Improved Targeting of Radiolabeled Streptavidin in Tumors Pretargeted with Biotinylated Monoclonal Antibodies through an Avidin Chase

Zhengsheng Yao, Meili Zhang, Hisataka Kobayashi, Harumi Sakahara, Hiroshi Nakada, Ikuo Yamashina and Junji Konishi

Department of Nuclear Medicine, Faculty of Medicine, Kyoto University, Kyoto and Department of Biotechnology, Faculty of Engineering, Kyoto Sangyo University, Kyoto, Japan

Radiolabeled streptavidin can be accumulated in tumors pretargeted with biotinylated anti-tumor antibodies. However, circulating biotinylated antibody and endogenous biotin may interfere with the tumor targeting of streptavidin. To reduce biotinylated antibody concentration in the blood, we injected avidin before streptavidin administration. The effects of avidin administration on the biodistribution and tumor targeting of radiolabeled streptavidin were examined. **Methods:** Biotinylated anti-human colon cancer monoclonal antibody (MAb) MLS128 was injected intravenously into nude mice bearing human colon cancer xenografts for pretargeting. After intraperitoneal injection of avidin, radioiodinated streptavidin was administered and its biodistribution and tumor accumulation was investigated. **Results:** Radioiodinated streptavidin specifically localized in the tumor pretargeted with biotinylated antibody. Avidin preadministration accelerated the tumor uptake and blood clearance of radioiodinated streptavidin. The tumor-to-blood radioactivity ratio at 6 and 24 hr after radiolabeled streptavidin injection were 1.23 ± 0.29 and 3.04 ± 0.86, respectively, in mice with avidin chase (mean ± s.d., n = 7), and 0.82 ± 0.17 and 2.29 ± 0.29, respectively, in those without chase (mean ± s.d., n = 7). **Conclusion:** Localization of radiolabeled streptavidin in tumors pretargeted with biotinylated MAb could be improved by avidin chase. This approach may be useful for tumor radioimmunoimaging and radioimmunotherapy.

Key Words: radiolabeled streptavidin; biotinylated monoclonal antibody; avidin chase; colon cancer xenografts

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The major problem in tumor imaging and therapy using radiolabeled monoclonal antibodies (MAbs) is the low level and slow uptake of the antibody in the tumor and slow clearance of the antibody from the blood and other nontumor tissues. Improvements may result from novel procedures where the unlabeled antibody is administered and allowed to clear from the circulation before administration of the labeled compound which binds the antibody.

Avidin, a 66-kD glycoprotein present in egg white, has a strong avidity for biotin, a 244-Dalton vitamin H. Because of the extremely high affinity of avidin to biotin (Kd = 10^{-15} *M*), the avidin-biotin system has been applied for in vivo tumor radiodetection (*1–11*). Avidin, when administered in vivo, rapidly accumulates in the liver (*12,13*). Streptavidin, a 60-kD protein obtained from *Streptomyces avidinii*, is similar to avidin with respect to its binding affinity to biotin but it shows less nonspecific binding to tissues and remains in the blood longer than avidin (*12*).

Two-step immunoscintigraphy, i.e., the administration of radiolabeled streptavidin after pretargeting of biotinylated MAb, has shown some interesting results (*6,7*). However, biotinylated antibody (*7*) and endogenous biotin (*8*) in blood may interfere with the binding of streptavidin to the tumor. As avidin accumulates in the liver rapidly, biotinylated antibody could be rapidly cleared from the circulation by avidin administration (*10,13–16*).

In this study, we examined the effects of an avidin chase on the accumulation of radiolabeled streptavidin in tumors pretargeted with biotinylated anti-tumor antibody. After pretargeting of unlabeled biotinylated MAb, avidin was administered to chase biotinylated antibody and endogenous biotin from the circulation, and then, radiolabeled streptavidin was injected for tumor targeting. In this way, tumor uptake and blood clearance of the label were improved.

MATERIALS AND METHODS

Monoclonal Antibody

MLS128 is a mouse IgG3 MAb with a kappa light chain, which was produced by immunizing mice with LS180 cells (*17*). The antibody recognizes Tn antigen, a cluster of tri-GalNAc α-Ser/Thr (*18,19*). The antibody was purified from ascitic fluid of hybrido-

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For correspondence or reprints contact: Harumi Sakahara, MD, Dept. of Nuclear Medicine, Faculty of Medicine, Kyoto University, Shogoin, Sakyo-ku, Kyoto 606-01, Japan.

TABLE 1
Experimental Protocol: Mice Killed 6 or 24 Hours Postinjection of Radiolabeled Proteins

Time (hr)	Substances and route of administration	Group 1	2	3	4
−48	Biotinylated MLS128 i.v.	+	+	−	−
−0.5	Avidin (60 μg) i.p.	+	−	−	−
0	Radioiodinated streptavidin or MLS128 i.v.	+	+	+	+
2.5	Avidin (60 μg) i.p.	+	−	−	−

ma-bearing mice using protein A affinity chromatography (Bio-Rad, Richmond, CA).

Biotinylation of Monoclonal Antibody

The antibody MLS128 was conjugated with biotin using NHS-LC-biotin (Pierce, Rockford, IL) (*20*). Briefly, in a 5-ml test tube, 5 mg/ml of antibody in 0.075 *M* phosphate-buffered saline, pH7.4, and 9 μl of freshly prepared NHS-LC-biotin solution (20 mg/ml) were incubated for 2 hr at 4°C, and unconjugated biotin was then removed by chromatography on a PD10 column (Pharmacia LKB Biotechnology, Uppsala, Sweden). The average number of biotin molecules coupled to each antibody was determined to be 1.7 by the method of Green et al. (*20*) using HABA solution (Pierce). More than 90% of biotinylated MLS128 bound to immobilized avidin (Pierce). Immunoreactivity of biotinylated MLS128 was compared with that of unmodified MLS128 by inhibition of ^{125}I-labeled MLS128 binding to LS180 cells. The biotinylated antibody was stored at 4°C until use.

Radioiodination of Proteins

MLS128, biotinylated MLS128, or streptavidin (Pierce) was radioiodinated with ^{125}I or ^{131}I using the chloramine T method (*21*). Twenty to forty micrograms of protein in 200 μl of 0.3 *M* phosphate buffer, pH 7.5, ^{125}I or ^{131}I (29.6–59.2 MBq) (Du Pont, Wilmington, DE) were mixed with 2.5 μg of chloramine T (Nacalai tesque, Kyoto, Japan) dissolved in 0.3 *M* phosphate buffer. After 5 min, radiolabeled proteins were separated from free iodine by chromatography through PD10 gel. Specific activities of radiolabeled proteins were 370–1230 MBq/mg.

Biodistribution Study and Immunoscintigraphy

The effect of avidin dose on the pharmacokinetics of ^{125}I-labeled biotinylated antibody after chase was studied using normal BALB/c mice.

The colon cancer xenograft was established by subcutaneous inoculation of LS180 cells into female BALB/c-nu/nu mice, and the xenograft was maintained by serial subcutaneous transplantation. Potassium iodide solution was administered to mice 1 day before the injection of radioiodinated proteins throughout the experiments. The effect of the streptavidin dose on its blood clearance and tumor uptake was examined and the most appropriate dose was determined. The experimental protocol for treatment of tumor-bearing mice is shown in Table 1. Nude mice, bearing LS180 xenografts, received 30 μg of unlabeled biotinylated MLS128 through the tail vein in Groups 1 and 2. Two days later, 37 kBq of ^{131}I-streptavidin (3 μg) were injected intravenously in Groups 1 and 2. In Group 1, two chases by intraperitoneal injection of avidin were performed at 30 min before and 2.5 hr after radiolabeled streptavidin injection. In Group 2, avidin was not administered. Mice received 3 μg of ^{131}I-streptavidin alone in Group 3, and 30 μg of ^{125}I-MLS128 alone in Group 4. Groups of 5–7 mice were killed 6 and 24 hr after injection of radiolabeled protein, organs were removed and weighed, and the radioactivity was counted. The protein dose of radiolabeled antibody and streptavidin was adjusted by adding unlabeled proteins. Data were expressed as percentages of the injected dose per gram of tissue (%ID/g). Statistical analysis was performed using Student's t-test.

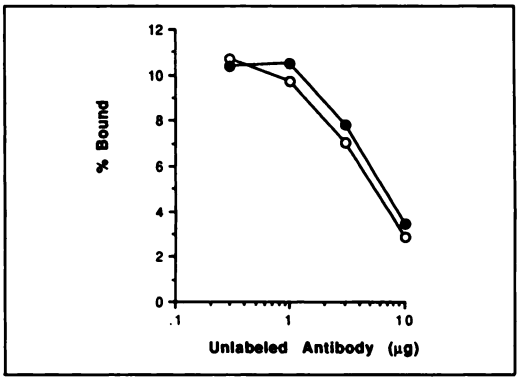

FIGURE 1. Inhibition of ^{125}I-MLS128 binding to LS180 cells by unmodified MLS128 and biotinylated MLS128. After the incubation of ^{125}I-MLS128 and increasing doses of unlabeled MLS128 with 1 × 10^6 cells for 1 hr at 4°C, the radioactivity bound to cells was counted (●:biotinylated MLS128, ○:unmodified MLS128).

For the imaging study, 3.7 MBq of ^{131}I-labeled streptavidin (3 μg) was administered intravenously after pretargeting with 30 μg of biotinylated MLS128. The mice were treated according to the same protocol as those in Group 1 except for the injected radioactivity. At 6, 24, 48, and 96 hr after injection of streptavidin, mice were anesthetized by intraperitoneal injection of sodium pentabarbital, and scintigrams were obtained using a gamma camera equipped with a pinhole collimator.

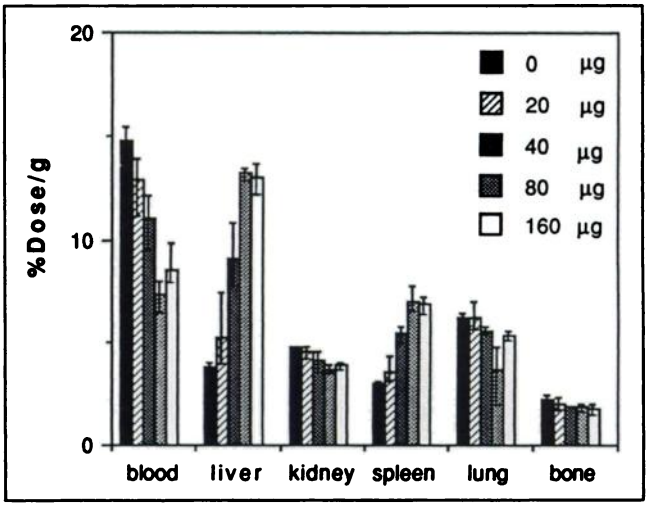

FIGURE 2. Two days after ^{125}I-biotinylated MLS128 intravenous injection into BALB/c mice, different doses of avidin were injected intraperitoneally and biodistribution of radioactivity was determined and expressed as %ID/g 1 hr later. Bars represent the mean and range of three mice.

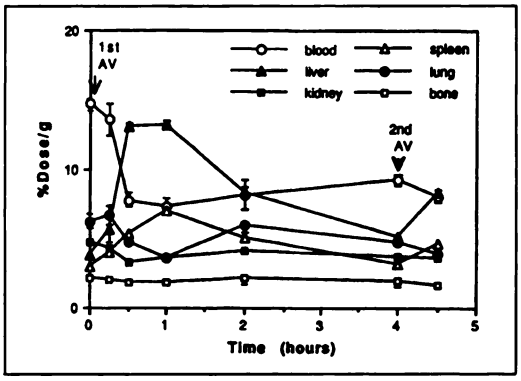

FIGURE 3. Two days after ^{125}I-biotinylated MLS128 intravenous injection into BALB/c mice, 80 μg of avidin were injected intraperitoneally at time 0. Biodistribution data are expressed as %ID/g (mean and range of three mice). In the second chase (2nd AV), 60 μg of avidin were administered intraperitoneally 4 hr after the first injection of avidin.

RESULTS

Biotinylation slightly reduced the immunoreactivity of MLS128 (Fig. 1); biotinylated MLS128 showed 90.1% of immunoreactivity compared with the unmodified antibody.

Two days after injection of ^{125}I-labeled biotinylated MLS128 (30 μg) into normal BALB/c mice, different doses of avidin were injected intraperitoneally and 1 hr later, the biodistribution of ^{125}I-labeled biotinylated MLS128 was determined. With increases in avidin dose, radioactivity decreased in the blood and lungs, and increased in the liver and spleen (Fig. 2). The 80-μg dose gave the maximum effect. Therefore, in the following experiments, 80 μg was used as the chase dose.

The pharmacokinetics of ^{125}I-biotinylated MLS128 after an 80-μg avidin chase were studied using BALB/c mice. Radioactivity in the blood decreased to about 50% at 30 min and continued low for 2 hr, rising a little at 4 hr after avidin injection (Fig. 3). The blood radioactivity decreased once again after the second intraperitoneal injection of 60 μg of avidin.

The effect of ^{125}I-labeled streptavidin dose on its biodistribution is shown in Table 2. The mice were treated according to the same protocol as those in Group 1. Compared with the 1-μg doses which resulted in a slower blood clearance and the 10-μg doses which showed low tumor uptake, the 3-μg dose seemed suitable for tumor targeting with 30 μg of biotinylated antibody pretargeting.

On the basis of these results, the pretargeting experiment was performed with 30 μg of biotinylated antibody followed by intraperitoneal administration of avidin chase twice, and 3 μg of radioiodinated streptavidin injection. The avidin chase resulted in a more rapid uptake of radiolabeled streptavidin in the tumor (Group 1 versus Group 2, Table 3). The blood clearance was also faster in Group 1. Therefore, the tumor-to-blood radioactivity ratio was improved significantly. Iodine-131-labeled streptavidin alone showed a significantly lower localization in the tumor than labeled streptavidin with pretargeting, which indicated a specific localization of the streptavidin in tumor in Groups 1 and 2. The tumor-to-blood ratio of the labeled antibody (Group 4) was lower than that of labeled streptavidin (Group 1).

Scintigraphic images were consistent with the results of biodistribution data (Fig. 4). A high tumor uptake was obtained as rapidly as 6 hr and lasted up to 96 hr after injection. Nonspecific uptake in the liver, kidney and spleen was observed.

DISCUSSION AND CONCLUSION

In tumor imaging with radiolabeled biotinylated antibody or liposomes, avidin chase significantly accelerated blood clearance of radioactivity, thus improving tumor-to-nontumor radioactivity ratio (*10,14–16*). In this case, however, the avidin chase cannot be performed until sufficient radioactivity has accumulated in the tumor. Pretargeting with unlabeled biotinylated antibody followed by radiolabeled streptavidin administration is also promising (*6–7,10,11*). However, biotinylated antibodies and endogenous biotin in the circulation may interfere with the tumor accumulation of radiolabeled streptavidin (*7,8*). In this study, we combined the two methods. The avidin chase reduced circulating biotinylated antibody which would bind radiolabeled streptavidin and interfere with its tumor targeting.

Multiple steps in imaging or therapy protocols greatly increase the complexity of optimization. The relative doses of the various reagents, the time interval between steps and the route of administration are among the most important considerations. In our study, several experimental vari-

TABLE 2
Effect of ^{125}I-Streptavidin on Its Biodistribution

	1 μg		3 μg		10 μg	
Tissue	6 hr	24 hr	6 hr	24 hr	6 hr	24 hr
Blood	11.5(10.5–13.0)	9.1(7.3–10.6)	10.7(10.0–11.2)	4.8(4.3–5.1)	11.2(9.8–12.3)	4.4(3.5–5.3)
Tumor	16.6(11.8–21.6)	19.1(16.2–22.5)	16.1(15.0–17.4)	18.3(15.3–21.2)	13.6(11.3–15.0)	13.8(12.5–16.4)
Tumor/blood	1.48 (0.99–1.99)	2.10(1.76–2.35)	1.51(1.34–1.62)	3.83(3.04–4.28)	1.22(1.15–1.28)	3.20(2.94–3.55)

Mean and range of %ID/g and its tumor-to-blood ratio (n = 3).

TABLE 3
Biodistribution of Radioiodinated Streptavidin and MLS128

Tissue	^{131}I-SA (group 1)*		^{131}I-SA (group 2)†		^{131}I-SA (group 3)‡		^{125}I-MLS128 (group 4)§	
	6 hr (n = 7)	24 hr (n = 7)	6 hr (n = 7)	24 hr (n = 7)	6 hr (n = 5)	24 hr (n = 5)	6 hr (n = 6)	24 hr (n = 6)
Blood	11.97 ± 1.44	5.74 ± 1.14	¶13.65 ± 1.48	***7.54 ± 0.77	11.38 ± 1.41	4.25 ± 0.76	12.21 ± 1.77	***8.75 ± 0.62
Liver	7.13 ± 1.26	6.84 ± 1.74	7.01 ± 0.59	6.45 ± 1.48	6.25 ± 0.88	6.20 ± 0.48	4.32 ± 1.04	2.20 ± 0.41
Kidney	22.18 ± 4.07	29.73 ± 11.2	18.04 ± 5.38	24.93 ± 12.9	30.69 ± 5.97	47.69 ± 4.32	5.33 ± 2.02	2.74 ± 0.16
Intestine	2.21 ± 0.27	1.24 ± 0.12	2.13 ± 0.24	1.21 ± 0.16	2.60 ± 0.23	1.51 ± 0.11	1.56 ± 0.53	0.96 ± 0.11
Stomach	2.49 ± 0.58	1.84 ± 1.08	3.03 ± 1.01	1.80 ± 0.65	3.81 ± 0.82	2.11 ± 0.52	6.77 ± 4.87	1.95 ± 0.63
Spleen	5.82 ± 1.67	6.56 ± 2.25	5.61 ± 0.68	6.97 ± 1.57	4.09 ± 0.48	5.57 ± 0.56	5.38 ± 3.30	3.91 ± 1.62
Lung	5.98 ± 1.06	4.05 ± 0.95	6.83 ± 1.15	4.67 ± 0.52	6.38 ± 0.40	3.67 ± 0.44	7.10 ± 2.05	4.75 ± 0.39
Muscle	1.07 ± 0.14	0.84 ± 0.11	0.94 ± 0.07	0.81 ± 0.11	1.25 ± 0.21	1.02 ± 0.11	0.66 ± 0.26	0.84 ± 0.11
Bone	2.10 ± 0.16	1.42 ± 0.22	2.20 ± 0.18	1.48 ± 0.21	2.34 ± 0.41	1.97 ± 0.24	1.83 ± 0.55	1.49 ± 0.37
Tumor	14.43 ± 2.11	16.75 ± 2.28	**11.04 ± 1.66	17.17 ± 1.87	***6.36 ± 0.97	***5.17 ± 0.82	**10.50 ± 2.23	16.66 ± 0.80
T/Blood	1.23 ± 0.29	3.04 ± 0.86	**0.82 ± 0.17	¶2.29 ± 0.29	***0.56 ± 0.10	***1.28 ± 0.46	¶0.86 ± 0.17	¶1.91 ± 0.16
T/Bone	6.96 ± 1.41	12.10 ± 3.10	**5.04 ± 0.85	11.92 ± 2.73	**2.80 ± 0.68	***2.70 ± 1.20	6.22 ± 2.50	12.11 ± 4.40

Mean ± s.d. of %ID/g and its tumor-to-blood or tumor-to-bone ratio.
*Radioiodinated streptavidin with pretargeting of biotinylated MLS128 and with avidin chase;
†Radioiodinated streptavidin with pretargeting of biotinylated MLS128 but without avidin chase;
‡Radioiodinated streptavidin alone;
§Radioiodinated MLS128 alone.
¶$p < 0.05$.
**$p < 0.01$.
***$p < 0.005$ compared with Group 1.

ables were examined to determine optimal conditions for the method. Radioiodinated streptavidin was administered 2 days after pretargeting because accumulation of MLS128 in the tumor peaked at 48 hr. To achieve an effective targeting of radiolabeled streptavidin, it is necessary not only to reduce circulating biotinylated antibody and biotin, but also to keep their levels low. For this purpose, repeated intraperitoneal administration of avidin was performed. The relatively low chase effect shown in Figure 2 could be due to the fact that we administered avidin intraperitoneally and performed the assay after 1 hr. However, the blood level of biotinylated antibody reached a nadir at 30 min (Fig. 3) and the chase by intravenous injection was not significantly more effective than that by intraperitoneal injection even at 30 min (data not shown). We suppose that there was another reason for the low chase effect in our experiments; i.e., the biotinylation level of the antibody used in the present experiments might have been much lower than that in previous studies (*10,13,16*), and it was suggested that the chase effect depends on the biotinylation level of the antibody (*10,13*).

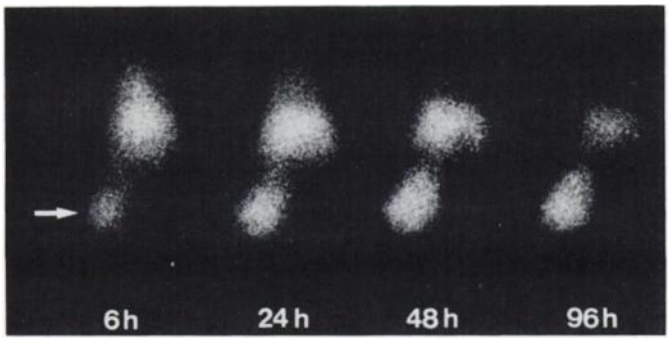

FIGURE 4. Scintigrams of a nude mouse bearing LS180 xenograft pretargeted with biotinylated MLS128 followed by avidin chases and radiolabeled streptavidin administration. The images were obtained 6, 24, 48 and 96 hr after injection of ^{131}I-streptavidin. Arrow indicates the xenografted tumor.

It could have been predicted that the avidin chase would saturate biotin binding sites at the tumor, hence lowering the uptake of labeled streptavidin. However, in the present study, this does not appear to have happened. Pimm et al. reported that the accumulation of avidin in tumors pretargeted with a biotinylated antibody was less than 1% of the injected dose per gram (*5*). In a preliminary experiment, we also found that only 0.4% of the injected radioactivity was recovered per gram of tumor when 80 μg of ^{111}In-labeled avidin was injected intraperitoneally into tumor-bearing mice pretreated with biotinylated antibody (data not shown). A simple calculation to determine the molar amounts of biotin in tumor relative to avidin and streptavidin was performed. Providing that 30% of the injected dose was accumulated in tumor at 48 hr postinjection of 30 μg of antibody (*22*), 0.06 nmole of biotinylated antibody was present in the tumor. Since the conjugation ratio of biotin in the antibody was 1.7, the tumor therefore accumulated 0.102 nmole of biotin. When 140 μg of avidin (80 μg plus 60 μg) was injected and its accumulation in the tumor was 0.4% dose per gram, the amount of avidin accumulated in the tumor was 0.0085 nmole. Assuming that the interaction between biotin in the tumor and avidin

or streptavidin is 1:1, less than 10% of biotin was used for binding with avidin and more than 90% of biotin was available for binding with streptavidin. This indicated that 5.6 μg of streptavidin could bind the biotin in 1 g of tumor tissue. Although the results of these experiments may be tumor-dependent, the avidin chase can improve the delivery of radiolabeled streptavidin to tumor pretargeted with biotinylated anti-tumor antibodies.

The blood clearance of proteins is roughly proportional to their molecular weight (*1*). The relatively low molecular weight of streptavidin (60 kD) enabled it to be cleared faster than antibody, but it was not as fast as we expected. Furthermore, radiolabeled streptavidin showed a high uptake in the kidney, liver and spleen (*23*). Streptavidin has a tripeptide sequence of Arg-Tyr-Asp (RYD) which may bind to the surfaces of many cell types (*24*). A reagent with faster blood clearance and less nonspecific binding to normal tissue is worth looking for.

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