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Enhanced Tumor Localization of Radiolabeled Fab Fragments of Monoclonal Antibody A7 in Nude Mice Bearing Human Pancreatic Carcinoma Xenografts

Eigo Otsuji,[1,3] Toshiharu Yamaguchi,[1] Nobuki Yamaoka,[1] Makoto Kato,[1] Tatsuya Kotani,[1] Kazuya Kitamura,[1] Nozomi Yamaguchi[2] and Toshio Takahashi[1]

[1]*The First Department of Surgery and* [2]*The Research Institute of Neurology and Geriatrics, Kyoto Prefectural University of Medicine, Kawaramachi, Hirokoji, Kamigyo-ku, Kyoto 602*

Much recent research has been directed toward the use of monoclonal antibodies (MAb) for the immunodetection of solid tumors. In pancreatic cancer, the results of conventional immunoscintigraphy using intact MAb remain disappointing. Clear immunoscintigraphy with radiolabeled MAb requires a high tumor tissue/blood ratio of radioactivity and a low normal tissue/blood ratio of radioactivity. In this study, ^{125}I-labeled Fab fragments produced by papain digestion of MAb A7 were injected intravenously into nude mice bearing a human pancreatic cancer (HPC-YS) xenograft previously shown to react specifically with MAb A7. The radioactivity of tumors and normal organs was subsequently measured. The tumor tissue/blood ratio of ^{125}I-labeled Fab fragments of MAb A7 was 1.00 ± 0.24 and 9.68 ± 2.54 at 2 and 24 h after injection, respectively. The tumor tissue/blood ratio of radioactivity was significantly higher than those of normal organs at 24 h after injection. Moreover, the tumor tissue/blood ratio of ^{125}I-labeled Fab fragments of MAb A7 was greater than that of intact MAb A7, although the ^{125}I-labeled Fab accumulation level was much less than that of ^{125}I-labeled intact MAb A7 in the tumor. When mice bearing tumors which did not react with MAb A7 were studied, ^{125}I-labeled Fab fragments did not specifically localize to the tumors. These results suggest that Fab fragments of MAb A7 may be suitable carriers of radionuclides for the immunodetection of human pancreatic cancer.

Key words: Pancreatic cancer — Tumor imaging — Monoclonal antibody A7 — Fab fragment

Radioimmunoscintigraphic applications of MAb[4] for noninvasive detection and visualization of targets such as tumor[1–4] or myocardial infarction[5] have grown immensely. The first reported *in vivo* application of MAb for target visualization was in experimental tumor imaging using ^{131}I as the radiolabel.[6] Fragments of monoclonal antibodies, particularly $F(ab')_2$ and Fab fragments are generally regarded as showing improved tumor imaging.[7–9] This is possibly due to their faster rate of extravasation, allowing quicker tumor localization. In addition, fragments are catabolized more quickly than an intact antibody, leading to accelerated removal of blood and normal tissue radiolabel.

Although successful immunoscintigraphy of pancreatic cancer in xenografted nude mice or in patients with pancreatic cancer has been reported,[10] there is no antibody available that is suitable for routine clinical use.

Kotanagi *et al.* produced the MAb A7 against a human colon carcinoma and demonstrated that it reacted with colonic carcinoma with high sensitivity.[11] MAb A7 has been reported to recognize an antigen distinct from other cancer-associated antigens.[12] In addition, we have reported reactivity of MAb A7 with human pancreatic cancers,[13] and MAb A7 has been applied clinically as a carrier of an anti-cancer antibiotic, NCS (Kayaku, Tokyo).[14] Good tumor localization by MAb A7 radiolabeled with ^{125}I has also been reported,[15] though to maximize its clinical utility in tumor imaging, a higher tumor tissue/blood ratio is desired. In this paper, we used a human pancreatic cancer xenograft in an attempt to achieve improved tumor localization with Fab fragments of MAb A7.

MATERIALS AND METHODS

Cell lines The human pancreatic carcinoma cell line, HPC-YS,[16] and the human squamous cell carcinoma, KB-2,[13] were used in this study. HPC-YS was established from a ductal cell adenocarcinoma of the human pancreas and obtained from N. Yamaguchi (Research Institute of Neurology and Geriatrics, Kyoto Prefectural University of Medicine). KB-2 cells were purchased from the American Type Culture Collection, Rockville, MD. Both of these cells were maintained in RPMI 1640 medium supplemented with 10% FBS (Flow Laboratories Inc., Rockville, MD).

[3] To whom requests for reprints should be addressed.

[4] Abbreviations: MAb, monoclonal antibody; NCS, neocarzinostatin; FBS, fetal bovine serum; EDTA, ethylenediaminetetraacetic acid; PBS, phosphate-buffered saline; %ID/g, % injected dose/g; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Tumor xenografts Cultured HPC-YS cells were harvested by brief treatment with EDTA, washed in PBS and resuspended in PBS. Approximately 5×10^6 viable cells were injected s.c. into the left flank of athymic eight-week-old male nude mice (BALB/C, nu-nu) (SLC Co., Shizuoka) weighing approximately 22.5 g. As a control, KB-2 cells were also inoculated into the athymic nude mice by the same method. A tumor mass was detected in all mice injected with the HPC-YS or KB-2 cells.

Monoclonal antibodies MAb A7 was produced by a hybridoma created by fusion of splenocytes from a mouse immunized against human colonic carcinoma cells with murine myeloma P3.X63.Ag8653 cells, as previously described.[11] Ascites was collected from mice injected with hybridoma cells producing MAb A7, and the antibody was purified by ammonium sulfate precipitation. MAb A7 belongs to the IgG_1 class and reacts with 73% of human pancreatic carcinoma cell lines tested, as well as with human colonic carcinoma.[13] MAb A7 does not react immunohistochemically with normal pancreatic tissues.[17]

Preparation of Fab fragments Papain (16–40 unit/mg, Sigma) was added to MAb A7 (A7/papain: 100/1) in 0.1 *M* phosphate buffer containing 0.01 *M* 2-mercaptoethanol, pH 7.2, and incubated at 37°C for 7 h. The reaction was stopped by the addition of 0.014 *M* iodoacetoamide, then the preparations were dialyzed in 5 m*M* disodium hydrogenphosphate buffer. Fab fragments were immediately separated by ion exchange chromatography (DEAE cellulofine) and gel filtration (G 3000 SW). Fab fragments of normal mouse IgG were purchased from Rockland, USA.

Preparation of radiolabeled antibody and Fab fragments Radiolabeling of MAb A7 with ^{125}I (Amersham Japan, Ltd., IMS 30, Tokyo) was performed by the chloramine-T method.[18] Iodinated MAb A7 was separated from excess reactants by gel filtration on a Sephadex G-25 column. Fab fragments of MAb A7 and of normal mouse IgG were also labeled with ^{125}I by the method described above. MAb A7, Fab fragments of MAb A7, and Fab fragments of normal mouse IgG were labeled with ^{125}I to specific activities of 5.9, 5.5, and 5.0 μCi/μg, respectively.

SDS-PAGE of MAb A7 and its Fab fragment The purity of MAb A7 and its Fab fragments obtained by the method described above was analyzed by 4–20% SDS-PAGE under nonreducing conditions according to the method of Laemmli.[19] After electrophoresis, the gel was stained with Coomassie Brilliant Blue.

Binding activities of Fab fragment of MAb A7 to HPC-YS cells The binding activities of Fab fragments were measured by a competitive radioimmunoassay using HPC-YS cells. Aliquots of HPC-YS cells (5×10^5) were incubated with ^{125}I-labeled MAb A7 (1×10^5 cpm) in the presence of serially 5-fold diluted antibody or Fab fragments in PBS at 37°C for 60 min. The antibody and Fab fragments ranged from $(1/5)^{11}\times10^{-5}$ to 1.0×10^{-5} mol/liter. After incubation, cell pellets were subjected to γ-scintillation counting and the percent inhibition was calculated as compared to the control.

Biodistribution of radiolabeled Fab fragments The distribution of Fab fragments was investigated in athymic nude mice bearing HPC-YS tumors and compared to those of MAb A7 of Fab fragments of normal mouse IgG. Three weeks after inoculation, the tumor-grafted mice were divided into three groups: eight mice were injected intravenously with 0.7 μCi of either ^{125}I-labeled MAb A7, ^{125}I-labeled Fab of MAb A7, or ^{125}I-labeled Fab of normal mouse IgG. Four mice from each group were killed at 2 and 24 h after injection and dissected. After dissection, the tumors, blood, and normal organs (lung, heart, liver, spleen, pancreas, stomach, colon, and kidney) were weighed. The mean weight of the tumors was 135 mg. The radioactivity in each tissue was then measured using a γ-scintillation counter. The results from the various tissues were expressed as cpm/g and compared to each other. To compare the specific localization of the three probes in the tumor to that in the blood or normal tissues, the ratio of radioactivity in these tissues to that in the blood was calculated. These ratios were derived by dividing the radioactivity in the various tissues per weight basis by that in total blood per weight basis. Student's *t* test was used to establish the statistical significance of differences.

The distribution of the three probes in the A7 antigen-negative tumors was also examined by the same method in mice bearing KB-2 tumors, which do not react with MAb A7 or Fab fragments of MAb A7.

RESULTS

SDS-PAGE of MAb A7 and its Fab fragment MAb A7 and Fab fragment preparations each appeared as a single band after electrophoresis in 4–20% gradient SDS-PAGE followed by Coomassie blue staining. The apparent molecular weights of the whole antibody and its Fab fragments were approximately 1.5×10^5 and 4.5×10^4, respectively (Fig. 1).

Binding activities of Fab fragment of MAb A7 to HPC-YS cells The binding activity of Fab fragments of MAb A7 was compared to that of intact MAb A7 by competitive radioimmunoassay. The results indicate that Fab fragments of MAb A7 retained a binding activity nearly identical to that to intact MAb A7 (Fig. 2).

Biodistribution of radiolabeled Fab fragments in nude mice bearing HPC-YS tumors The accumulation of ^{125}I-labeled Fab fragments of MAb A7 in the blood and HPC-YS tumors of mice after intravenous injection was

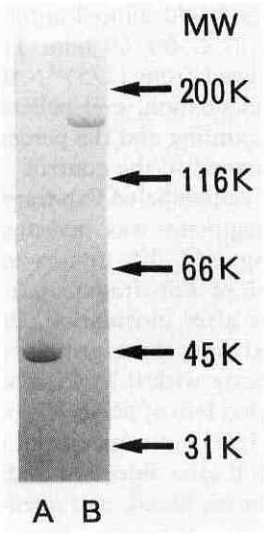

Fig. 1. The 4–20% SDS-PAGE of MAb A7 and its Fab fragment. The molecular weights of the whole antibody and its Fab fragments were approximately 1.5×10^5 and 4.5×10^4, respectively. A, Fab fragments of MAb A7; B, MAb A7.

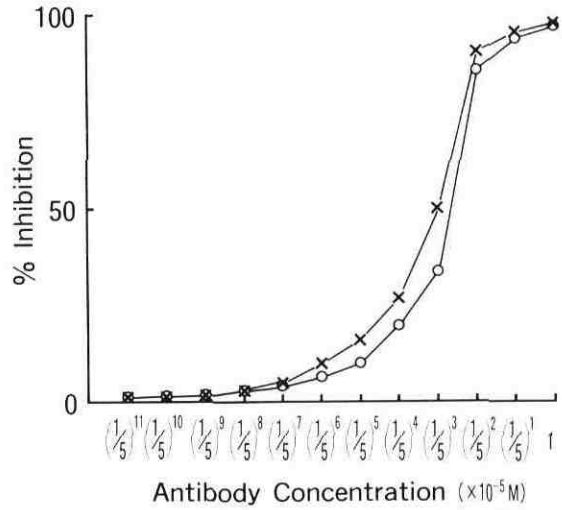

Fig. 2. The binding activities of Fab fragments of MAb A7 were compared to that of intact MAb A7 by a competitive radioimmunoassay. The binding activity was retained by Fab fragments of MAb A7 and was nearly identical to that of intact MAb A7. ○, ^{125}I-labeled Fab fragments of A7; ×, ^{125}I-labeled MAb A7; Points, mean values.

examined and compared to that of ^{125}I-labeled MAb A7 and ^{125}I-labeled Fab fragments of normal mouse IgG. The %ID/g values of the radiolabeled Fab fragments of MAb A7 accumulated in the tumor at 2 and 24 h after injection were 4.34 ± 0.93 (mean $\pm$SD) and 2.10 ± 0.53, respectively. In blood, 4.46 ± 0.81 and 0.22 ± 0.01 %ID/g of ^{125}I-labeled Fab fragments of MAb A7 accumulated at 2 and 24 h after injection, respectively. In contrast, 2.39 ± 0.70 and 0.42 ± 0.11 %ID/g of ^{125}I-labeled Fab fragments of normal mouse IgG were localized in the tumor at 2 and 24 h after injection. In blood, 5.49 ± 0.49 and 0.42 ± 0.11 %ID/g of ^{125}I-labeled Fab fragments of normal mouse IgG accumulated. The amount of ^{125}I-labeled Fab fragments of MAb A7 accumulated in the tumor was significantly greater than that of normal mouse IgG at both 2 ($P<0.05$) and 24 ($P<0.005$) h after injection. On the other hand, the accumulation levels of ^{125}I-labeled MAb A7 in the tumor increased with time (3.50 ± 0.77 at 2 h and 6.12 ± 0.79 at 24 h) after injection. The level of ^{125}I-labeled MAb A7 in blood was much higher than those of the Fab fragments and decreased with time (Fig. 3).

Fig. 4 shows the tissue/blood ratio of the tumors and normal organs at 2 h after injection. The ^{125}I-labeled Fab fragments of MAb A7 gave a tumor tissue/blood ratio of 1.00 ± 0.24 at 2 h. In contrast, the values for normal organs varied from 0.24 ± 0.01 (spleen) to 2.76 ± 0.78 (kidney). The tumor tissue/blood ratio using ^{125}I-labeled normal mouse IgG was 0.43 ± 0.10 and the normal tissue/blood ratios varied from 0.21 ± 0.07 (pancreas) to 2.26 ± 0.15 (kidney). The ^{125}I-labeled MAb A7 gave a tumor tissue/blood ratio of 0.12 ± 0.02. The tumor tissue/blood ratio of Fab fragments of MAb A7 was significantly greater than that of MAb A7 ($P<0.005$) or Fab fragments of normal mouse IgG ($P<0.05$) at 2 h after injection (Fig. 4).

Fig. 5 shows the tissue/blood ratios of ^{125}I-labeled Fab fragments of MAb A7 and normal mouse IgG and intact MAb A7 at 24 h after injection. The tumor tissue/blood ratio for ^{125}I-labeled Fab fragments of MAb A7 reached 9.68 ± 2.54. In contrast, those of normal organs ranged from 0.38 ± 0.05 (liver) to 1.09 ± 0.10 (kidney). The tumor tissue/blood ratio of Fab fragments of MAb A7 was much greater at 24 h after injection than it was at 2 h after injection. The Fab fragments of normal mouse IgG had a tumor tissue/blood ratio of 1.04 ± 0.32 and normal tissue/blood ratios varied from 0.37 ± 0.05 (colon) to 1.05 ± 0.22 (stomach). The MAb A7 tumor tissue/blood ratio at 24 h was slightly increased compared to the value at 2 h after injection. The differences between Fab fragments of MAb A7 and other groups were statistically significant ($P<0.005$).

Biodistribution of radiolabeled Fab fragments in nude mice bearing KB-2 tumors Nude mice bearing KB-2 tumors, which are non-reactive with MAb A7, were studied in a similar fashion. The %ID/g values of the radiolabeled Fab fragments of MAb A7 accumulated in KB-2 tumors at 2 and 24 h after injection were $4.29\pm$

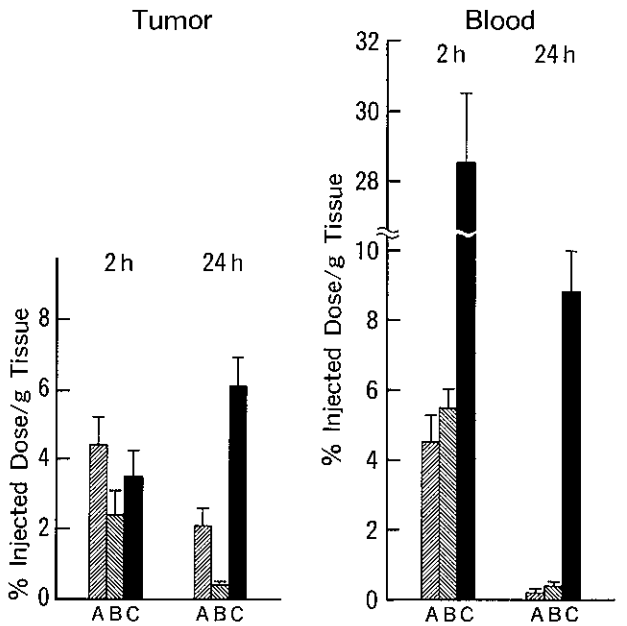

Fig. 3. The accumulation of ^{125}I-labeled Fab fragments of MAb A7 in the blood and HPC-YS tumors of mice after intravenous injection. Although the accumulation of ^{125}I-labeled Fab fragments of MAb A7 in HPC-YS tumors decreased with time, it was significantly greater than that of normal mouse IgG at both 2 ($P<0.05$) and 24 h ($P<0.005$) after injection. The accumulation level of ^{125}I-labeled MAb A7 in the tumor increased time-dependently. The accumulation level of ^{125}I-labeled MAb A7 in blood was much higher than those of the Fab fragments and decreased time-dependently. A, ^{125}I-labeled Fab fragments of MAb A7; B, ^{125}I-labeled Fab fragments of normal mouse IgG; C, ^{125}I-labeled MAb A7; bars, SD.

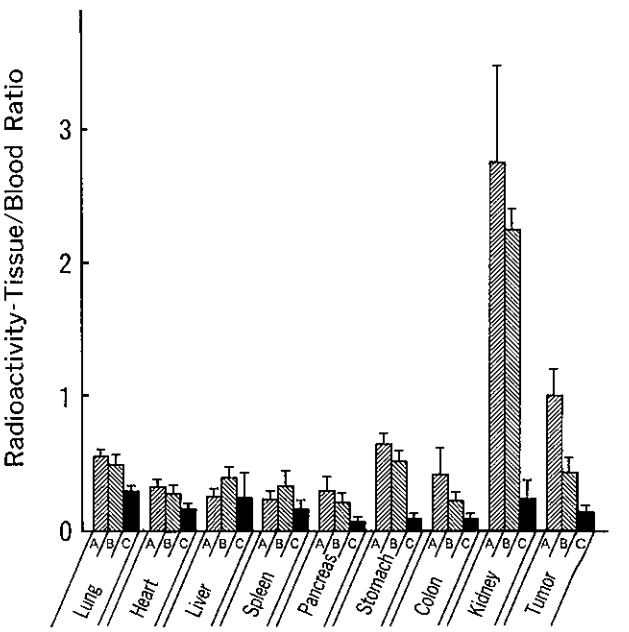

Fig. 4. The values of tissue/blood ratio of the HPC-YS tumors and normal organs at 2 h after injection. The tumor tissue/blood ratio of Fab fragments of MAb A7 was significantly greater than that of MAb A7 ($P<0.005$) or Fab fragments of normal mouse IgG ($P<0.05$). A, ^{125}I-labeled Fab fragments of MAb A7; B, ^{125}I-labeled Fab fragments of normal mouse IgG; C, ^{125}I-labeled MAb A7; bars, SD.

0.95 and 0.23±0.09, respectively. In blood, 8.43±1.39 and 0.36±0.05 %ID/g of ^{125}I-labeled Fab fragments of MAb A7 accumulated at 2 and 24 h after injection, respectively. The %ID/g values of ^{125}I-labeled Fab fragments of normal mouse IgG localized in the tumor at 2 and 24 h after injection were 3.25±0.73 and 0.40±0.08. In blood, 9.67±1.21 and 0.69±0.05 %ID/g of ^{125}I-labeled Fab fragments of normal mouse IgG accumulated. Accumulation of the fragments of both MAb A7 and normal mouse IgG in the tumors was low with no significant differences between the amounts accumulated (Fig. 6).

The values of tumor tissue/blood ratio were 0.50±0.06 and 0.33±0.04 2 h after injection, respectively, for ^{125}I-labeled Fab fragments of MAb A7 and normal mouse IgG. At 24 h after injection, the values of tumor tissue/blood ratio were 0.61±0.16 and 0.58±0.12 for ^{125}I-labeled Fab fragments of MAb A7 and normal mouse IgG. No significant difference was observed between the value for the Fab fragments of MAb A7 and normal

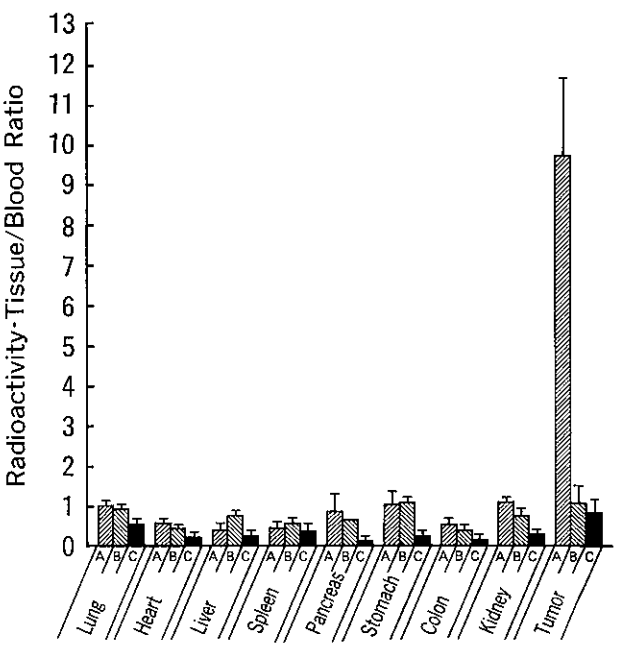

Fig. 5. The values of tissue/blood ratio of the HPC-YS tumors and normal organs at 24 h after injection. The tumor tissue/blood ratio of Fab fragments of MAb A7 was significantly greater than that of MAb A7 or Fab fragments of normal mouse IgG ($P<0.005$). Bars, SD.

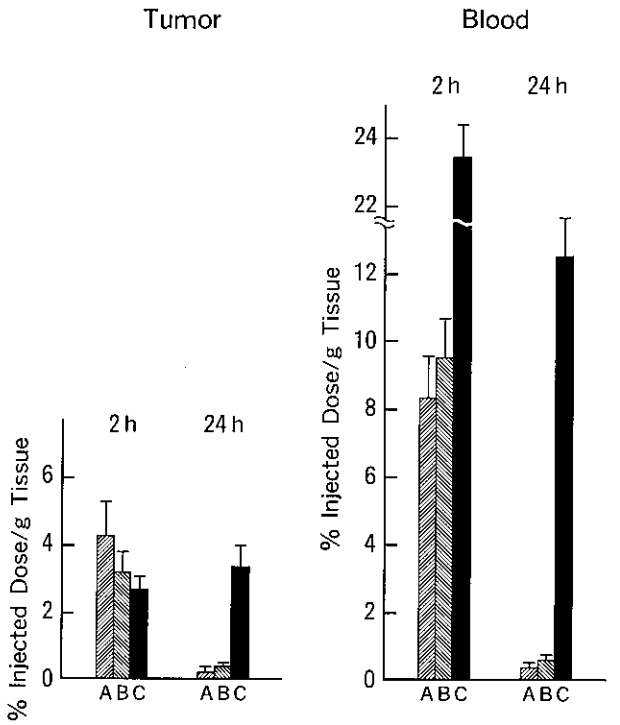

Fig. 6. The accumulation of ^{125}I-labeled Fab fragments of MAb A7 in blood and KB-2 tumors of mice after intravenous injection. Accumulation of the fragments of both MAb A7 and normal mouse IgG in the tumors was low and there was no significant difference. A, ^{125}I-labeled Fab fragments of MAb A7; B, ^{125}I-labeled Fab fragments of normal mouse IgG; C, ^{125}I-labeled MAb A7; bars, SD.

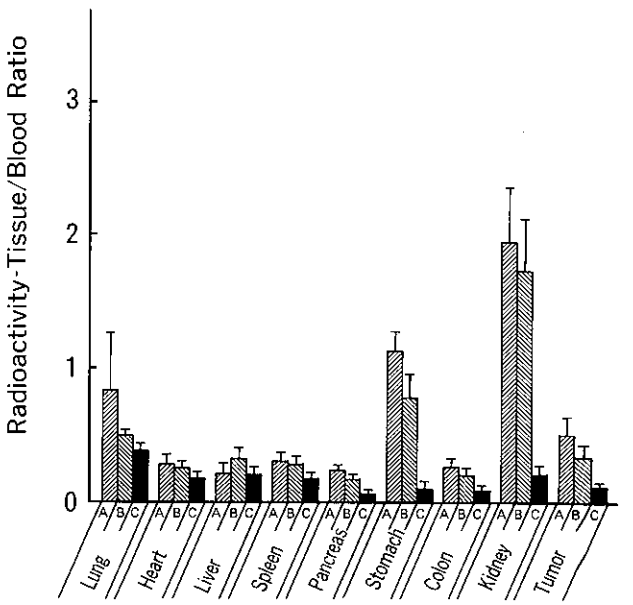

Fig. 7. The values of tissue/blood ratio of KB-2 tumors and normal organs at 2 h after injection. No significant difference was observed between the value for the Fab fragments of MAb A7 and that for the Fab fragments of normal mouse IgG. A, ^{125}I-labeled Fab fragments of MAb A7; B, ^{125}I-labeled Fab fragments of normal mouse IgG; C, ^{125}I-labeled MAb A7; bars, SD.

mouse IgG at either 2 or 24 h after injection. The levels of radioactivity in normal organs was also low for mice injected with ^{125}I-labeled Fab fragments of MAb A7 and normal mouse IgG, and they were similar to each other (Figs. 7 and 8).

DISCUSSION

In this study, we evaluated the potential utility of radiolabeled Fab fragments of MAb A7 in the imaging of pancreatic cancer. To be useful, any probe must have high *in vivo* specificity and rapid clearance from the vascular space. To address these issues, we studied the distribution of Fab fragments of MAb A7 in nude mice bearing human pancreatic carcinoma xenografts. The Fab fragments of MAb A7 were produced by papain digestion, with retention of binding activity. We expressed the distribution of ^{125}I-labeled Fab fragments to tumor and normal tissues in terms of the values of tissue/blood ratio of radioactivity. The values of HPC-YS tumor tissue/blood ratio of ^{125}I-labeled Fab fragments of MAb

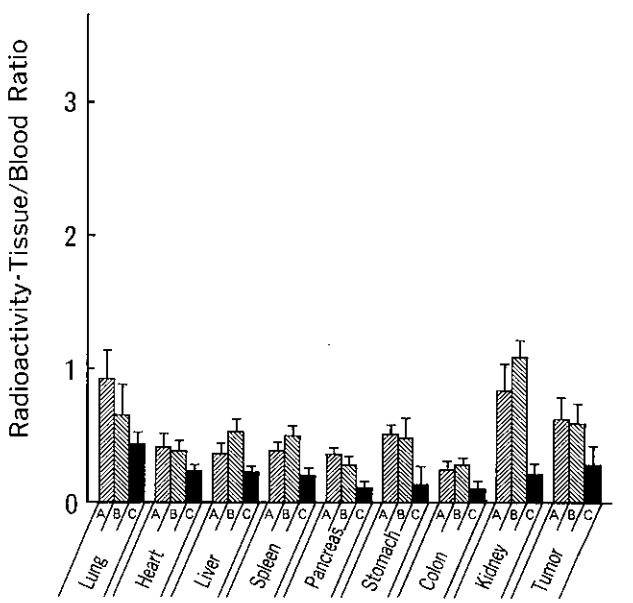

Fig. 8. The values of tissue/blood ratio of the KB-2 tumors and normal organs at 24 h after injection. No significant difference was observed between the value for the Fab fragments of MAb A7 and that for the Fab fragments of normal mouse IgG. A, ^{125}I-labeled Fab fragments of MAb A7; B, ^{125}I-labeled Fab fragments of normal mouse IgG; C, ^{125}I-labeled MAb A7; bars, SD.

A7 were approximately 2.3 and 9.3 times greater than those of ^{125}I-labeled Fab fragments of normal mouse IgG at 2 and 24 h after injection, respectively. In contrast, the values of normal tissue/blood ratio of ^{125}I-labeled Fab fragments of MAb A7 and normal mouse IgG were low and comparable to each other, except for kidney. Hansson *et al.* demonstrated that clearance of non-Fe-bearing antibody fragments is rapid and may be mainly via the kidney, while removal of intact Ig occurred mainly through interaction with Fc-receptor-bearing cells followed by a slow clearance via the reticuloendothelial system.[20] Furthermore, Pimm *et al.* reported that radioiodine accumulating in the kidneys was partially due to the metabolism of fragments elsewhere, probably in the liver and intestine.[21] Although the kidney tissue/blood ratios of ^{125}I-labeled Fab fragments of MAb A7 and normal mouse IgG were relatively high, these values decreased in a time-dependent manner and were considered to be due to non specific binding. When mice with KB-2 tumors, to which MAb A7 does not bind, were used, the accumulation of ^{125}I-labeled Fab fragments of MAb A7 in the tumor was low and there was no significant difference between the values for Fab fragments of MAb A7 and of normal mouse IgG. These results suggest that the localization of ^{125}I-labeled Fab fragments of MAb A7 to the pancreatic carcinoma was due to antigen-antibody specific binding.

MAb A7 has been covalently linked to NCS, and this conjugate has been used to treat more than 70 patients with colorectal or pancreatic cancer. Some of the patients who have been treated with this conjugate have had an apparent regression of their tumors.[14] In the animal experiments, MAb A7 was reported to localize specifically in pancreatic cancer xenografted in nude mice.[15] In our studies, a larger amount of ^{125}I-labeled MAb A7 accumulated in the HPC-YS tumors as compared with ^{125}I-labeled Fab fragments of MAb A7 at both 2 and 24 h after injection. Generally, the tumor tissue/blood ratio is more important than the absolute accumulation value in the tumors for tumor imaging. The tumor tissue/blood ratio of ^{125}I-labeled Fab fragments of MAb A7 was higher than that of ^{125}I-labeled MAb A7 at both 2 and 24 h after injection.

The tumor accumulation of ^{125}I-labeled MAb A7 increased with time, while that of ^{125}I-labeled Fab fragments of MAb A7 decreased. Moreover, the tissue/blood ratio of ^{125}I-labeled Fab fragments of MAb A7 increased rapidly, while that of ^{125}I-labeled MAb A7 increased slowly. These findings suggest that Fab fragments of MAb A7 rapidly leave the vascular space and penetrate tumor tissue, making their radiolabeled conjugate useful for rapid visualization of a tumor.

Reintgen *et al.* have reported that an antibody tumor/blood ratio must be greater than 2 to 10 for reliable tumor imaging,[22] and Tsuda *et al.* have successfully performed immunoscintigraphy of pancreatic cancer xenografts in nude mice after injection of an ^{131}I-labeled MAb against pancreatic cancer which achieves a tumor tissue/blood ratio of 3.1.[10] Therefore, the tumor tissue/blood ratio of Fab fragments of MAb A7 should be adequate for tumor imaging. Moreover, the HPC-YS tumor tissue/blood ratio of ^{125}I-labeled Fab fragments of MAb A7 was approximately 12 times greater than the pancreas/blood ratio at 24 h after injection. Although relatively large amounts of Fab fragments of MAb A7 were localized in the kidney at 2 h after injection, this value decreased rapidly, becoming almost equal to that in blood. Furthermore, the kidneys can be distinguished easily from pancreatic cancer by their location.

Another potential advantage of Fab fragments over intact MAbs is that Fab fragments lack the Fc portion of the molecule, which is the most immunopotent region of intact MAbs.[23] We could not evaluate the significance of this difference in these experiments because we were injecting a mouse antibody and its derivatives into mice.

Our results suggest that Fab fragments of MAb A7 may be useful for the noninvasive immunodetection of human pancreatic carcinomas by scintigraphy.

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