Improved Intratumoral Penetration of Radiolabeled Streptavidin in Intraperitoneal Tumors Pretargeted with Biotinylated Antibody

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Inefficient intratumoral penetration of pharmaceuticals is one of the major limiting factors against effective tumor-targeting therapy. This study investigated the effect of the distribution pattern of the binding site in tumors on the penetration of target material. **Methods:** In the first experiment, radiolabeled biotinylated monoclonal antibody, MLS128, was injected intraperitoneally or intravenously into nude mice bearing intraperitoneal human colon cancer xenografts. In the second experiment, radiolabeled streptavidin was injected intraperitoneally in the tumor-bearing mice after the pretargeting with the unlabeled biotinylated antibody. Intratumoral distribution of radioactivity was examined with quantitative autoradiography. **Results:** There was no difference in the biodistribution of biotinylated antibody between intraperitoneal and intravenous administrations, but autoradiography showed a higher uptake in the margin and a lower uptake in the center of radioactivity in tumor nodules with intraperitoneal injection and a more uniform intratumoral radioactivity distribution with intravenous injection. In the two-step method, radioactivity in a low dose of streptavidin with intraperitoneal pretargeting primarily localized at the tumor margin. By increasing the dose, streptavidin penetrated more deeply. In tumors with intravenous pretargeting, a more uniform intratumoral distribution of streptavidin was obtained. The biodistribution of radiolabeled streptavidin was the same between different pretargeting routes. **Conclusion:** The better intratumoral penetration of radiolabeled streptavidin after intravenous pretargeting than intraperitoneal pretargeting with biotinylated antibody may be the result of different intratumoral distribution of the binding site for the radiolabel.

Key Words: intratumoral penetration; radiolabeled streptavidin; biotinylated antibody; intraperitoneal tumor

J Nucl Med 1998; 39:30–33

For effective tumor-targeting therapy using armed macromolecules, such as antibodies, specific and high tumor uptake are essential. In addition, a homogeneous intratumoral distribution of pharmaceuticals are also important, especially when short-range radioisotopes, drugs or toxins are conjugated. However, previous studies with radiolabeled antibodies showed that the intratumoral distribution of a labeled antibody was quite heterogeneous and the penetration of the antibody was not deep enough (*1–8*). Various factors are attributed to the heterogeneous distribution of the antibody, such as heterogeneous antigen expression, heterogeneous vascular supply and increased interstitial pressure within the tumor tissue (*8,9*). Furthermore, the administered dose of the antibody, the binding affinity of the antibody to antigen and the antigen density in the tumor cell also affected the distribution of the radiolabeled antibody (*1–7*). Lower dose, higher affinity and higher antigen density sequester the antibody with antigen and prevent the antibody from diffusing deeply, which is known as the binding site barrier hypothesis (*10–12*). The barrier effect was demonstrated in human melanoma and experimental tumor nodules of subcutaneous or intraperitoneal xenografts and even micrometastases as small as 300 μm in diameter (*1–7*).

Since specific antibody distribute in tumors with a concentration gradient from high to low along the penetration route (*11,12*), making the antigen distribution a proper adverse gradient (i.e., from low to high) would help the antibody penetrate more deeply. Changing the distribution pattern of antigen may be difficult. However, a different distribution pattern of the binding site could be obtained by using the intraperitoneal tumor model and pretargeting technique (*13–19*). In intraperitoneal tumors, the antibody can approach the tumor nodules from either the peritoneal surface or the newly formed vessels within the tumor, thus producing different distribution patterns (*5,20*). The antibody, if conjugated with biotin and used as the first step in multistep methods, could serve as the binding site for the following step, such as streptavidin (*16–19*).

In this study, a two-step method of biotinylated antibody and radiolabeled streptavidin was used to study the effect of the intratumoral distribution pattern of the antibody as the binding site for the radiolabel on the intratumoral penetration of radioactivity in intraperitoneal tumors.

MATERIALS AND METHODS

Monoclonal Antibody

MLS128 is a mouse IgG3 monoclonal antibody with a kappa light chain, which recognizes Tn antigen, a cluster of tri-GalNAc α-Ser/Thr (*21–22*). The antibody was purified from ascitic fluid of hybridoma-bearing mice using protein A affinity chromatography (Bio-Rad, Richmond, CA). Radiolabeled MLS128 was bound to human colon cancer cell line LS180 in vitro and accumulated in LS180 tumors subcutaneously inoculated in nude mice (*23*).

Biotinylation of the Antibody

MLS128 was conjugated with biotin using NHS-LC-biotin (Pierce, Rockford, IL) (*24*). Five mg/ml of antibody in 0.075 M phosphate buffered saline (PBS), pH 7.4, and 10 μg/μl of freshly prepared NHS-LC-biotin at a molar ratio of 1:5 were incubated for 2 hr at 4°C, and then unconjugated biotin was removed by chromatography on a PD10 column (Pharmacia Biotech, Uppsala, Sweden).

Radioiodination and Reactivity of Biotinylated Antibody and Streptavidin

Biotinylated MLS128 and streptavidin (Pierce, Rockford, IL) were radioiodinated with ^{125}I using the chloramine T method (*25*). Labeled proteins were separated from free iodine by chromatogra-

Received Mar. 11, 1997; revision accepted Jun. 13, 1997.

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TABLE 1
Biodistribution of Radioactivity in Mice Bearing LS180 Intraperitoneal Xenografts

	^{125}I-MLS128*		^{125}I-streptavidin		
	Intravenous	Intraperitoneal	5 μg†	50 μg†	50 μg‡
Tumor	23.81 ± 5.83	23.69 ± 3.55	32.13 ± 4.00	20.89 ± 1.68	18.74 ± 2.08
Tumor/blood	2.36 ± 0.70	2.07 ± 0.18	1.53 ± 0.38	1.45 ± 0.16	1.45 ± 0.09
Tumor/liver	11.18 ± 2.22	13.67 ± 2.54	4.49 ± 0.53	3.17 ± 0.44	3.31 ± 0.63
Tumor/kidney	7.91 ± 2.22	7.09 ± 0.61	2.69 ± 0.64	0.43 ± 0.07	0.46 ± 0.12
Tumor/intestine	20.11 ± 4.96	20.31 ± 1.66	19.82 ± 3.96	12.87 ± 1.91	11.28 ± 1.42
Tumor/stomach	12.88 ± 3.29	18.11 ± 6.62	17.22 ± 3.89	12.02 ± 3.46	8.94 ± 3.29
Tumor/spleen	12.98 ± 3.11	12.81 ± 2.48	6.08 ± 0.95	4.69 ± 0.35	5.01 ± 1.07
Tumor/lung	4.00 ± 1.28	3.14 ± 0.40	3.56 ± 1.15	2.85 ± 0.47	2.89 ± 0.20
Tumor/muscle	28.88 ± 6.82	26.67 ± 1.12	48.04 ± 18.4	21.98 ± 2.34	19.73 ± 2.25
Tumor/bone	13.13 ± 3.21	14.07 ± 1.49	22.34 ± 4.36	14.99 ± 2.34	13.50 ± 1.23

*Radioiodinated biotinylated MLS128 (300 μg, 48 hr).
†Radioiodinated streptavidin (intraperitoneal, 6 hr) with biotinylated MLS128 pretargeting (300 μg, intraperitoneal, 48 hr).
‡Radioiodinated streptavidin (intraperitoneal, 6 hr) with biotinylated MLS128 pretargeting (300 μg, intravenous, 48 hr).
Mean ± s.d. (n = 5) of tumor %ID/g or tumor-to-nontumor ratios.

phy through a PD10 column. The reactivity of radiolabeled biotinylated MLS128 with avidin was measured by avidin agarose gel (Pierce, Rockford, IL). The reactivity of radiolabeled streptavidin with biotin was measured with polystyrene beads (6.4 mm in diameter), which were coated with biotinylated antibody by the method reported previously (*26*).

Intraperitoneal Tumor Model

LS180 human colon cancer cells were grown in RPMI 1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% fetal calf serum (GIBCO Laboratories, Grand Island, NY) and 0.03% L-glutamine. Subconfluent cells were harvested, and 3×10^6 cells in 0.2 ml PBS was injected intraperitoneally into female BALB/c-nu/nu mice. After 10 to 12 days, many tumor nodules were found in the peritoneal cavity.

Biodistribution Study

In the first experiment, 300 μg of ^{125}I-labeled biotinylated MLS128 antibody was injected intraperitoneally or intravenously into nude mice bearing intraperitoneal tumor xenografts. Biodistribution of radioactivity was examined 48 hr postinjection.

In the second experiment, 300 μg of unlabeled biotinylated MLS128 was given intraperitoneally or intravenously in the tumor-bearing mice. After 48 hr, 5 or 50 μg of ^{125}I-labeled streptavidin was given intraperitoneally. Biodistribution of radioactivity was examined 6 hr postinjection. Statistical analysis was performed using the Student's t-test.

Intratumoral Distribution of Radioactivity

Intratumoral distribution of radioactivity was determined by autoradiography. Immediately after removal, tumors were quickly frozen in Tissue-Tek OCT-compound (Miles Inc., Eikhart, IN). Sections of 16-μm thickness were cut from frozen tumor tissues with a criomycrotome. Dried sections were placed in a light-tight cassette in direct contact with Kodak X-OMAT XAR film (Eastman Kodak, Rochester, NY). Films were exposed at room temperature for 7–10 days according to the estimated tumor uptake of radioactivity and processed through an automatic film processor.

From each group, autoradiograms of tumor nodules more than 2 mm in the shortest diameter were chosen and digitized with a videodensitometric program (Carl Zeiss Vision, München, Germany). For each nodule, the shortest diameter was set from its surface to center, and the densities were measured. The relative density along the line was determined by setting the background as zero and the highest density as 100. The mean density was plotted against the depth in millimeters to create a penetration profile. Magnification was adjusted to give a spatial resolution of 33 μm/pixel.

Semiquantitative analysis of radioactivity intratumoral distribution also was performed. All nodules more than 2 mm in the shortest diameter were examined visually (x^2 test).

RESULTS

Reactivity of Biotinylated Antibody and Streptavidin

The average number of biotin molecules coupled to each antibody was determined to be 1.5 by the method of Green et al. (*24*) using HABA solution (Pierce, Rockford, IL), and the biotinylation did not affect antigen-binding activity of the antibody (*27*). About 90% of the radiolabeled biotinylated MLS128 bound to avidin gel, and more than 80% of the radiolabeled streptavidin bound to biotinylated antibody-coated beads.

Biodistribution of Antibody and Streptavidin

There was no difference in tumor uptake or tumor-to-nontumor ratios between intraperitoneal and intravenous injections of biotinylated antibody (Table 1). With 300 μg of biotinylated antibody pretargeting, the radiolabeled streptavidin markedly and rapidly localized to the tumor. The radioactivity percentage of injected dose per gram of tissue (%ID/g) in tumor decreased when the dose of radiolabeled streptavidin increased from 5 μg to 50 μg ($p < 0.001$). There was no difference in the biodistribution of radiolabeled streptavidin with the same dose (50 μg) between intraperitoneal and intravenous pretargeting routes ($p > 0.1$). Without the pretargeting, radioactivity accumulation to the tumor was significantly lower (data not shown).

Intratumoral Distribution of Iodine-125-Labeled MLS128

Intratumoral distribution of radioactivity was different between intraperitoneal and intravenous injections of the biotinylated antibody (Fig. 1). The density was almost constant from the surface to the center of tumor nodules with intravenous administration. On the other hand, the density was high in the margin and decreased toward the center with intraperitoneal injection. The two administration routes of biotinylated antibody provided different distribution patterns of the binding site for streptavidin in tumors.

Intratumoral Distribution of Iodine-125-Labeled Streptavidin

After intraperitoneal pretargeting of biotinylated MLS128, 5 μg of radiolabeled streptavidin localized at the margin of the

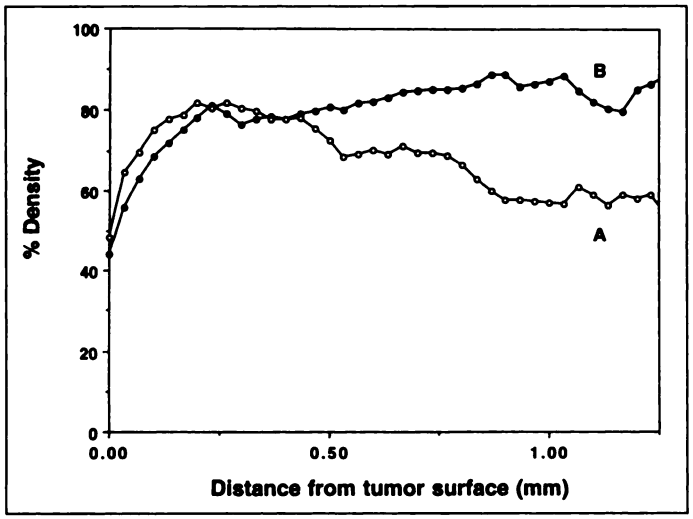

FIGURE 1. Average videodensitometric profiles of all tumor nodules more than 2 mm in the shortest diameter taken 48 hr after injection of radiolabeled biotinylated MLS128. (A) Intraperitoneal administration. (B) Intravenous administration.

tumor as shown in Figure 2A. On videodensitometric analysis, a sharp increase of density was found at less than 0.2 mm from the tumor surface, and the density decreased steeply toward the center of tumor nodules (Fig. 3). By increasing the dose from 5 μg to 50 μg, radiolabeled streptavidin penetrated more deeply as shown in Figures 2B and 3. In intravenously pretargeted tumors, 50 μg of radiolabeled streptavidin showed a more uniform pattern of intratumoral distribution of radioactivity (Figs. 2C, 3). The density was constant between 0.3 mm and 1.2 mm from the surface of tumor nodules. Without the pretargeting, the labeled streptavidin showed diffused but rather faint distribution of radioactivity within the tumor (data not shown).

Results of semiquantitative analysis revealed significantly more nodules showing homogeneous radioactivity distribution with 50 μg of streptavidin in intravenous pretargeting (87.5%, 14/16) than intraperitoneal pretargeting (33.3%, 5/15, $p < 0.01$). There were no nodules showing homogeneous radioactivity distribution with 5 μg of streptavidin.

DISCUSSION

This study shows the binding-site barrier effect in the two-step targeting of intraperitoneally xenografted tumors, similar with previous reports (*18,19*) and demonstrated that, by changing intratumoral distribution pattern of biotinylated antibody, intratumoral penetration of radiolabeled streptavidin was improved without loss of targeting specificity.

We did not examine the internalization of MLS128 in this experimental model. In our previous study (*23*), however, radioactivity of both ^{125}I- and ^{111}In-labeled MLS128 in the tumor maintained at the same level 2 to 4 days postinjection. Together with the effective targeting demonstrated in this study and previous studies (*17,27*), it is likely that the fraction of the MLS128 antibody internalized is very small. Therefore, MLS128 is suitable for pretargeting. In this study, with 300 μg of biotinylated antibody pretargeting, there were about 8×10^{13} antibody, in other words, 1.2×10^{14} biotin molecules in 0.3 g of tumor (the average tumor weight), which could bind a maximal 12 μg of streptavidin (the doses would actually be somewhat less due to metabolism of antigen-antibody complex though very slow for this antibody and steric hindrance of biotin). With the 5-μg administration dose, only 0.45 μg of streptavidin could be accumulated to the tumor, which is far from saturating the binding site. Therefore, a marginal intratumoral distribution resulted. Increasing dose of radiolabeled streptavidin partially improved the penetration but at a cost of specificity (*1,3,4,18*). Theoretically, the administered 50 μg of streptavidin would have 3 μg in the tumor. Though this was still not enough to saturate the binding site, the penetration was better than the low-dose group. The findings also suggest that 50 μg of streptavidin, in this situation, were sensitive enough to see the effect of the distribution pattern of the binding site on the intratumoral penetration of the radiolabel.

As mentioned previously, the density of the binding site also may affect the intratumoral penetration of target material. Theoretically, a lower density of the binding site favors the penetration but worsens the specificity (*10–12,19*). In this experiment, by using pretargeting, a different distribution pattern of binding site was produced, but the amount was not decreased as shown by the same tumor %ID/g of biotinylated antibody with intraperitoneal and intravenous injections, similar with previous reports (*20,28*). Intraperitoneal pretargeting of the biotinylated antibody caused high uptake in periphery and low uptake in the center within the tumor nodule. Therefore, intraperitoneally injected radiolabeled streptavidin would be sequestered at the margin of tumors, slowing its deeper penetration. However, in intravenous biotinylated antibody pretargeting, the distribution of the binding site was different from intraperitoneal pretargeting. Thus, the intraperitoneally injected radiolabeled streptavidin, in the same dose, penetrated more deeply into tumor nodules. That is, the favorable distribution pattern of the binding site improved the intratumoral penetration without decreasing the target specificity.

Molecular size is also one of the factors affecting intratumoral penetration of tumor-specific agents (*29,30*). In this aspect, streptavidin was expected to penetrate more rapidly than the antibody. However, the binding-site barrier principle sug-

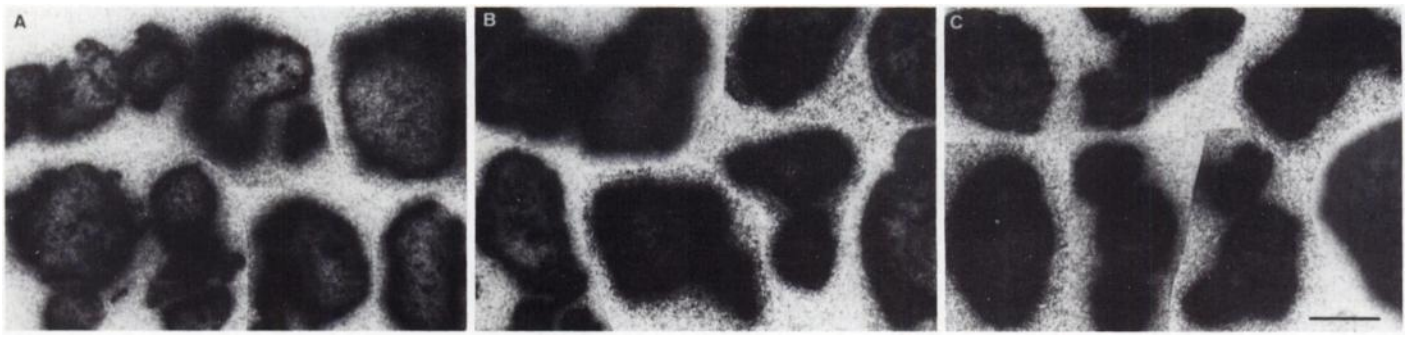

FIGURE 2. Autoradiograms of tumor nodules 6 hr after intraperitoneal injection of radiolabeled streptavidin. (A) 5 μg of streptavidin was injected after intraperitoneal pretargeting with 300 μg of biotinylated MLS128. (B) 50 μg of streptavidin was injected after intraperitoneal pretargeting with 300 μg of biotinylated MLS128. (C) 50 μg of streptavidin was injected after intravenous pretargeting with 300 μg of biotinylated MLS128. Bar = 2 mm.

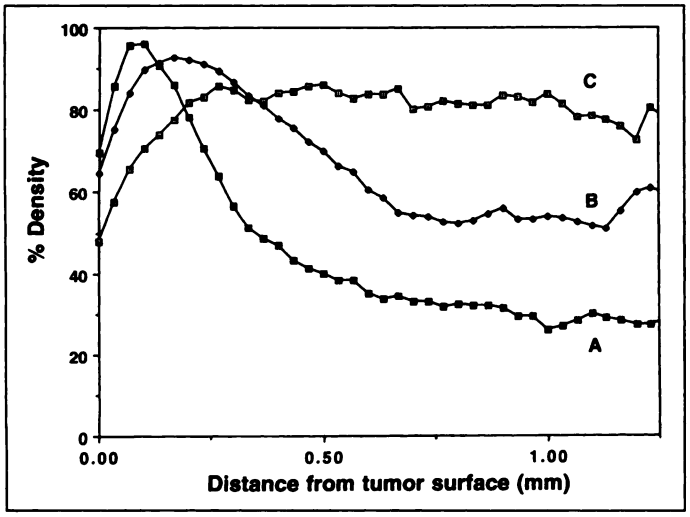

FIGURE 3. Average videodensitometric profiles of all tumor nodules more than 2 mm in the shortest diameter taken 6 hr after injection of radiolabeled streptavidin. (A–C) See Figure 2.

gests that higher affinity decreases the rate of penetration (*10,12*).

For intraperitoneal tumors using the two-step targeting, intraperitoneal administration of radiolabeled streptavidin obtained significantly higher tumor uptake and tumor-to-nontumor ratios of radioactivity than intravenous injection, regardless of the biotinylated antibody administration route (our unpublished observation). Therefore, intraperitoneal injection was used for radiolabeled streptavidin administration in this experiment.

CONCLUSION

The intraperitoneally injected radiolabeled streptavidin had a more homogeneous intratumoral distribution after intravenous pretargeting than intraperitoneal pretargeting with the biotinylated antibody, which might have resulted from the different intratumoral distribution of the antibody, in other words, the binding site for the radiolabel. These findings suggest that intratumoral penetration of target material could be improved by modifying the distribution pattern of its binding site.

ACKNOWLEDGMENTS

We are grateful to the Kato Asao International Scholarship Foundation for their help. This work was supported in part by a Grant-in-Aid for Scientific Research (08266230, 08671024) from the Ministry of Education, Science and Culture, and a Grant-in-Aid from the Sankyo Foundation of Life Science, Japan.

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