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Therapy and Imaging of Pancreatic Carcinoma Xenografts with Radioiodine-labeled Chimeric Monoclonal Antibody A10 and Its Fab Fragment

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Recombinant mouse/human chimeric monoclonal antibody A10 (ch-A10) and its Fab fragment (ch-Fab) react with carcinoembryonic antigen on various gastrointestinal carcinomas. We performed biodistribution studies with ^{125}I-labeled ch-A10 and ch-Fab in an antigen-positive human pancreatic carcinoma (BxPC-3) xenograft model. We also evaluated the anti-tumor effect of ^{131}I-labeled ch-A10 and studied the detection of BxPC-3 xenografts with ^{123}I-labeled ch-Fab in whole body scintigraphy. In comparative biodistribution studies, the tumor uptake of ^{125}I-labeled ch-A10 was significantly greater than that of ^{125}I-labeled ch-Fab 24 h post-injection. However, the tumor-to-blood ratio was 46.8 for ch-Fab at 24 h post-injection, while it was only 1.4 for ch-A10. Microautoradiography studies showed that ch-Fab penetrated more uniformly into the tumor nodules than did ch-A10. In mice given a therapeutic dose of ^{131}I-labeled ch-A10, a significant inhibition of tumor growth was seen, while control ^{131}I-labeled human IgG did not affect tumor growth. Leukocyte toxicity was observed within 3 weeks after injection of ^{131}I-labeled ch-A10, but leukocyte counts recovered to normal levels at 8 weeks post-injection. In whole-body scintigraphy, clear and rapid tumor imaging was obtained with 200 μCi of ^{123}I-labeled ch-Fab 24 h post-injection. These results suggest that radioiodine-labeled chimeric A10 antibodies could potentially be useful candidates for radioimmunotherapy and radioimmunodetection of pancreatic carcinomas.

Key words: Pancreatic carcinoma — Chimeric monoclonal antibody — Chimeric Fab — Carcinoembryonic antigen

Radiolabeled murine monoclonal antibodies (MAbs) against tumor-specific antigens are being used in clinical therapy[1,2] or diagnosis[3–5] of human malignancies. However, multiple injections of murine MAb often induce development of human anti-mouse antibodies.[6,7] In an attempt to reduce the immunogenicity of murine MAbs, we previously produced recombinant mouse/human chimeric MAb A10 (ch-A10) constructed with the variable regions of the parental murine A10 MAb (m-A10) against carcinoembryonic antigen (CEA) and the constant regions of human IgG1.[8] Recently, functional recombinant chimeric Fab fragments have been expressed in *Escherichia coli (E. coli)* without proteolytic digestion of parental MAb.[9,10] We also developed a large-scale production of recombinant mouse/human chimeric A10 Fab (ch-Fab) from *E. coli* in a mini-jar fermentation system.[8] Ch-Fab demonstrated specific immunohistochemical reactions with most of the gastrointestinal cancers tested, such as pancreatic, colon and gastric carcinomas. The ch-Fab reacted very specifically with CEA without cross-reactivity to CEA-related antigens.

For therapy with radiolabeled MAb, it may be important that antibodies can deliver higher doses of β-irradiating isotopes for longer periods of time to the surface of tumor nodules.[11] We have previously reported that significantly greater antibody uptake was demonstrated in the tumors when mice were treated with m-A10 as compared with chimeric and murine A10 Fabs.[12] For clear imaging, however, the tumor specificity, especially tumor-to-blood ratio, may be more important than tumor uptake.[13,14] The antibody fragments, Fab or $F(ab')_2$, were eliminated faster from the bloodstream and showed higher tumor-to-background ratios than whole IgG.[12–14] Our previous report showed that clearer radiolocalization was obtained without visible uptake in normal organs by ch-Fab as compared with m-A10 in sagittal autoradiographic studies.[12] For clinical imaging, ^{123}I and ^{99m}Tc are often used, since the gamma-ray energy of these isotopes is more suitable for whole-body scintigraphy than that of ^{111}In or ^{131}I.[15,16] Furthermore,

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radioiodines are easy to conjugate to antibodies, while ^{90}Y or ^{111}In are conjugated to antibodies by coupling with bifunctional chelating agents.[17, 18]

Using a mouse model of human pancreatic carcinomas, we investigated whether our ch-A10 and ch-Fab could be used as therapeutic or diagnostic agents. In the present report, we used ^{125}I-, ^{131}I- or ^{123}I-labeling to examine the biodistribution of chimeric A10 antibodies and their *in vivo* efficacy for radioimmunotherapy and radioimmunodetection, respectively.

MATERIALS AND METHODS

Production of ch-A10 and ch-Fab Development and characterization of ch-A10 (IgG1) and ch-Fab was previously described.[8] The mammalian cell expression vectors for ch-A10 light-chain (pING2252) and heavy-chain (pING2254) were constructed at Xoma Corp. (Berkeley, CA). The maps of the two vectors are shown in Fig. 1A. Each plasmid contains the immunoglobulin heavy chain enhancer element, the Abelson LTR promoter, the simian virus 40 16S splice junction, the chimeric immunoglobulin light or heavy chain gene, the genomic immunoglobulin polyadenylation signals and a selectable marker (gpt or neo). Transfection was performed in the following manner. The light-chain plasmid was introduced into a murine myeloma cell-line (Sp2/0) by electroporation, and then the heavy-chain plasmid was introduced into high productivity chimeric light-chain transfectants. Ch-A10 was purified from the culture supernatant of transfectant cells at Xoma Corporation. Ch-Fab expression vector, pING3204 was also constructed at Xoma Corporation. The plasmid pING3204 was derived from pIT106,[9] in which kappa and Fd genes were placed under the control of the *Salmonella typhimurium araB* promoter[19] (Fig. 1B). Ch-Fab was produced directly in a secretory *E. coli* clone, E101/pING3204, with induction of L-arabinose in mini-jar fermentation systems. Purified ch-Fab was obtained from culture supernatants using ion-exchange chromatography as previously described.[8]

Cell line The human pancreatic carcinoma cell line, BxPC-3,[20] which produces CEA, was purchased from the American Type Culture Collection (through Dainippon Pharmaceutical Co., Tokyo). BxPC-3 cells were grown in continuous culture in RPMI 1640 medium containing 2 m*M* L-glutamine, 100 units/ml of penicillin, 100 μg/ml of streptomycin and 10% heat-inactivated fetal bovine serum (Intergen, New York, NY) at 37°C in a humidified 5% CO_2 atmosphere. Subconfluent cells were removed using phosphate-buffered saline (PBS) containing 0.25% trypsin and 1m*M* EDTA.

Animal models Congenitally athymic 5-week-old female BALB/c mice (*nu/nu*) (Japan SLC Inc., Shizuoka) were inoculated subcutaneously with approximately 2×10^7 BxPC-3 cells in 0.1 ml of PBS. Mice bearing pancreatic carcinoma xenografts were used for the studies of biodistribution, radioimmunotherapy and radioimmunodetection when the tumor reached about 10–12 mm in maximal dimension, five weeks after subcutaneous inoculation. To minimize radioiodine uptake into the thyroid, the animals were given water containing 0.2% potassium iodide.

Comparative biodistribution in pancreatic carcinoma xenograft models Ch-A10 and ch-Fab were radiolabeled with $Na^{125}I$ using the Iodogen method.[21] Each antibody was injected into the tail vein to a total amount of 4.0 μg per mouse (0.6 mCi/mg). At 3, 12 and 24 h after injection, the tumor-bearing animals were killed and radiolabel uptakes in the tumor and normal tissues were

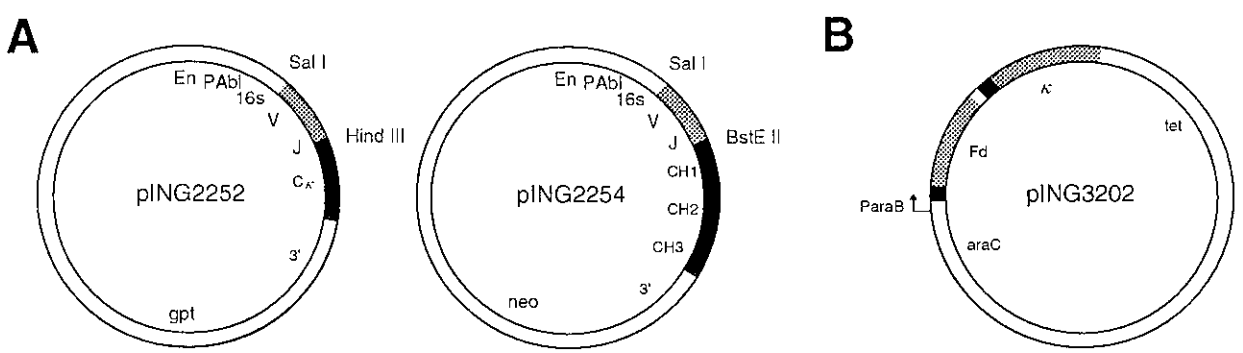

Fig. 1. Maps of expression plasmids for chimeric A10 antibodies. A: The expression vectors for chimeric A10 IgG1 light-chain (pING2252) and heavy-chain (pING2254). En, enhancer; PAbl, Abelson promoter; 16s, SV40 splice junction; V-J, mouse immunoglobulin V-J regions from murine A10; $C_{\kappa,H1,H2\text{ and }H3}$, human immunoglobulin constant regions; 3′, polyadenylation signal; gpt, mycophenolic acid-resistance gene; neo, G418-resistance gene. B: The expression vector for chimeric A10 Fab fragment. ParaB, araB promoter; Fd, chimeric Fd gene; κ, chimeric light chain gene; tet, tetracycline-resistance gene; araC, the arabinose C gene.

measured. There were four animals in each group. The results for uptake in each tumor and tissue were expressed as %ID/g (percentage of the injected dose per gram tissue). To compare the specific localization, the ratio of %ID/g in the tumor relative to that in the blood was calculated for each tumor-bearing animal injected with ^{125}I-labeled antibodies.

Immunostaining and microautoradiography The distribution of A10 antigen in the BxPC-3 tumor was tested by immunohistochemistry. Frozen sections of BxPC-3 tumor were stained by the biotin-avidin method using biotinylated ch-Fab (4 μg/ml) as previously described.[8] The sections were also stained with biotinylated ch-Fab (4 μg/ml) in the presence of excess unlabeled ch-Fab (200 μg/ml). Microautoradiography was performed to investigate the penetration patterns of ch-A10 and ch-Fab. Frozen sections of tumor obtained from tumor-bearing animals injected with radiolabeled A10 antibodies were dipped in emulsion type NR-M2 (Konica, Tokyo). Five weeks later, the sections were fixed with

Table I. Biodistribution of ^{125}I-labeled Chimeric A10 IgG in Nude Mice Bearing BxPC-3 Tumors

Tissue	Time after injection (h)		
	3	12	24
Tumor	4.92±0.78[a]	12.92±1.61	12.35±2.00[b]
Blood	18.36±0.44	13.30±1.29	9.19±2.00
Liver	4.31±0.18	3.00±0.31	1.91±0.29
Spleen	3.37±0.28	2.03±0.24	1.68±0.09
Pancreas	1.61±0.16	1.27±0.08	1.04±0.16
Kidney	4.75±0.09	2.91±0.45	2.55±0.72
Lung	14.21±3.50	5.76±0.39	4.02±0.44
Muscle	0.60±0.05	0.99±0.13	0.90±0.13

a) Data are expressed as the mean percentage of injected dose per gram of tissue ± SEM for four animals.
b) Significantly greater compared with uptake in normal tissues.

Table II. Biodistribution of ^{125}I-labeled Chimeric A10 Fab Fragment in Nude Mice Bearing BxPC-3 Tumors

Tissue	Time after injection (h)		
	3	12	24
Tumor	2.92±0.49[a]	1.01±0.26[b]	0.91±0.20[b]
Blood	1.95±0.38	0.10±0.04	0.03±0.02
Liver	0.77±0.07	0.06±0.01	0.02±0.004
Spleen	1.00±0.15	0.05±0.01	0.02±0.003
Pancreas	1.11±0.16	0.04±0.01	0.01±0.004
Kidney	3.06±0.33	0.25±0.04	0.11±0.02
Lung	2.52±0.85	0.23±0.03	0.19±0.17
Muscle	0.49±0.09	0.02±0.004	0.01±0.002

a) Data are expressed as the mean percentage of injected dose per gram of tissue ± SEM for four animals.
b) Significantly greater compared with uptake in normal tissues.

Table III. Tumor-to-blood Ratio for BxPC-3 Tumor-bearing Nude Mice Injected with ^{125}I-labeled Chimeric A10 IgG and Fab Fragment

Antibody	Time after injection (h)		
	3	12	24
Ch-A10	0.3±0.04[a]	1.0±0.2	1.4±0.3
Ch-Fab	1.5±0.2[b]	10.5±2.1[b]	46.8±12.5[b]

a) Data are shown as means ± SEM for four animals.
b) Significantly higher compared with the ratio for animals given chimeric A10 IgG.

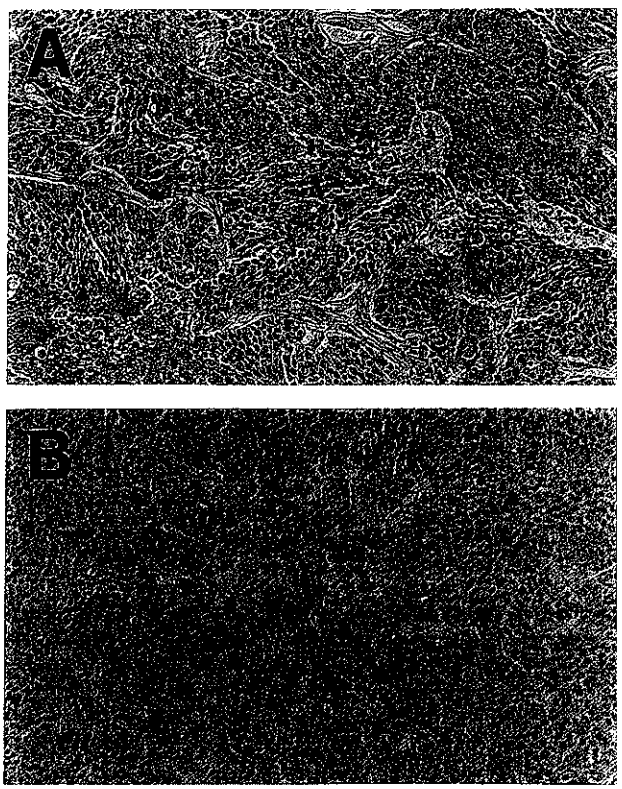

Fig. 2. Immunohistochemical reactions of chimeric A10 Fab to BxPC-3 tumors. Strong staining was demonstrated on the apical side in frozen sections from xenografted pancreatic carcinoma (A). Specific binding of biotinylated A10 Fab was inhibited with an excess of native chimeric Fab (B). Both sections; ×90.

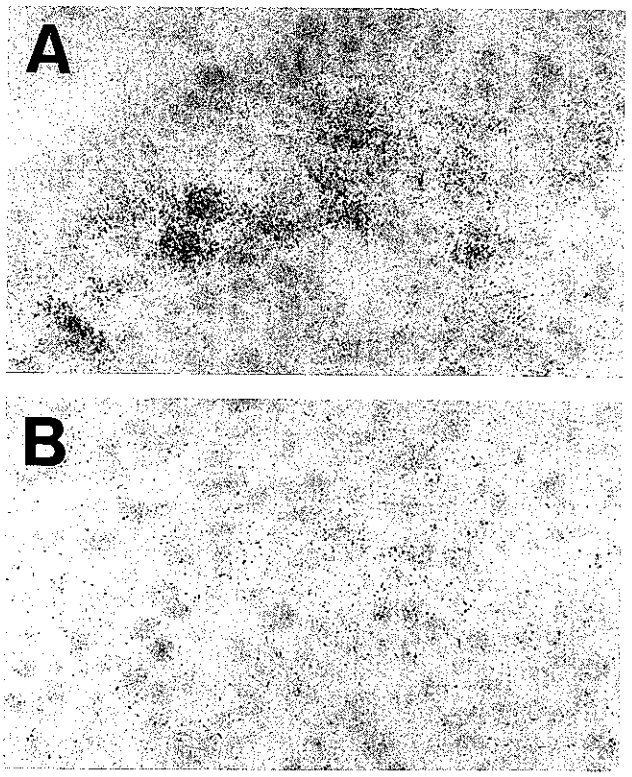

Fig. 3. Penetration patterns of ^{125}I-labeled chimeric A10 antibodies. Sections of BxPC-3 xenografted into nude mice were obtained after injection of ^{125}I-labeled chimeric A10 IgG (A) and Fab fragment (B). Both sections; ×400.

ethanol and diethylether mixture. The sections were developed in KONICADOL-X for 4 min and KONICAFIX for 5 min. After washing and drying, the sections were counterstained with hematoxylin-eosin.

Radioimmunotherapy with ^{131}I-labeled ch-A10 Radioimmunotherapy of pancreatic carcinoma was studied with ^{131}I-labeled ch-A10. Ch-A10 was radiolabeled with $Na^{131}I$ by the Iodogen method.[21] Animals bearing BxPC-3 xenografts were injected with 200 μCi of ^{131}I-labeled ch-A10 (1.0 mCi/mg). As the control, animals were infused with 200 μCi of radiolabeled human IgG. The tumor diameters were measured twice weekly using vernier calipers. Untreated BxPC-3 tumor-bearing mice were studied as a separate control. The tumor volume was calculated by using the following formula;

$$\text{longitudinal diameter} \times \text{short diameter}^2/2.$$

To evaluate toxicity, 20 μl of peripheral blood was collected from the tail vein at 7, 14, 21 and 56 days after injection of radiolabeled antibodies. The leukocyte number was counted with a microcell counter and expressed as a percentage of that in the untreated group.

Radioimmunodetection by ^{123}I-labeled ch-Fab We performed scintigraphic imaging of pancreatic carcinoma tumor xenografts with radiolabeled ch-Fab. Ch-Fab was radiolabeled with $Na^{123}I$ by the Iodogen method.[21] Each mouse was infused intravenously with 200 μCi of ch-Fab (1.3 mCi/mg). Whole-body scintigraphs of mice bearing pancreatic xenografts were obtained by a gamma camera with a pin-hole collimator, 24 h after injection of the radiolabeled fragment.

Statistical analysis All data are presented as mean$\pm$SEM. Statistical analysis was performed by the use of Student's t test for unpaired data; $P<0.05$ was considered statistically significant.

RESULTS

Comparative biodistributions of chimeric A10 antibodies Tissue uptakes of ^{125}I-labeled ch-A10 and ch-Fab were compared 3, 12 and 24 h after injection. The results are summarized in Tables I and II. Significantly higher

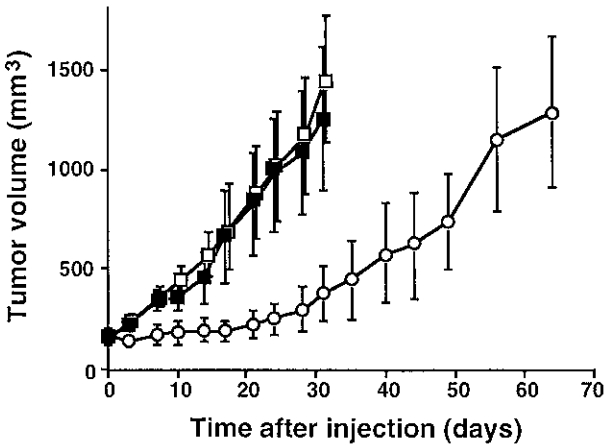

Fig. 4. Targeted therapy with ^{131}I-labeled chimeric A10 IgG in nude mice bearing BxPC-3 tumors. Tumor-bearing mice received a single i.v. injection of 200 μCi of radiolabeled antibodies. ○, ^{131}I-labeled chimeric A10 IgG; ■, ^{131}I-labeled human IgG; □, untreated. Each group included five animals. Vertical bars indicate SEM.

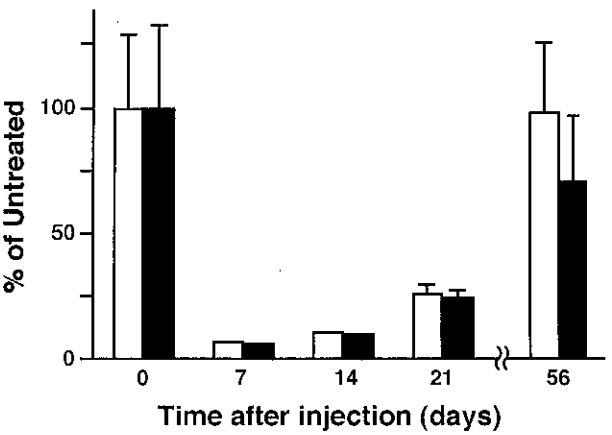

Fig. 5. Leukocyte toxicity in BxPC-3 tumor-bearing mice injected with ^{131}I-labeled antibodies. Data were calculated as the percentage of peripheral white blood cells relative to the untreated group at each time point (n=5). □, ^{131}I-labeled chimeric A10 IgG; ■, ^{131}I-labeled human IgG. Vertical bars indicate SEM.

tumor uptakes were obtained in mice injected with ch-A10 than in those injected with ch-Fab 3, 12 and 24 h after administration (4.9 vs. 2.9, 12.9 vs. 1.0 and 12.4 vs. 0.9 %ID/g, respectively). However, ch-Fab was cleared faster from the bloodstream than ch-A10. The highest uptake of Fab in normal tissues was found in the kidney and the lung, while whole IgG was found mainly in the blood.

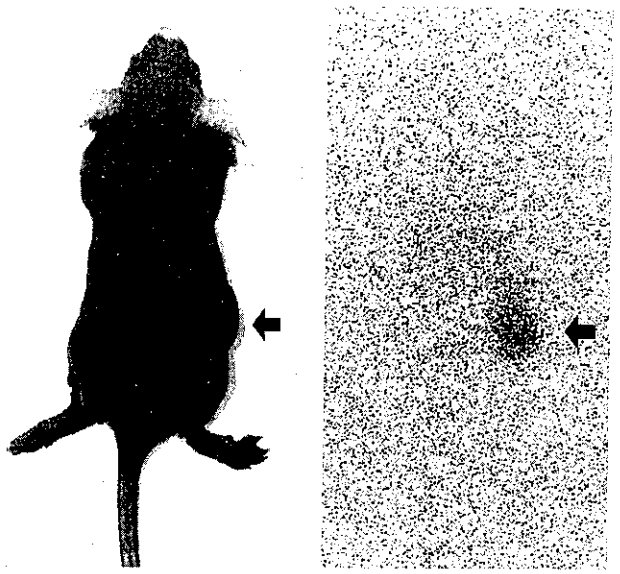

Fig. 6. Whole-body scintigraphy of BxPC-3 xenograft-bearing nude mice with ^{123}I-labeled chimeric A10 Fab at 24 h post-injection. Clear tumor detection (arrow) was obtained without accumulation of radiolabel in normal tissues.

The tumor-to-blood ratios for ^{125}I-labeled chimeric A10 antibodies are shown in Table III. For mice given ch-Fab, the tumor-to-blood ratios were 1.5, 10.5 and 46.8 at 3, 12 and 24 h post-injection, respectively. The tumor-to-blood ratios for ch-A10 were significantly lower than those for ch-Fab at all times and the ratio was only 1.4 at 24 h after injection.

Antigen distribution and microautoradiography Immunohistochemical studies with biotinylated ch-Fab showed that the antigen recognized by the A10 Fab was distributed mainly in tumor nodules and not in normal connective tissues (Fig. 2). However, the A10 antigen was not expressed in all the tumor cells. Strong reactivity was shown at the apical side of the tumor nodules, while the cytoplasm was stained weakly. With an excess of native ch-Fab, binding of biotinylated ch-Fab to cancer cells was inhibited and no staining was seen.

In the microautoradiographic studies, the tumors were excised from nude mice at 3 and 12 h after injection of ch-Fab or ch-A10, respectively, when the highest tumor uptake was observed. Microautoradiographs showed that ch-Fab was distributed homogeneously in the tumor nodules. However, tumor penetration patterns of ch-A10 were more heterogeneous than those observed with ch-Fab (Fig. 3). Both chimeric antibodies were localized in the tumor nodules and not in the stromal connective tissues, yet the intensity of the tumor uptake of ch-A10 was significantly higher than that of ch-Fab.

Anti-tumor effect of ^{131}I-labeled ch-A10 The efficacy of radioimmunotherapy with ^{131}I-labeled ch-A10 is shown in Fig. 4. When tumor-bearing animals were injected with 200 μCi of ^{131}I-labeled ch-A10, the tumor growth was significantly inhibited (85.7% at 28 days after injection) compared with that in the control mice injected with human IgG. Both radiolabeled ch-A10 and normal human IgG caused a decrease in leukocyte count during the first 3 weeks post-injection (Fig. 5). There was no difference in toxicity between radiolabeled ch-A10 and control human IgG. The leukocyte count recovered to a normal level 8 weeks after injection in mice given ^{131}I-labeled ch-A10, while lower leukocyte counts were obtained for mice injected with ^{131}I-labeled control IgG.

Scintigraphic detection by ^{123}I-labeled ch-Fab Whole-body scintigraphic imaging of pancreatic carcinoma xenografts was performed using ^{123}I-labeled ch-Fab. When tumor-bearing mice were injected with 200 μCi of ^{123}I-labeled ch-Fab, clear tumor imaging was obtained at 24 h post-injection, without accumulation of radioiodine in normal tissues (Fig. 6).

DISCUSSION

We previously reported that ch-Fab specifically accumulated in antigen-positive human pancreatic carcinoma (BxPC-3) xenografts in nude mice.[12] Additionally, ch-Fab showed specific immunohistochemical reactions with human pancreatic carcinomas.[8] In the present report, specific accumulation of radiolabeled chimeric A10 antibodies was observed in the BxPC-3 tumors, even though the A10 antigen was not expressed in all the BxPC-3 cells. These results support the idea that our chimeric A10 antibodies could potentially be useful agents for therapy and diagnosis of human pancreatic carcinoma, if cancer cells express the A10 antigen.

To use ch-A10 or ch-Fab as a therapeutic agent against tumors, the antibodies could be conjugated with cytotoxic drugs, protein toxins or radionuclides, since A10 antibodies lack cytotoxic activity towards cancer cells.[8] For targeted therapy, theoretically, the drug- or toxin-conjugated MAbs should reach all viable cancer cells and be internalized to mediate cell killing.[22] Thus, the penetration of antibodies or fragments into the tumors is an important factor in targeted therapy using MAbs. The penetration of antibodies into the central part of tumor nodules can be increased if Fab or F(ab')$_2$ fragments are used instead of intact IgG.[23, 24] Distribution patterns of MAb or fragments in the tumor change time-dependently and the intact IgG demonstrated maximum tumor penetration at 48 to 96 h post-injection.[25] The penetration pattern of ch-A10 was heterogeneous at 12 h post-injection, when the highest tumor uptake was observed. However, ^{131}I-labeled ch-A10 has the potential to destroy distant cancer cells despite its heterogeneous percolation in the tumor nodules, since ^{131}I can deposit its energy over a range of fifty cells by emitting β particles.[11] Our radioimmunotherapy study showed that ^{131}I-labeled ch-A10 was efficacious in targeted therapy of pancreatic carcinoma, while no anti-tumor effect was found in mice injected with ^{131}I-labeled control IgG. The anti-tumor effect of radiolabeled MAb with no antibody-dependent cell-mediated cytotoxicity (ADCC) was reported to be mainly due to targeted radiation.[26] This might also be the case for ^{131}I-labeled ch-A10, since ch-A10 lacks ADCC activity.

The hematological toxicity of therapies using radiolabeled MAb was indicated as one of the major side effects in animal models or humans.[1, 27] In the present study, we also found leukocyte toxicity in mice treated with ^{131}I-labeled ch-A10. In future studies, multiple lower-dose injections of ^{131}I-labeled ch-A10 may be necessary in order to reduce toxicity. For radioimmunotherapy, it is also important that MAb can deliver a greater dose of radionuclides into the tumors. Because of the lower tumor uptake with F(ab')$_2$ than with IgG, a 4- to 6-fold greater injected dose of ^{131}I-labeled F(ab')$_2$ may be needed to match the radiation dose of ^{131}I-labeled parental IgG.[27] The tumor uptake of ch-Fab was less than 10% of that of ch-A10 at 12 and 24 h post-injection. Therefore, a bigger dose of ^{131}I-labeled ch-Fab may be necessary to obtain an anti-tumor effect similar to that afforded by ^{131}I-labeled ch-A10. However, radiolabeled ch-Fab would not be suitable for radioimmunotherapy, since such a bolus injection could elicit considerable toxicity in humans.

To obtain clear and rapid imaging with radiolabeled MAb, high tumor-to-background ratios are necessary early after injection of antibodies. Previous studies showed successful and rapid imaging of pancreatic carcinomas by ^{123}I-labeled anti-CEA F(ab')$_2$ in humans.[15] In biodistribution studies, our ch-Fab showed the highest tumor-to-background ratios at 24 h after injection over a period of 48 h.[12] Here, we show clear tumor detection with 200 μCi of ^{123}I-labeled ch-Fab at 24 h after injection. Furthermore, there was no notable radiolabel uptake in normal tissues, in contrast to injections of radiolabeled IgG MAbs, which show radiolabel uptakes in normal organs after 48 h post-injection.[28, 29] In biodistribution studies, the tumor-to-blood ratio 24 h after injection was only 1.4 when mice were injected with ^{125}I-labeled ch-A10. Chimeric IgG had a longer half-life in the blood circulation, as compared with parental murine MAb in humans.[30] Thus, ^{123}I-labeled ch-Fab was superior to whole IgG for rapid and clear diagnosis of pancreatic carcinomas. The optimal dose to obtain clear tumor imaging with ^{123}I-labeled ch-Fab remains to be determined.

In conclusion, our results show that ^{131}I-labeled ch-A10 may be useful in radioimmunotherapy of pancreatic carcinomas. Whole-body scintigraphy with ^{123}I-labeled ch-Fab demonstrated clear and rapid tumor imaging. However, vascularity, vascular permeability and histological structure in the tumor are limiting factors for clinical use of MAb.[22] The most common type of human pancreatic carcinoma is scirrhous, while the BxPC-3 xenograft was medullary carcinoma in the present animal models. Thus, further investigations will focus on the therapeutic or diagnostic effects of radioiodine-labeled chimeric antibodies in human scirrhous carcinomas. Further studies are also required to determine suitable dose schedules of radioiodine-labeled ch-A10 and ch-Fab for clinical use in various gastrointestinal carcinomas. Such clinical trials should also explore the immunogenicity of our recombinant chimeric antibodies.

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