Jpn. J. Cancer Res. 85, 530–535, May 1994

Biodistribution of Neocarzinostatin Conjugated to Chimeric Fab Fragments of the Monoclonal Antibody A7 in Nude Mice Bearing Human Pancreatic Cancer Xenografts

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In this study, we conjugated chimeric Fab fragments of the monoclonal antibody (MAb) A7, which reacts with pancreatic cancers, to the antitumor drug neocarzinostatin (chA7Fab-NCS) and intravenously injected ^{125}I-labeled chA7Fab-NCS into nude mice bearing a human pancreatic cancer xenograft. We compared the tumor localization of ^{125}I-labeled chA7Fab-NCS with that of conventional ^{125}I-labeled A7-NCS, which was produced by conjugation of MAb A7 and NCS. ^{125}I-Labeled chA7Fab-NCS accumulated in the tumor earlier than ^{125}I-labeled A7-NCS, and significantly larger amounts of ^{125}I-labeled chA7Fab-NCS had accumulated in the tumor 1 hour after injection. The results suggest that chA7Fab may be a suitable carrier for NCS in immunotargeting therapy against pancreatic cancer.

Key words: Pancreatic cancer — Targeting chemotherapy — Monoclonal antibody A7 — Chimeric antibody

The incidence of pancreatic adenocarcinoma has increased steadily over the last decade. Ninety-five percent of pancreatic cancer patients die within three years of their initial diagnosis.[1, 2] However, the development of hybridoma technology by Köhler and Milstein[3] offered the possibility of an effective means of delivering chemotherapy specifically to cancer cells. A number of MAbs[2] have been linked to various antitumor drugs, cytotoxins and enzymes in an attempt to increase the effectiveness of chemotherapy.[4–6]

We generated the MAb A7, using human colonic carcinoma as the antigen, and covalently conjugated it to the antitumor antibiotic NCS (A7-NCS).[7] The conjugate A7-NCS has been used clinically to treat patients with colorectal and pancreatic carcinomas.[8]

Murine MAbs administered to humans induce an HAMA response[9–11] that may reduce the tumor localization of the MAb and can lead to anaphylactic reactions. One way to avoid this problem is the use of human MAbs. Although some human MAbs have been reported to react with gastrointestinal cancers,[12] they have been of the IgM subclass and have shown limited tumor localization.[13, 14] Another approach that has been taken to avoid the HAMA response has been to produce Fab fragments of MAbs. Fab fragments lack the Fc portion of the molecule, which is the most immunopotent region of intact MAbs.[15] The third approach that can reduce HAMA responses, has been to produce chimeric human-mouse antibodies composed of the antigen-binding variable region of a murine MAb and the constant regions of human immunoglobulin.[16–18] We have recently produced chimeric Fab fragments of MAb A7 and conjugated them to the anticancer drug NCS. In this study, we investigate the *in vivo* distribution of NCS conjugated to chimeric Fab fragments of MAb A7 compared with those of NCS conjugated to whole MAb A7 and free NCS as a preclinical study for the application of NCS conjugated to chimeric Fab fragments of MAb A7 in the treatment of human pancreatic carcinoma.

MATERIALS AND METHODS

Cell lines The human pancreatic carcinoma cell line HPC-YS[19] (the kind gift of Dr. Yamaguchi, Research Institute of Neurology and Geriatrics, Kyoto Prefectural University of Medicine) was used in this study. HPC-YS was established from a human ductal cell pancreatic adenocarcinoma. The cells were maintained in RPMI 1640 medium supplemented with 10% FBS (Flow Laboratories Inc., Rockville, MD).

Tumor xenograft Approximately 5×10^6 viable cells were injected subcutaneously into the left flanks of athymic eight-week-old male mice (BALB/c, nu-nu) (SLC Co., Shizuoka) weighing approximately 22.5 g. Tumor masses were detected in all mice injected with the HPC-YS cells.

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[2] Abbreviations: MAb, monoclonal antibody; NCS, neocarzinostatin; HAMA, human anti-mouse antibody; FBS, fetal bovine serum; SPDP, N-succinimidyl-3-(2-pyridyldithio)-propionate; chA7Fab-NCS, chimeric A7 Fab fragment-NCS conjugate; %ID/g, % injected dose/g; SD, standard deviation.

MAb MAb A7 was produced from spleen cells of a mouse immunized against a human colon carcinoma, Colon 6, as previously described.[20] MAb A7 has been reported to react with 73% of the human pancreatic carcinomas tested, in addition to human colonic carcinoma.[21] MAb A7 does not react immunohistochemically with normal human pancreatic tissues.[22] MAb A7 is an IgG_1 and does not possess any intrinsic antitumor activity.[21]

Preparation and purification of chimeric Fab fragments of MAb A7 A murine light chain variable region gene was joined to a human κ light chain constant region gene. A murine heavy chain variable region gene was joined to the human γ_1 heavy chain constant region gene to construct a human-mouse chimeric heavy chain gene. The plasmid DNAs were linearized and introduced into AH22 yeast cells as described previously.[23] After incubation in YPD medium for three days, the cellular debris was removed from the medium by centrifugation, and purified using a CM Sepharose 4B anti-human IgG column.

NCS conjugation to MAb A7 and chimeric Fab fragments of MAb A7 MAb A7 was conjugated to NCS with SPDP as described previously by Fukuda.[7] Briefly, MAb A7 and NCS were separately reacted with SPDP, introducing 2-pyridyl-disulfide groups, and then unreacted SPDP was removed by gel filtration using Sephadex G-25 columns. The modified NCS was treated with dithiothreitol (DTT) (Wako Chemical Co., Tokyo) and mixed with the modified MAb A7 containing the 2-pyridyldisulfide group. For further purification, Sephacryl S-200 columns were used. The conjugation ratio used was 2 mol of NCS per mol of MAb A7, i.e., 7.5 mg of MAb A7 was bound to 1 mg of NCS. Chimeric Fab fragments of MAb A7 were conjugated to NCS by the same method. The conjugation ratio used was 1 mol of NCS per mol of chimeric Fab fragments of MAb A7.

Preparation of radiolabeled antibodies and Fab fragments Radiolabeling of chA7Fab-NCS with ^{125}I (Amersham Japan, Ltd., IMS 30, Tokyo) was performed by the chloramine-T method.[24] Iodinized chA7Fab-NCS was separated from excess reactants by gel filtration on a Sephadex G-25 column. A7-NCS and free NCS were also labeled with ^{125}I by the same method. chA7Fab-NCS, A7-NCS and free NCS were labeled with ^{125}I to specific activities of 4.2, 3.1, and 5.0 μCi/μg, respectively.

Biodistribution of radiolabeled chA7Fab-NCS, A7-NCS, and free NCS in nude mice bearing tumors The distribution of chA7Fab-NCS, A7-NCS, and free NCS was investigated in athymic nude mice bearing HPC-YS tumors. Three weeks after inoculation, the tumor-grafted mice were divided into five groups. Groups of eight mice were injected intravenously with 0.7 μCi of ^{125}I-labeled chA7Fab-NCS, ^{125}I-labeled A7-NCS, ^{125}I-labeled free NCS, ^{125}I-labeled chA7Fab-NCS with an excess amount of chA7Fab (1×10^3 times as much chA7Fab as chA7Fab-NCS), or ^{125}I-labeled chA7Fab-NCS with an excess amount of normal mouse IgG Fab fragments (1×10^3 times as much Fab as chA7Fab-NCS). Four mice from each group were killed and dissected 1 h after injection, and the remaining four mice were killed 24 h after injection. 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 (Auto-Gamma 5000, Packard). The radioactivities in the various tissues were expressed as cpm/g, and were compared with each other. To compare the kinetics of the five probes in the tumors, and blood, the results were expressed as %ID/g. Student's t test was used to check for statistically significant differences. A P value of less than 0.005 was considered to be significant.

RESULTS

Biodistribution of radiolabeled murine and chimeric Fab fragments of MAb A7 in nude mice bearing tumors The %ID/g values of the radiolabeled chA7Fab-NCS in the tumors 1 h and 24 h after injection were 4.16 ± 0.19 (mean$\pm$SD) and 4.20 ± 0.24, respectively. In blood, the %ID/g values of ^{125}I-labeled chA7Fab-NCS were 16.54 ± 2.65 and 0.36 ± 0.06 1 h and 24 h after injection, respectively. In normal tissues, the values ranged from 1.38 ± 0.05 (colon) to 19.49 ± 3.79 (kidney) at 1 h and from 0.12 ± 0.01 (pancreas) to 0.34 ± 0.06 (lung) 24 h after injection. In contrast, the %ID/g values of radiolabeled A7-NCS in the tumors 1 h and 24 h after injection were 2.24 ± 0.34 at 1 h and 5.86 ± 2.55 at 24 h. Those in blood were 25.17 ± 2.10 at 1 h and 8.63 ± 1.24 at 24 h after injection. In normal tissues, the values ranged from 1.01 ± 0.20 (colon) to 6.68 ± 2.01 (lung) at 1 h and from 0.74 ± 0.02 (pancreas) to 3.21 ± 0.27 (lung) 24 h after injection. The tumor accumulation of ^{125}I-labeled chA7Fab-NCS was significantly greater than that of ^{125}I-labeled A7-NCS 1 h after injection. Tumor accumulations of ^{125}I-labeled chA7Fab-NCS and ^{125}I-labeled A7-NCS were not significantly different 24 h after injection. The accumulation of ^{125}I-labeled free NCS was low in all tissues 1 h and 24 h after injection (Tables I, II and III). The values of ^{125}I-labeled chA7Fab-NCS tumor tissue/blood ratio were 0.26 ± 0.03 and 11.71 ± 1.20 1 h and 24 h after injection, respectively. In contrast, the values for ^{125}I-labeled A7-NCS were 0.09 ± 0.01 and 0.69 ± 0.32, respectively, 1 h and 24 h after injection (Fig. 1). The tumor tissue/blood ratio of ^{125}I-labeled chA7Fab-NCS was significantly greater than that of ^{125}I-labeled A7-NCS 1 and 24 h after injection.

Table I. Accumulation (%ID/g) of ^{125}I-Labeled ChA7Fab-NCS in Nude Mice Bearing Human Pancreatic Cancer Xenografts

	Blood	Lung	Heart	Liver	Spleen	Pancreas	Stomach	Colon	Kidney	Tumor
1 h	16.54±2.65	6.22±0.93	3.86±0.58	4.20±0.90	3.27±0.34	1.56±0.13	2.49±0.52	1.38±0.05	19.49±3.79	4.16±0.19
24 h	0.36±0.06	0.34±0.05	0.17±0.02	0.14±0.03	0.13±0.01	0.11±0.01	0.23±0.05	0.12±0.01	0.29±0.14	4.20±0.24

Table II. Accumulation (%ID/g) of ^{125}I-Labeled Free NCS in Nude Mice Bearing Human Pancreatic Cancer Xenografts

	Blood	Lung	Heart	Liver	Spleen	Pancreas	Stomach	Colon	Kidney	Tumor
1 h	2.21±0.30	1.32±0.29	0.64±0.10	1.61±0.41	0.68±0.09	0.57±0.08	3.31±0.97	0.68±0.16	3.99±0.32	1.14±0.18
24 h	0.13±0.01	0.12±0.01	0.09±0.01	0.25±0.02	0.11±0.02	0.08±0.01	0.09±0.01	0.07±0.01	0.51±0.05	0.13±0.03

Table III. Accumulation (%ID/g) of ^{125}I-Labeled A7-NCS in Nude Mice Bearing Human Pancreatic Cancer Xenografts

	Blood	Lung	Heart	Liver	Spleen	Pancreas	Stomach	Colon	Kidney	Tumor
1 h	25.17±2.10	6.68±2.10	5.56±1.68	6.24±1.25	3.85±0.29	1.32±0.13	1.85±0.35	1.01±0.20	5.08±0.60	2.23±0.34
24 h	8.63±1.24	3.21±0.27	1.49±0.25	1.82±0.03	1.44±0.08	0.74±0.02	1.01±0.08	0.80±0.06	1.79±0.19	5.86±2.55

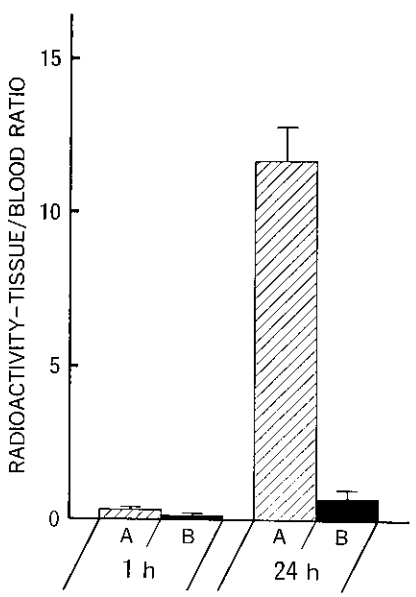

Fig. 1. The tumor tissue/blood ratio of ^{125}I-labeled chA7Fab-NCS or ^{125}I-labeled A7-NCS in nude mice bearing HPC-YS tumors 1 h and 24 h after injection. The tissue/blood ratio of ^{125}I-labeled chA7Fab-NCS was significantly greater than that of ^{125}I-labeled A7-NCS ($P<0.005$). A, ^{125}I-labeled chA7Fab-NCS; B, ^{125}I-labeled A7-NCS; bars, SD.

When an excess of chimeric Fab fragments of MAb A7 was added to ^{125}I-labeled chA7Fab-NCS, tumor accumulation of ^{125}I-labeled chA7Fab-NCS was inhibited more than when an excess amount of normal mouse IgG Fab fragments was added to ^{125}I-labeled chA7Fab-NCS. The difference was statistically significant both 1 h and 24 h after injection (Figs. 2 and 3).

DISCUSSION

One of the reasons for the high mortality of pancreatic cancer is the lack of an effective chemotherapeutic regimen. There are some reports[8] on the successful use of a murine MAb-drug conjugate for immunochemotherapy in patients with visceral malignant disease. However, there is little information about the application of such immunoconjugates to pancreatic cancer.

We have used the A7-NCS immunoconjugate to treat more than 70 patients with colorectal carcinoma. Some of the patients who have been treated with this conjugate have had regression of their tumors.[8] In a previous study, the radiolabeled MAb A7 was shown to be localized in a pancreatic cancer that expressed the antigen recognized by MAb A7, and not in a tumor negative for this antigen.[25] Moreover, in a previous study, the *in vivo* antitumor activity of the A7-NCS conjugate on an antigen-positive human pancreatic carcinoma was greater than that of free NCS. However, the pancreatic cancer was able to grow in the presence of A7-NCS, although the A7-NCS conjugate inhibited the growth of the pancreatic carcinoma xenograft.[26] One of the reasons for this insufficient *in vivo* antitumor activity of A7-NCS against grafted pancreatic cancer tumor was the rapid inactivation of NCS in the blood. Fujita *et al.*

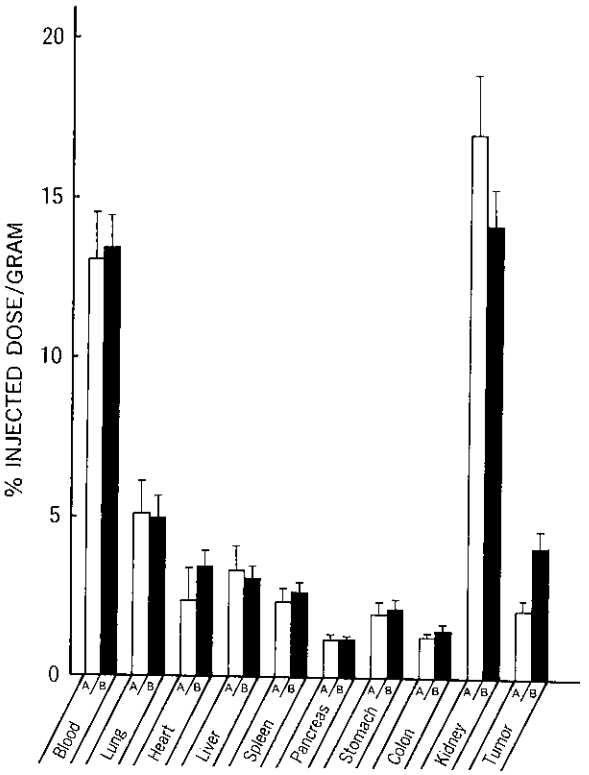

Fig. 2. The accumulation of ^{125}I-labeled chA7Fab-NCS injected with excess chA7Fab-NCS or normal mouse IgG Fab fragments into nude mice bearing HPC-YS tumors 1 h after intravenous injection. The tumor accumulation of ^{125}I-labeled chA7Fab-NCS given with excess chA7Fab was significantly less than when it was given with excess normal mouse IgG Fab fragments ($P<0.005$). A, ^{125}I-labeled chA7Fab-NCS with excess chA7Fab; B, ^{125}I-labeled chA7Fab-NCS with excess normal mouse IgG Fab fragments; bars, SD.

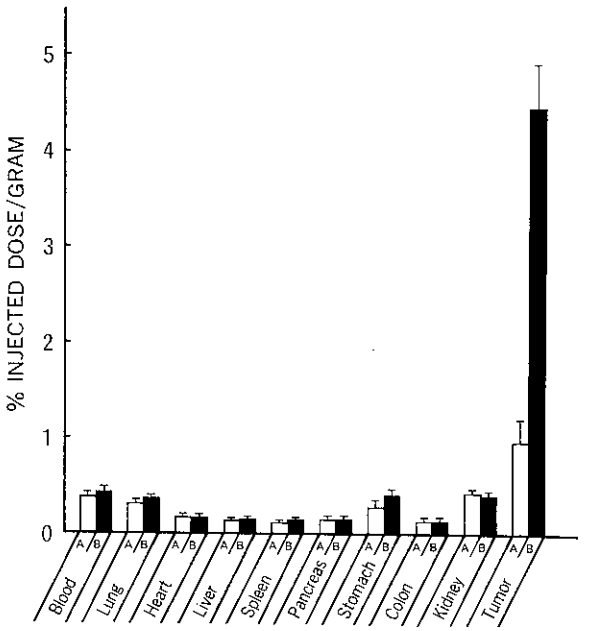

Fig. 3. The accumulation of ^{125}I-labeled chA7Fab-NCS injected with excess chA7Fab-NCS or normal mouse IgG Fab fragments into nude mice bearing HPC-YS tumors 24 h after intravenous injection. The tumor accumulation of ^{125}I-labeled chA7Fab-NCS with excess chA7Fab was significantly less than with excess normal mouse IgG Fab fragments ($P<0.005$). A, ^{125}I-labeled chA7Fab-NCS with an excess amount of chA7Fab; B, ^{125}I-labeled chA7Fab-NCS with an excess amount of normal mouse IgG Fab fragments; bars, SD.

reported that more than 70% of antitumor activity of NCS was inactivated by 10% mouse serum within 120 min *in vitro*.[27] ^{125}I-Labeled chA7Fab-NCS accumulated in tumors earlier than ^{125}I-labeled A7-NCS, and significantly larger amounts of ^{125}I-labeled chA7Fab-NCS accumulated in the tumors 1 h after injection, a time at which NCS is still active.

The clearance of ^{125}I-labeled chA7Fab-NCS from the blood was more rapid than that of ^{125}I-labeled A7-NCS. In the previous study using nude mice, the clearance of ^{125}I-labeled chA7Fab from the blood was almost the same as that of ^{125}I-labeled murine Fab fragments of MAb A7, which does not contain xenogenic antigen, though the chimeric antibody contained a human component which was a xenogenic antigen for mice (unpublished data). From these results, the reason for this more rapid clearance of chA7Fab-NCS from the blood than that of A7-NCS was the smaller molecular weight of chA7Fab-NCS than that of A7-NCS. The tumor tissue/blood ratio of ^{125}I-labeled chA7Fab-NCS was significantly higher than that of ^{125}I-labeled A7-NCS. In contrast, the normal tissue/blood ratios of ^{125}I-labeled chA7Fab-NCS and ^{125}I-labeled A7-NCS were low, and comparable to each other, except for in the kidney. Hansson *et al.* demonstrated that the clearance of non-Fc bearing antibody fragments is rapid, and mainly via the kidney, while the removal of intact Ig occurred mainly through interaction with Fc-receptor-bearing cells, followed by a slow clearance via the reticuloendothelial system.[28] Fujita *et al.* reported that NCS is rapidly inactivated in the kidney while it is hardly inactivated in the blood,[27] and therefore, the adverse effect of NCS in the kidney may be minimized. Moreover, when murine immunoconjugates are administered to humans, HAMA, which sometimes causes allergic reactions and reduces the efficacy of targeting chemotherapy, is produced. Takahashi *et al.*[29] previously reported that HAMA was produced in all patients who received A7-NCS. Because the origin of the shortened Fc portion of the chimeric Fab fragments

of MAb A7, which is the most immunopotent region of intact MAbs,[15] is human, HAMA production should decrease when this conjugate is administered to humans.

In this study, the tumor distribution of ^{125}I-labeled chA7Fab-NCS injected with excess chA7Fab was significantly lower than when it was injected with excess normal mouse IgG Fab fragments. This suggests that the enhanced tumor localization of ^{125}I-labeled chA7Fab-NCS was due to antigen-antibody binding. We conclude that chA7Fab may be a suitable carrier for NCS in the immunotargeting therapy of pancreatic cancer.

(Received November 12, 1993/Accepted February 4, 1994)

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