid nanoparticles (5-FU-Ab-NPs), and the 5-FU-loaded anti-VEGF polyclonal antibody polylactic acid NPs were prepared as previously described[4,5].

^{99m}Tc-labeling of the 5-FU immunological nanoparticles

Newly prepared 5-FU-Ab-NPs (3 ml) in phosphate-buffered solution (PBS), along with 2 mg of mouse anti-VEGF MCAB and an excess of 2-mercaptoethanol (2-ME), ranging from 20~40 μl, was held at 30 ℃ for 30 min to reduce the antibody. Excess 2-ME was removed by dialysis in PBS overnight at 4℃. Then 20 μg of $SnCl_2{\cdot}2H_2O$ was added per ml of the 5-FU-Ab-NPs in PBS, and newly produced 99mTc ranging from 550 to 740 MBq was eluted and added to the 5-FU-Ab-NPs in PBS, which was held at room-temperature for 30 min. The ^{99m}Tc-labeled murine multiclonal IgG nanoparticles (^{99m}Tc-5-FU-IgG-NPs) used for the control group were prepared using the same method.

Determination of the labeling rate: Xinhua No. I filter paper was used as a support for paper chromatography which was developed first with a sodium chloride solution. The Rf values of ^{99m}Tc-5-FU-Ab-NPs, ^{99m}Tc-IgG-nanoparticles and Tc colloid were all 0.0. The second system was developed with a solution of water: alcohol: ammonia at a ratio of 5:2:1. A solution of bovine serum albumin (BSA, 10 g/L) was first applied at the time of sample application, and it was conducted once more following an open-air drying. So the Tc colloid remained at the original point, and the other constituents developed to the solvent front. When the solvent was at 10 cm, open-air drying was performed, and a scan conducted using a TLC-MINI-Scan, and Laura 3.0 software used for analysis. Employing column chromatography[5], the double bed volume was washed using a 0.01 $mol{\cdot}L^{-1}$ PBS on the Sephadex G250 (10 cm × 1 cm) chromatographic column, and a total of 20 test tubes of eluant were collected, with 0.5 ml/tube. Radioactivity intensity

and ultraviolet absorption at 280 nm were measured for each tube.

To assay the immunocompetence of the tumor marker, an enzyme-labeled ELISA indirect method was used to determine the tumor marker and the dilution of the pre-labeled antibody. The immunohistochemical test showed that there was a immunoreaction between the tumor marker and paraffin sections of the gastric carcinoma. A sufficient quantity of labeled 5-FU-Ab-NPs in PBS was used for bacterial culture, and 0.3 ml of the labeled 5-FU-Ab-NPs in PBS was injected into the aural vein of two rabbits. The effects on the 24-h heart rate, respiration and body temperature, were noted.

Distribution of the ^{99m}Tc-5-FU-Ab-NPs in the SCID mouse tissues and tumor

In the experimental group, each SCID mouse was injected via the caudal vein with 0.3 ml of the ^{99m}Tc-labeled 5-FU Ab NPs having 4.81 MBqs of ^{99m}Tc. The control mice were injected in the same way with 0.3 ml of the ^{99m}Tc-IgG-labeled 5-FU NPs having 4.81 MBqs of ^{99m}Tc. The experimental and control groups were both examined at 2 and 6 h after injection of their respective NPs. Specific methods were as follows: the mice of each group underwent planar imaging by SPECT (single photon emission computed tomography) at 2 and 6 h after the injection. Then at 2 and 6 h following the injection, blood was sampled from the orbital venous sinuses, and the SCID mice were sacrificed by cervical dislocation. The mouse heart, liver, spleen, kidney and tumor tissues were removed, and weight recorded. Radioactive levels for each tissue at the 2 and 6-h time periods were measured using a gamma counter. The values of the %ID/g and T/NT specific values were calculated.

Determination of the 5-FU concentration in the tumor tissue and blood by HPLC

The tumor tissue and 0.5 ml of blood from each SCID mouse were removed, and the adipose tissue and connective tissue separated from the tumor. The tumors were cut into small pieces, weighed, and 0.5 g was removed and ground using a tissue homogenizer. PBS buffer was added to a volume of 2 ml and the tissue sonicated to break the cells. The suspension was centrifuged, and 0.5 ml of the supernatant fluid added to a test tube containing 2 g of $(NH)_2SO_4$. Then 7 ml of a solution containing acetic acid and isopropanol (85:15, V:V) was added, with stirring for 5 min followed by centrifugation at 2000 rpm for 10 min. Some of the supernatant fluid was removed and blow-dried. The residue was added to 0.4 ml of the mobile phase, and was filtered. A 20 μl sample was taken for the 5-FU determination by HPLC. Blood plasma (0.2 ml) was placed in a test tube containing 0.5 g of $(NH_4)_2SO_4$. Other procedures were the same as described. The HPLC (HP1100, Agilent) conditions were as follows: chromatographic column, YWGC18, 46×250 mm, 5 μmol/L; mobile phase: 0.01 mol/L phosphate buffered saline (pH 2.8), final concentration of phospho-triethylamine was 5 mmol/L; flow rate: 0.5 ml/min; determination wavelength: 270 nm. The chromatographic peak of the samples was recorded.

Statistical analysis

SPSS 10.0 software was used for the *t* test between two means.

RESULTS

Characteristics of the ^{99m}Tc-5-FU-Ab-NPs

The labeling percentage of ^{99m}Tc-labeled 5-FU-Ab-NPs and 5-FU-IgG NPs was 95.1% and 93.3%, respectively, producing particles with a radiochemical purity of over 95%. An immunological activity assay was conducted after labeling the nanoparticles. The indirect method of enzyme-labeled immunosorbent assay showed that the titer of the tumor marker was roughly the same as that with the pre-labeled antibody. Immunohistochemical analysis showed that the tumor marker reacted on paraffin sections of the gastric carcinoma, and staining of cancer cells was the same as that with the pre-labeled antibody, see Fig.1. Paper chromatography assessment indicated that the labeling percentage ranged from 90% to 95%.

Development of the distribution of ^{99m}Tc-5-FU-Ab-NPs in the mouse tissues and the gastric tumor

SPECT planar imaging was performed at 2 and 6 h after injections of the experimental group and the control mice. A gamma counter was employed to determine the tissue radioactivity, the values of the %ID/g and T/NT ratio were calculated (Tables 1,2). After injection of the ^{99m}Tc-5-FU-Ab-NPs, the %ID/g value of the tumor tissues at both times was significantly higher compared to mice receiving the control ^{99m}Tc-5-FU-IgG-NPs (P<0.05). At 2 and 6 h after injection of the 99mTc-5-FU-Ab-NPs, the %ID/g value of the tumor tissue and ratio of radioactivity between the tumor and blood (T/NT) were both raised from 4.11 ±1.47 and 1.36±0.83 to 7.31±0.62 and 2.87±0.56, respectively, resulting in a significant difference at each time (P<0.05). The 5-FU concentration in the tumor

Table 1. Tissue distribution of ^{99m}Tc-5-FU-Ab-NPs and 99mTc-5-FU-IgG-NPs in SCID mice bearing the transplanted human gastric carcinoma (n=6) (%ID/g, $\bar{x}$±SD).

Tissues	Experimental group (2 h) ^{99m}Tc-5-FU-Ab-NPs	Control group (2 h) ^{99m}Tc-5-FU-IgG-NPs	Experiment group (6 h) ^{99m}Tc-5-FU- Ab-NPs	Control group (6 h) ^{99m}Tc-5-FU- IgG-NPs
Blood	4.02 ± 1.05	3.87 ± 1.34	2.06 ± 0.83#	2.43 ± 0.40
Liver	4.72 ± 1.23	3.92 ± 1.62	3.32 ± 1.35#	3.29 ± 0.51
Spleen	3.56 ± 1.60	5.12 ± 1.53	1.51 ± 0.74#	1.65 ± 0.62
Kidney	2.71 ± 1.78	5.34 ± 0.93	3.31 ±1.06#	3.16 ± 0.54
Heart	2.68 ± 1.39	4.12 ± 0.93	1.87 ± 0.90#	1.96 ± 1.25
Tumor	4.11 ± 1.47*	3.24 ± 1.01	7.31 ± 0.62*	1.31 ± 0.34

* vs. ^{99m}Tc-5-FU-IgG-NPs, P<0.05; # vs. tumor group, P<0.05.

Table 2. Tissue distribution of ^{99m}Tc-5-FU-Ab-NPs and 99mTc-5-FU-IgG-NPs in SCID mice bearing the transplanted human gastric carcinoma (n=6) (T/NT, $\bar{x}$±SD).

Tissues	Experimental group (2 h) ^{99m}Tc-5-FU-Ab-NPs	Control group (2 h) ^{99m}Tc-5-FU-IgG-NPs	Experiment group (6 h) ^{99m}Tc-5-FU- Ab-NPs	Control group (6 h) ^{99m}Tc-5-FU- IgG-NPs
Blood	1.36 ± 0.83*	0.88 ± 0.26	2.87 ± 0.56*	1.03 ± 0.32
Liver	1.45 ± 0.79*	0.93 ± 0.34	2.81 ± 0.65*	1.12 ± 0.36
Spleen	1.62 ± 0.81*	0.72 ± 0.28	2.58 ± 0.71 *	0.72± 0.22
Kidney	1.53 ± 0.83*	0.79 ± 0.33	1.86 ±0.36*	0.76 ± 0.24
Heart	1.47 ± 0.92*	0.86 ± 0.19	2.06 ± 0.57 *	0.96 ±0.15

* vs. ^{99m}Tc-5-FU-IgG-NPs, P<0.05.

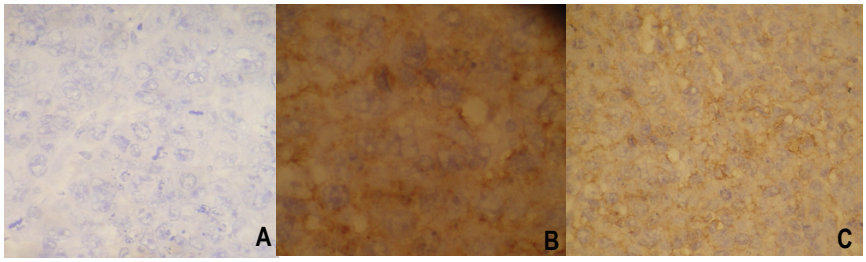
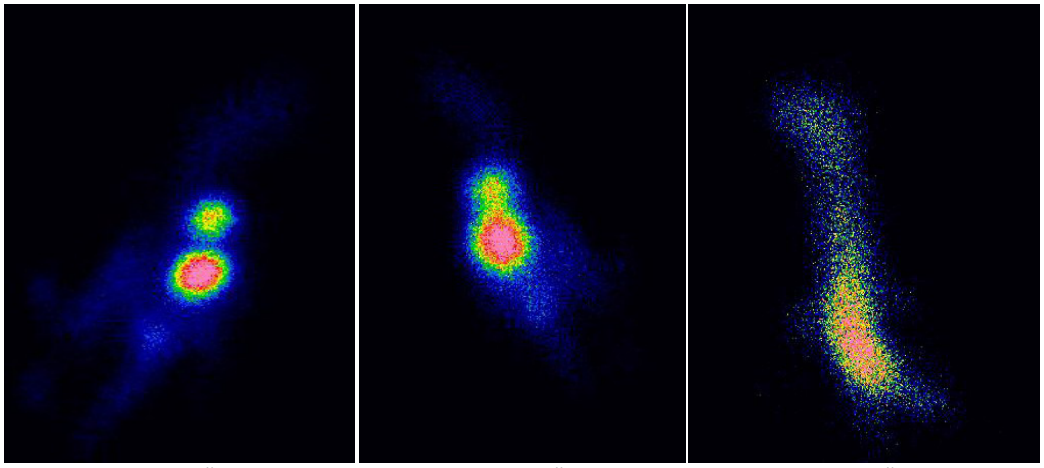


Fig.1. Relationship between the tumor marker and paraffin section of gastric carcinoma shown by immunohistochemical analysis × 200. A: The blank control group (^{99m}Tc-5-FU-IgG-NPs) B: The experimental group (^{99m}Tc-5-FU-Ab-NPs) C: The positive control group (anti-VEGF-MCAB)



Two hours after injection of ^{99m}Tc-5-FU-Ab-NPs | Six hours after injection of ^{99m}Tc-5-FU-Ab-NPs | Six hours after injection of ^{99m}Tc-5-FU-IgG-NPs

Fig.2. Planar imaging of a transplanted gastric carcinoma in a SCID mouse using SPECT.

was higher in the experimental group compared to the control group. The tumor image of the experimental group developed and became clearer with time at 6 h following the injection. However in the controls a diffuse distribution occurred, so no tumor image was found (Fig.2).

Concentration of 5-FU in the tumor and blood

Measurement of 5-FU concentration demonstrated that at 2 h after injection of the nanoparticles, the concentration of 5-FU in the tumor and blood was respectively 2.26±0.15 μg/g and 1.17±0.13 μg/ml in the experimental group, while in the control group, it was 1.08±0.10 μg/g and 1.32±0.12 μg/ml. At 6 h the respective concentrations were 3.59±0.12 μg/g and 0.97±0.13 μg/ml in the experimetal group, and 1.02 ±0.14 μg/g and 1.01±0.13 μg/ml in the controls. At 2 and 6 h after treatment, there was a significant difference in the 5-FU concentration between the tumor tissue of the experimental group and the control group (P<0.05). There also was a significant difference in the tumor 5-FU concentration in the experimental group comparing the 2 and 6 h time periods (P<0.05).

DISCUSSION

Anti-tumor MCAB may combine with tumor cells enabling radioisotope labeled antibodies to be used to conduct tumor RII or treatment. Apart from CT and MR, what the RII reflected is not only the anatomical functional imaging, but also the physiological, biochemical and pathological states. It has been confirmed previously by empirical studies that RII can aid in the diagnosis and assessment of tumors, especially, for differential diagnosis of obscure, recurrent and metastatic tumors[1]. The anti-gastric carcinoma VEGF MCAB used in our research did not combine with normal gastric mucosa, but it strongly reacted with the gastric carcinoma cells.

Anti-gastric carcinoma VEGF MCAB and 5-FU-Ab-NPs, the immune conjugate,combined with the 5-FU nanoparticles, only had a specific targeted orientating function on the gastric carcinoma cells, suggesting that anti-gastric carcinoma VEGF MCAB can play a role in tissue targeting. The 5-FU is the choice drug for treating malignant gastrointestinal tumors, being a time-dependent drug that needs a delayed time to exert its optimal anti-cancer effect[6,7]. The findings of our study showed that following intravenous administration, the concentration of 5-FU-Ab-NPs in the tumor increased 2 or more times, compared to that of the peripheral blood. With time, the 5-FU tumor concentration gradually increased. It has been confirmed that employment of 5-FU for treating gastrointestinal cancer is a rational therapy, and is consistent with the 5-FU pharmacokinetics[8].

Over-expression of VEGF is common in malignant solid tumors, and repression of the VEGF activity can significantly inhibit tumor growth[9]. With the anti-tumor antibodies as the targeting agent, and the polylactic acid (PLA) NPs as the vehicles, the NPs can deliver 5-FU to the foci of the tumor as well as inhibit angiogenesis. In our study, the anti-VEGF MCAB and 5-FU polylactic acid NPs immune conjugates were prepared in advance, and we had shown that this immune conjugate maintained its immunological activity[5]. Paper chromatography analysis showed the labeling percentage of the ^{99m}Tc-5-FU-Ab-NPs to be over 90%, indicating that the prepared ^{99m}Tc-5-FU-Ab-NPs can meet the needs of RII. By using the ELISA indirect method, we showed that the titer of the tumor marker was roughly the same as that of the antibody before labelling. It was confirmed by immunohistochemical staining that there was no obvious alteration in activity of the anti-VEGF MCAB after labelling. Based on %ID/g indices of the tumor marker taken up by the tissues, our study demonstrated that the marker is mainly distributed in the blood, liver, kidney, spleen and tumor. At 2 and 6 h after injection of the ^{99m}Tc-5-FU-Ab-NPs, the percentage of radioactivity taken up by the tumor, i.e. the value of ID%/g, in the treatment group was significantly higher, compared to the control group which received the ^{99m}Tc-IgG-NPs (P<0.05). The value of the ID%/g and the ratio of activity in the tumor to that in the blood at 2 and 6 h were respectively raised from 4.11±1.47 and 1.36±0.83 to 7.31±0.62 and 2.87±0.56, with a significant difference between the two times (P<0.05). In the treatment group, the ratio of activity between the tumor and the blood, at 6 h after injection, was 2.87 ±0.56, while in the control group, it was 1.03±0.32. There was a significant difference between the two groups (P<0.05).

The ^{99m}Tc has a predominant nuclear physical /nucleo-physical characteristics, a strong chelating capacity and an ideal half life, and thus can be adapted for low-energy γ–ray imaging. ^{99m}Tc as a labeling agent can be employed for imaging nearly all the organs[10]. The imaging time of the ^{99m}Tc–MCAB ranged from 2 to 24 h in our SCID mouse bearing a human gastric carcinoma, which is earlier compared to that using ^{131}I-labeled MCAB which ranges from 72 to 96 h [11]. Our study showed the body distribution and enrichment of the shadow in the tumor of the experimental group at 2 and 6 h compared to the controls receiving ^{99m}Tc-monoclonal IgG. With the elapse of time,

the amount entering the tumor tissue also increased. Our findings suggest a definite pragmatic value for localization, assessment, diagnosis, and treatment of tumorous lesions.

In our study, HPLC was used to determine the concentration of 5-FU in the blood and local tumor tissue, to indicate the level of 5-FU-Ab-NPs uptake by anti-VEGF antibody targeting. At 2 and 6 h after injection of the mice, the concentration of the 5-FU in tumor was respectively 2.26±0.15 μg/g and 3.39 ±0.12 μg/g, values which were significantly higher compared to that of the peripheral blood. With time, the 5-FU concentration in the tumor significantly increased, $P<0.05$.

Our results provide an experimental basis for clinical applications using 5-FU-Ab-NPs. These NPs deliver a slow 5-FU release, and a rather high 5-FU tumor concentration following intravenous injection.

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