The Relationship of Glycosylation and Isoelectric Point with Tumor Accumulation of Avidin

Zhengsheng Yao, Meili Zhang, Harumi Sakahara, Yuji Nakamoto, Tatsuya Higashi, Songji Zhao, Noriko Sato, Yasushi Arano and Junji Konishi

Department of Nuclear Medicine, Faculty of Medicine and Department of Radiopharmaceutical Chemistry, Faculty of Pharmaceutical Science, Kyoto University, Kyoto, Japan

Radiolabeled avidin markedly accumulated in intraperitoneal tumors and was cleared rapidly from circulation when given intraperitoneally. This study investigated the mechanisms of the tumor localization of avidin. **Methods:** Avidin was deglycosylated through endoglycosydase-H digestion and/or neutralized by acetylation of its lysine amino acids with acetic acid N-hydroxysuccinimide ester. Avidin and modified avidins were analyzed using sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS/PAGE) and isoelectric focusing. A tumor model was established by intraperitoneal injection of human colon cancer cells, LS180, in nude mice. Avidin and modified avidins were labeled with ^{111}In using diethyleneamine pentaacetic acid-biotin and were administered intraperitoneally into the tumor-bearing mice. The biodistribution of radioactivity was examined 2 and 24 h postinjection. **Results:** Deglycosylated avidins revealed a major band of smaller molecules on SDS/PAGE. The isoelectric point of neutralized avidins was reduced to less than 5, whereas that of unneutralized avidins was more than 9.5. Biodistribution study demonstrated that liver uptake was decreased by deglycosylation and kidney accumulation was decreased by neutralization, respectively. The blood clearance was remarkably slowed by combined modification of deglycosylation and neutralization. The tumor uptake of radioactivity was reduced by either deglycosylation or neutralization and was further decreased with combined modification. **Conclusion:** Both high glycosylation and positive charge of avidin contributed to its accumulation in tumor. This study may facilitate development of a new vehicle for the delivery of therapeutic agents to intraperitoneal tumors.

Key Words: avidin modification; glycosylation; isoelectric point; tumor accumulation

J Nucl Med 1999; 40:479–483

Intraperitoneal dissemination is a common event in gastrointestinal and ovarian cancer patients. The treatment is very difficult with the many clinically used alternatives, such as systemic or intraperitoneal chemotherapy, external radiotherapy, radioactive colloidal instillation, as well as targeting therapy with intraperitoneal injection of antitumor monoclonal antibodies conjugated with radionuclides, chemotherapeutic agents or toxins (*1–5*). The insufficient efficacy and/or the high blood and nonspecific uptake of therapeutics are the main obstacles to the therapy, therefore, a method with higher tumor-to-nontumor targeting ability needs to be investigated.

Avidin, a 66-kD, highly glycosylated and positively charged protein found in egg white, shows extremely high binding affinity with biotin. Avidin-biotin system has been used in biochemical techniques and also in tumor targeting studies (*6–9*). Because avidin, when given systemically, has been shown to clear rapidly from circulation and accumulate in the liver and kidneys, it has been used to remove biotinylated antibodies from the circulatory system in tumor-imaging studies (*10–13*). Furthermore, our previous study (*14*) showed that radiolabeled avidin localized highly and rapidly to intraperitoneal tumors, and with the rapid blood clearance, markedly high tumor-to-nontumor targeting ratios were obtained.

It has been reported that the carbohydrate of avidin contributed to its liver accumulation the positive net charge related to its kidney accumulation and both played a role in its rapid blood clearance (*15–16*). To investigate the mechanisms of high tumor localization of avidin, we studied the relationship of the high glycosylation and the high electric point of avidin with its high tumor uptake by deglycosylation or neutralization of avidin.

MATERIALS AND METHODS

Biochemical Modification of Avidin

Avidin (Pierce Chemical Co., Rockford, IL) was modified by deglycosylation and neutralization. Deglycosylation was performed through digestion of the sugar of avidin with endoglycosydase-H (Sigma Chemical Co., St. Louis, MO). Briefly, 5 mg/mL avidin in 0.05 mol/L sodium citrate buffer, pH 5.5, was digested with 0.1 U/mL endoglycosydase-H for 24 h at 37°C. The solution was washed with 0.05 mol/L phosphate-buffered saline (PBS), pH 7.5, on a V-20 Centricut mini filter (Biofield Inc., Tokyo, Japan)(*15*).

Neutralization of avidin was performed through acetylation of the lysine amino acids with acetic acid N-hydroxysuccinimide ester (Sigma Chemical Co.) (*15,17*). Briefly, 5 mg/mL avidin in 0.05 mol/L sodium phosphate/0.15 mol/L NaCl buffer, pH 8.0 and 10 μg/μL freshly prepared acetic acid N-hydroxysuccinimide ester at molar ratios of 1:50 were incubated for 1 h at room temperature

Received Apr. 14, 1998; revision accepted July 8, 1998.

For correspondence or reprints contact: Zhengsheng Yao, MD, Department of Nuclear Medicine, National Institutes of Health, Building 10, Rm. 1C-401, 10 Center Dr., MSC 1180, Bethesda, MD 20892-1180.

(*15*), and then, the mixture was applied to a PD10 column (Pharmacia Biotech, Uppsala, Sweden) for purification of avidin by removing unconjugated acetic acid N-hydroxysuccinimide ester.

For combined modification, avidin was deglycosylated first, and the deglycosylated avidin was neutralized with the same protocol.

Electrophoresis

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS/PAGE) was performed with Bio Rad mini-Protein II Ready Gels (Bio Rad Labs, Hercules, CA) (*15,17*). The solutions of avidin and modified avidins are diluted four times with sample buffer and reduced by heating at 95°C for 4 min. Samples (6 μL) containing 3–5 μg of proteins, along with standard solution, were applied to the gel. After running at 200 V for 30 min, the gel was stained with 0.1% Coomassie brilliant blue R-250 in 40% methanol/10% acetic acid for 0.5–1 h at room temperature and followed by being destained with 40% methanol/10% acetic acid for 3 h .

Polyacrylamide isoelectric focusing (IEF) was performed with a Bio Rad mini-IEF cell (Bio Rad Labs) with ampholytes, ranging from 3 to 10 (*15*). Samples (3 μL) containing 3–5 μg of proteins, along with standard solution, were applied to the gel. IEF was performed at 100 V for 15 min, 200 V for 15 min and 450 V for 60 min. The IEF gel was fixed with 12.5% trichloroacetic acid for 30 min at room temperature and then stained with 0.04% Coomassie brilliant blue R-250 in 27% ethanol/10% acetic acid for 1–2 h at room temperature. The IEF gel was destained with 12%–25% ethanol/7% acetic acid for 3 h.

Tumor Cell and Intraperitoneal Tumor Model

Human colon cancer cell line, LS180, supplied by the American Type Culture Collection (Rockville, MD) was used in the experiment. The cells were grown in RPMI-1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% fetal calf serum (GIBCO BRL; Life Technologies, Inc., Gaithersburg, MD) and 0.03% L-glutamine. Subconfluent cells were harvested by use of calcium- and magnesium-buffered saline that contained 0.02% ethylenediamine tetraacetic acid.

The tumor model was established by intraperitoneal injection of 3×10^6 LS180 cells in 0.2 mL PBS into female BALB/c nu-nu mice (Japan SLC, Inc., Hamamatsu, Japan). Many tumor nodules were found in the peritoneal cavity 11 d after injection. The combined tumor weight per mouse was 0.1–0.4 g at the time of experimentation.

Radiolabeling of Avidin

Avidin was radiolabeled with ^{111}In using diethylenetriamine pentaacetic acid α,ω-bis[biocytinamide] (DTPA-biotin) (Sigma Chemical Co.) as a bifunctional chelating agent (*18*).

At first, DTPA-biotin was labeled with ^{111}In using the conjugation of DTPA and indium. Three micrograms DTPA-biotin dissolved in 0.3 mol/L Tris-HCl buffer (pH 7.6) were incubated with 18.5 MBq (0.5 mCi) $^{111}InCl_3$ (Nihon Medi-Physics Co., Nishinomiya, Japan) for 30 min at room temperature. More than 99% of the radioactivity bound to the immobilized avidin gel.

Avidin was then radiolabeled with ^{111}In-DTPA-biotin using the high binding affinity of avidin-biotin. Avidin or modified avidins were mixed with the labeled biotin at a molar ratio of 10:1 and left standing for 30 min at room temperature (*18*). Unconjugated biotin was then removed by means of chromatography on a PD10 column (Pharmacia Biotech Inc.).

Biodistribution Study

All mice were assigned randomly to treatment groups and there were five mice in each group. The radiolabeled proteins (74 kBq [2 μCi]) were injected intraperitoneally into mice bearing intraperitoneal tumor xenografts, and the administered dose was adjusted to 20 μg per mouse by the addition of unlabeled counterparts to radiolabels. One group of mice received radiolabeled avidin as control. At 2 or 24 h after injection of the labels, mice were killed and the biodistribution of radioactivity was examined. The data were expressed as the percentage of the injected dose per gram of tissue (%ID/g) and as tumor-to-normal tissue ratios.

All procedures involving animal controls were performed in accordance with the regulations for animal welfare in Japan.

Statistical Analysis

All values are shown as the mean ± SD. Statistical analysis were performed by using unpaired Student *t* test to evaluate the biodistribution data and tested at the nominal 0.05 significance level. Because multiple comparisons were performed, Bonferroni correction was implemented for each individual comparison to control for the overall type 1 error rate. Statistic significance would be determined by a *P* value of less than 0.05/3 (i.e., 0.0167) because the data of modified avidins (three groups) were compared with those of unmodified avidin. Reported *P* values are from 2-sided tests.

RESULTS

In Vitro Characterization

Subunits from native (Avid) and neutralized avidin (Avid-N) showed the same monomeric weight as demonstrated by SDS/PAGE (Fig. 1A); however, those from deglycosylated avidin (Avid-E) or avidin with combined modification (Avid-E/N) showed a main band of decreased molecular weight due to the removal of oligosaccharide. On the other hand, native avidin showed a very high isoelectric point (pI), around 9.5, as did deglycosylated avidin, as shown in the polyacrylamide IEF (Fig. 1B). Neutralized avidin and avidin with combined modification had very low pIs and migrated to the bottom of the gel at pH 5. Deglycosylation had no effect on the pI of avidin and neutralization had no effect on its molecular weight.

Biodistribution Study

The biodistribution of radiolabeled proteins in nude mice bearing intraperitoneal LS180 tumor xenografts are shown in Tables 1 and 2. When injected intraperitoneally, radiolabeled avidin (Avid) rapidly localized to tumor. Two hours after injection, the tumor uptake of radioactivity reached 69.33 %ID/g, and a high level of radioactivity was also observed at 24 h. After being absorbed from the peritoneal cavity, radiolabeled avidin cleared from circulation rapidly and accumulated in the liver, kidneys and spleen. The blood concentration of radiolabeled avidin was 0.20 %ID/g at 2 h and decreased thereafter. The radioactivities in liver, kidneys and spleen were 10–20 %ID/g at 2 and 24 h.

The modified avidins, however, showed different biodistribution patterns with avidin. The accumulation of deglyco-

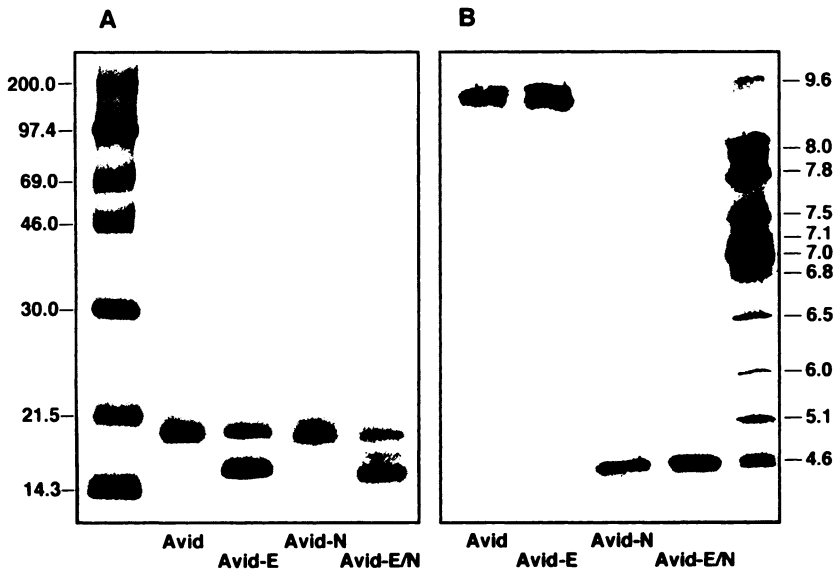

FIGURE 1. SDS/PAGE (A) and IEF (B) of avidin (Avid), deglycosylated avidin (Avid-E), neutralized avidin (Avid-N) and avidin with both deglycosylation and neutralization (Avid-E/N). SDS/PAGE shows decrease in subunit molecular weight with removal of oligosaccharide from avidin, and IEF shows decrease in pI of neutralized avidins.

sylated avidin (Avid-E) in the liver and spleen were decreased significantly ($P < 0.005$ for both 2 and 24 h), but kidney accumulation was still high. The tumor uptake of radiolabeled Avid-E was comparable with that of avidin at 2 h postinjection but decreased, with the difference being significant at 24 h ($P < 0.005$). The neutralized avidin (Avid-N) showed significantly lower tumor uptake ($P < 0.005$ for both 2 and 24 h) and lower kidney accumulation ($P < 0.0001$ for both 2 and 24 h) of radioactivity compared with those of avidin. Avidin with both modifications (Avid-E/N) showed even lower tumor uptake ($P < 0.0005$) and higher blood level ($P < 0.0001$) of radioactivity than those of avidin. As a result, tumor-to-blood ratio was significantly lower, being 1.5 and 10.66 for 2 and 24 h, respectively, compared with more than 300 of avidin. Kidney uptake of radiolabeled Avid-E/N remained low ($P < 0.0001$), but liver uptake increased and was the same at 24 h ($P > 0.2$) after injection though low at 2 h ($P < 0.01$).

DISCUSSION

Findings from this study showed that both high glycosylation and positive charge contributed to the marked tumor uptake of avidin. The biodistribution of avidin and modified avidins in normal organs was similar with previous reports (*15,16,18*). pI of avidin related to its accumulation in the kidneys and carbohydrate residues related to its accumulation in the liver, and both play a role in the rapid blood clearance of avidin.

Cationization of proteins, such as albumin and immunoglobulin molecules, raises their pI and enhances their absorptive-mediated endosytosis in cells (*19–23*). Polycatonic charges on the proteins interact electrostatically with

TABLE 1
Biodistribution of ^{111}In-Labeled Proteins in Mice Bearing Intraperitoneal LS180 Xenografts (2 hours)

	Avidin	Avid-E	Avid-N	Avid-E/N
Blood	0.20 ± 0.02	0.28 ± 0.04	0.44 ± 0.14	7.72 ± 2.71
Liver	18.15 ± 2.44	10.16 ± 1.53	22.81 ± 3.24	10.93 ± 3.41
Kidney	20.22 ± 3.03	36.41 ± 7.74	1.03 ± 0.55	2.87 ± 0.88
Intestine	1.55 ± 0.57	1.24 ± 0.31	0.67 ± 0.09	1.76 ± 0.85
Stomach	5.95 ± 4.03	3.08 ± 1.25	2.04 ± 0.79	1.70 ± 0.82
Spleen	14.97 ± 4.52	7.28 ± 1.76	12.75 ± 2.65	4.74 ± 1.55
Lung	1.17 ± 0.76	0.80 ± 0.39	0.59 ± 0.43	3.43 ± 1.07
Muscle	0.10 ± 0.04	0.09 ± 0.06	0.06 ± 0.05	0.30 ± 0.06
Bone	0.84 ± 0.18	0.75 ± 0.44	1.18 ± 0.26	1.40 ± 0.52
Tumor	69.33 ± 16.58	53.39 ± 16.17	30.38 ± 7.05	11.71 ± 4.78
Tumor-to-blood	343.52 ± 95.26	194.44 ± 54.53	77.49 ± 45.83	1.50 ± 0.15
Tumor-to-liver	3.87 ± 1.05	5.46 ± 2.31	1.35 ± 0.37	1.05 ± 0.16
Tumor-to-kidney	3.54 ± 1.17	1.55 ± 0.69	37.46 ± 22.32	4.03 ± 0.71
Tumor-to-spleen	5.02 ± 1.89	8.03 ± 4.39	2.47 ± 0.80	2.44 ± 0.41

Mean ± SD (n = 5) of %ID/g or tumor-to-organ ratios.

TABLE 2
Biodistribution of ^{111}In-Labeled Proteins in Mice Bearing Intraperitoneal LS180 Xenografts (24 hours)

	Avidin	Avid-E	Avid-N	Avid-E/N
Blood	0.18 ± 0.12	0.05 ± 0.01	0.55 ± 0.06	1.07 ± 0.88
Liver	16.39 ± 4.41	4.12 ± 1.26	23.06 ± 5.21	18.29 ± 3.68
Kidney	18.69 ± 3.74	14.58 ± 1.91	4.70 ± 0.70	1.62 ± 0.22
Intestine	2.52 ± 1.03	0.55 ± 0.19	1.53 ± 0.43	0.96 ± 0.12
Stomach	2.33 ± 0.63	0.99 ± 0.44	2.28 ± 0.72	0.99 ± 0.16
Spleen	11.44 ± 1.31	2.83 ± 0.66	16.56 ± 3.13	9.41 ± 3.07
Lung	1.32 ± 0.34	0.65 ± 0.33	1.55 ± 0.49	1.79 ± 0.34
Muscle	0.12 ± 0.07	0.05 ± 0.01	0.24 ± 0.03	0.55 ± 0.07
Bone	0.83 ± 0.12	0.30 ± 0.11	2.16 ± 0.27	1.97 ± 0.47
Tumor	54.29 ± 19.42	19.26 ± 6.28	26.83 ± 13.32	10.66 ± 1.31
Tumor-to-blood	357.27 ± 125.64	392.57 ± 156.76	50.13 ± 28.53	10.44 ± 0.89
Tumor-to-liver	3.76 ± 2.58	5.15 ± 2.49	1.19 ± 0.54	0.61 ± 0.19
Tumor-to-kidney	3.11 ± 1.75	1.33 ± 0.46	5.58 ± 2.26	6.76 ± 1.74
Tumor-to-spleen	4.90 ± 2.34	7.08 ± 2.60	1.65 ± 0.81	1.24 ± 0.44

Mean ± SD (n = 5) of %ID/g or tumor-to-organ ratios.

negatively charged residues on the cell surface, and this electrostatic interaction is believed to trigger the endocytosis process. The results of this study showed that the tumor uptake of radiolabeled neutralized avidin was significantly lower than that of avidin, suggesting the positive charge of avidin contributed to some extent to its uptake.

Avidin contains terminal N-acetylglucosamine and mannose residues that bind some lectins (*24,25*) and, when given systematically, rapidly accumulates in the liver because the liver contains binding sites for a variety of glycosylated ligands (*26–28*). Although the expression of lectins on LS180 tumor cells has not been studied, it has been reported that lectins are expressed at various levels on the surface of various tumor cells (*29–32*) and that they act to internalize their ligands (*33*). Therefore, glycoconjugates specifically recognized by these lectins could be used as carriers of therapeutics for the management of tumors (*29–33*). In this study, radiolabeled deglycosylated avidin showed significantly lower tumor uptake than that of avidin, suggesting that some carbohydrate residues of avidin also contributed to the uptake. Deglycosylated avidins still showed somewhat high liver accumulation, which may be related to the incompleteness of deglycosylation of avidin as shown on SDS/PAGE.

Avidin with both modifications showed still lower tumor localization and higher blood concentration, which is similar to the results from neutravidin (*14*), a modified avidin derivative that does not contain carbohydrate and has a relatively neutral pI (pI = 6.3). Once again, it suggested that both the positive charge and glycosylation of avidin contributed to its tumor uptake.

From this study, it could be hypothesized that, after intraperitoneal injection, avidin rapidly finds and binds to tumor cells and is internalized (*14*). Unbound avidin, after being absorbed into circulation, is rapidly cleared from circulation and accumulates in the liver and kidneys. Therefore, avidin could be used for tumor imaging and therapy because it can be conjugated with radioisotopes or other therapeutics like drugs (*14–16,26*). A two-step approach, in which avidin was injected for pretargeting and radiolabeled biotin was injected subsequently as the second step (*14*), decreased radioactivities in the liver, spleen and kidneys and improved the tumor-to-organ ratios.

Clarification of the mechanisms of tumor uptake of avidin, on the other hand, would be helpful in finding vehicles suitable for clinical applications. Because avidin is a foreign protein, the development of human antiavidin immunoglobulin antibody is a problem for clinical application (*34*). On the basis of the investigation of the mechanisms of the high tumor localization of avidin, we conjugated human serum albumin with mannose, which avidin contains. Levels of mannosyl-albumin were high in the tumor and low in the normal organs, resulting in high tumor-to-nontumor target ratios (our unpublished observation). The pI modification of the mannosyl-albumin should further enhance tumor uptake of modified albumin.

CONCLUSION

The high glycosylation and high pI of avidin relate to its marked accumulation in the tumor. It is hoped that this study will facilitate the development of a new vehicle for the delivery of therapeutics to intraperitoneal tumors.

ACKNOWLEDGMENT

$^{111}InCl_3$ was generously supplied by Nihon Medi-Physics Co., LTD.

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