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Biodistribution Study of ^{99m}Tc-Labeled Succinic Acid-Conjugated Low pI Avidin

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＝국문초록＝

낮은 등전점을 갖는 ^{99m}Tc 표지 Succinic Acid 결합 Avidin의 생체내분포에 관한 연구

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Avidin과 biotin의 높은 결합력을 이용하여 종양 영상을 개선하는 방법이 많이 연구되고 있다. 본 실험에서는 이러한 목적으로 쓰기 위하여 적당한 ^{99m}Tc 표지 avidin을 제조하였다. Avidin을 표지하기 위하여 우선 ^{99m}Tc과 안정한 킬레이트를 형성할 수 있는 benzoylmercaptoacetyltriglycine (Bz-MAG3)과 biocytin을 화학적으로 결합시킨 Bz-MAG3-biocytin을 합성하였다. 이 화합물을 Tc-99m으로 표지시켜 avidin 또는 streptavidin을 1：1로 섞어 줌으로서 Tc-99m으로 표지된 avidin과 streptavidin을 제조하였다. 이들의 생쥐 생체내 분포를 조사한 결과 avidin의 경우 높은 간(56.6%, 10 min)과 신장(28.5%, 10 min) 축적을 보였고 streptavidin의 경우 높은 신장 축적(28.9%, 21 hr)을 보였다. Avidin의 높은 정상 조직 축적을 줄이기 위하여 succinic acid를 결합시켜 등전점(pI)을 낮춘 다음 같은 실험을 하여 본 결과 신장 축적율은 pI가 7.0~9.3, 5.5~6.2, 4.0~4.8로 낮아졌을 경우 19.0%, 3.1%, 1.7%로 각각 떨어졌지만 간에의 축적은 pI 변화에 따른 상관성을 찾아 볼 수가 없었다. 체내 제거율을 측정하여 본 결과 pI를 변화시킨 avidin과 변화시키지 않은 avdin들은 반감기가 13.5에서 16.0 시간 사이로 큰 차이점을 보이지 않았는데 streptavidin은 반감기 61.5시간 정도로 느리게 제거된다는 것을 알았다. 이 실험의 결과 1. Avidin을 ^{99m}Tc-MAG3-biocytin으로 안정하게 표지할 수 있었고, 2. pI가 낮아진 avidin은 신장에의 축적율이 크게 감소되었으며, 3. ^{99m}Tc으로 표지된 avidin과 streptavidin은 먼저 간으로 흡수된 후 대사된 다음 신장으로 배설된다는 사실을 알았다.

주요 단어：Avidin, biotin, ^{99m}Tc, Isoelectric point

INTRODUCTION

Avidin is a basic glycoprotein (pI=10) with a molecular weight of 66 kDa and is composed of 4 subunits. Each subunit has one biotin-binding site, and the avidin-biotin binding constant, 10^{15} M^{-1}, is known to be the highest in the biological system. Streptavidin also has 4 subunits those bind to biotin with similar affinity with avidin. Unlike avidin, streptavidin is a neutral protein with a molecular weight of 60 KDa and the possibility that one can increase the binding site concentration by conjugating either avidin or biotin molecules to antibody, this avidin-biotin binding system has been applied to enhance tumor to normal tissue ratios for scintigraphic imaging of tumors.

In spite of a potential complication due to the modulation of target antigens upon binding to antibody or possible interference of endogenous biotin, a two-step or three-step approach using In-111 labeled biotin has been applied to visualize tumors in nude mice[1~3] and also in human patients with some success[4~8].

Since In-111 labeled biotin or avidin was reported to clear from whole body faster than intact antibody, we thought that ^{99m}Tc coupled to this avidin-biotin system also might be useful for a two-or three-step approach for the scintigraphic detecton of tumors. We have first labeled biotin with ^{99m}Tc after conjugating *Nε-biotinyl-L-lysine with* benzoyl-MAG3, expecting that the polar nature of the core of ^{99m}Tc-MAG3 complex would have made the conjugate be excreted through kidney. However, it accumulated rapidly in liver (>86% ID/g at 5 min) and was excreted through hepatobiliary system, similar to ^{99m}Tc diisopropyliminodiacetic acid (^{99m}Tc-DISIDA)[9]. This paper describes the modification of avidin with succinic acid, and finally their biodistribution in normal mice.

MATERIALS AND METHODS

1. Conjugation of Succinic Acid to Avidin

To reduce the pI of avidin (Fluka Chemika-BioChemika), succinic anhydride (1.2 ml of 2.4×10^{-2} M) dissolved in DMSO was added to avidin solution (12 ml of 3.8×10^{-5} M) in 0.08 M sodium bicarbonate buffer, pH 8.2. The solution was mixed well and the conjugation reaction was continued at room temperature for 1 hr. The succinic acid-conjugated avidin was fractionated based on differences in isoelectric point using a preparative isoelectric focusing instrument (RF3, Rainin Instrument, 1000 V, 15 to 24 mA, 1% Ampholyte pI 3~10 with 10% glycerol, 4 hr run at 8°C). The pI's of fractionated succinic acid conjugated avidin were measured by analytical isoelectric focusing instrument (PhastSystem, Pharmacia-LKB). The samples were isoelectric focused on a Phast Gel (IEF 3~9, 2,000 V, 2.5 mA, 15℃, 40 min) and stained with Coomassie blue.

2. Synthesis of Bz-MAG3-Biocytin

Benzoylmercaptoacetylglycylglycylglycine (Bz-MAG3) was synthesized by the method of Fritzberg, et al[10]. The carboxy group of MAG3 was activated to the corresponding N-hydroxysuccinimide ester by reacting Bz-MAG3 (197.3 mg, 0.537 mmol) with N-hydroxysuccinimide (61.8 mg, 0.537 mmol) using dicyclohexylcarbodiimide as a coupling agent in 3.2 ml DMF. *The esterification was performed with* gentle stirring at 4°C overnight. Dicyclohexylurea precipitated out of the reaction solution was removed by centrifugation (3000xg, 30 min). To the supernatant solution, biocytin (200 mg, 0.537 mmol in 1 ml of 0.5 M sodium bicarbonate, pH 8.4) was added dropwise and the acylation reaction was continued at room temperature for 2 hr. The product was precipitated by adding tetrahydrofuran. The crude product was then washed thoroughly with distilled water to remove polar reagents and dried.

The melting point was 171~173°C. Elemental analysis calculated for $C_{31}H_{44}O_9N_7S_2$: C, 51.47%; H, 6.14%; N, 13.62%; S, 8.86%. Found: C, 50.75%, H, 6.01%; N, 13.36%; S, 8.55%.

3. Radiolabeling of MAG3-Biocytin with ^{99m}Tc

Bz-MAG3-Biocytin (10 μl of 20 mg/ml DMSO) and $SnCl_2 \cdot 2H_2O$ (3 μl of 1.5 mg/ml 0.02 M HCL) were added to a vial containing 40 μl of 0.4 M sodium glucarate pH 5.6. To this solution, 300 μl (10 mCi) of ^{99m}Tc-pertechnetate was added. The reaction vial was evacuated and placed in boiling water for 10 min. The labeling yield and purity were determined by reverse phase TLC (Analtech) developed with 0.01 M sodium phosphate at pH 6.7 and ITLC (Gelman) developed with a solvent mixture (50% methanol in 10% aqueous ammonium acetate solution). The Rf values of ^{99m}Tc-MAG3-biocytin, ^{99m}Tc-MAG3 and ^{99m}Tc pertechnetate were 0, 0.6 and 0.9, respectively on the reverse phase TLC. These products moved to the solvent front on the ITLC and no activity remained at the origin, indicating that ^{99m}Tc-colloids were not formed in this labeling condition, The labeled product was further pruified by a C_{18} SEP-PAK cartridge (Waters, Milford, MA). The cartridge was first eluted with a solvent mixture containing 5% ethanol and 95% 0.01 M sodium phosphate at pH 6.7 to eliminate possible polar ^{99m}Tc impurities from the cartridge. The desired product was eluted out with ethanol and the ethanolic solution was diluted 500 times with saline for the biodistribution studies.

4. Radiolabeling of Avidin with Biocytinyl MAG3-^{99m}Tc

Ten μl (400 μCi) of ^{99m}Tc-MAG3-biocytin (7.8×10^{-4} M) was mixed with 260 μl of avidin or avidin-succinic acid conjugate (3.0×10^{-5} M). The mixture was incubated at room temperature for 1 hr (Fig. 1). The ^{99m}Tc labeling yields were higher than 95%. The ^{99m}Tc labeled avidin and each avidin-succinic acid was further purified by size exclusion HPLC (equipped with TSK-3000, eluted with 0.1 M sodium sulfate/0.01 M sidium phosphate (pH 6.7) with 1 ml/min) for the biodistribution studies.

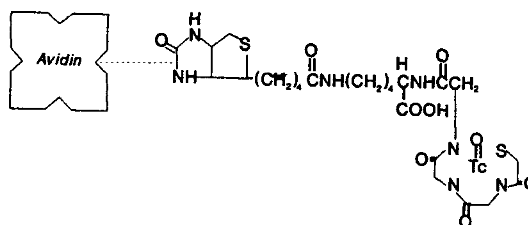

Fig. 1. Labeling of avidin using ^{99m}Tc-MAG3-biocytin.

5. Competitive Binding Test

Avidin conjugated agarose (Sigma) was diluted with Sepharose 4B (Pharmacia) to adjust the biotin-binding capacity (about 0.1 μg biotin binding/100 μl packed beads). To 100 μl of the packed beads, 100 μl of PBS, 200 μl of biotin with varing amount containing upto 24.4 μg and 100 μl of ^{99m}Tc-MAG3-biotin (2.5 ng, 330,000 cpm) were added. After 1 hr incubation at room temperature the test tubes were centrifuged at 2,500 xg for 30 min. The supernatants were aspirated and the agarose beads were washed with 1 ml of PBS. The radioactivities of agarose beads were measured by gamma counter (Packard AutoGamma 5650).

6. In Vitro Stability Test

^{99m}Tc-MAG3-biocytin (5 ng, 660,000 cpm) was bound to 100 μl of packed avidin-agarose beads in a disposable pipette. The beads were thoroughly washed with 3 ml of PBS and then added to 200 μl of mouse serum. After 2 hr and 24 hr incubation at 37°C, the mixture was vortexed and filtered through a Ultrafree-MC (Millipore) 0.45 μm filter unit. The radioactivity of the filtrate and the radioactivity bound to the beads were measured by gamma counter.

7. Biodistribution

^{99m}Tc labeled avidin or ^{99m}Tc labeled avidin-succinic acid (38 μg, 30 μCi/0.2 ml) was injected into the tail vein of normal female mice (about 10 g, 5 mice per group, female Balb/c from Harlan Sprague Dawley, Inc). The mice were sacrificed by exanguination after halothane anaesthesia at 0.25, 1, 6 and 21 hr after injection. The organs were excised, blotted with tissue papers, weighed prompltly and counted with gamma counter.

RESULTS

To radiolabel the biotin derivative with ^{99m}Tc, alpha amino group of biocytin was reacted with the activated ester of Bz-MAG3. This biotin conjugate was then radiolbeled using ^{99m}Tc glucarate.

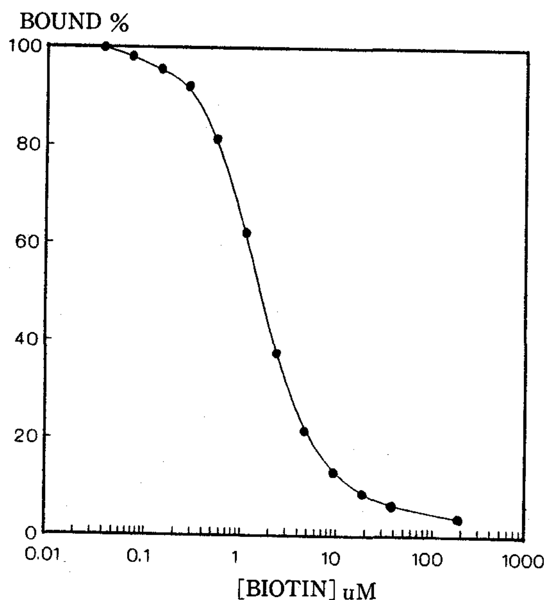

Fig. 2. Competitive binding of ^{99m}Tc-MAG3-biocytin to avidin-agarose beads using cold biotin as a competing ligand. ^{99m}Tc-MAG3-biocytin (2.5 ng, 330,000 cpm) and cold biotin at a concentration up to 24.4 μg were incubated with avidin-agarose beads (capacity of binding 0.1 μg biotin) in 0.5 ml PBS at pH 7.4 for 1 hr.

Biocytin, which we used for this study, is a commercially available biotin analogue derived from acylation of the carboxy end of biotin to the ε-amino group of lysine. The high affinity constant of the avidin-biotin binding also resulted in a quantitative binding of the ^{99m}Tc labeled biotin to avidin when the agents at a 1: 1 molar ratio were combined (Fig. 1).

Competitive binding with cold biotin to avidin conjugated agarose shows that the binding activity of ureido group of ^{99m}Tc-MAG3-biocytin to avidin was not altered by harsh labeling condition (Fig. 2).

The in vitro stability test serum showed greater than 97% and 95% of radioactivity was bound to avidinagarose after 2 hr and 24 hr incubation at 37°C, respectively.

A drawback for the use of the ^{99m}Tc labeled avidin was that the radioactivity was eliminated from blood very rapidly and taken up primarily by liver (56.6%) and kidneys (28.9%) with .8% ID remained in the whole blood at 15 min. The liver and kindey activities decreased to 13.1% and 2.1%, respectively

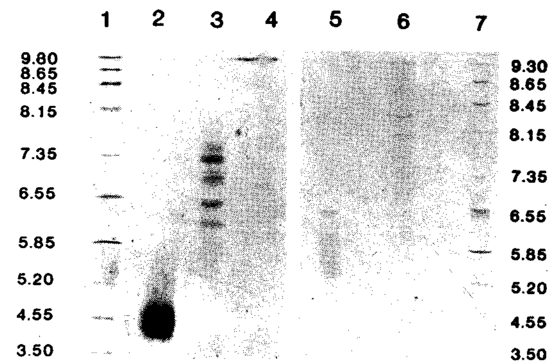

Fig. 3. Isoelectric focusing of avidin, modified avidin and streptavidin. The samples were isoelectric-focused on a PhastGel (IEF 3~9, Pharmacia) using the following pI standard samples; amyloglucosidase (3.50), methyl red dye (3.75), soybean trypsin inhibitor (4.55), beta-lactoglobulin A (5.20), bovine carbonic anhydrase B (5.85), human carbonic anhydrase B (6.55), horse myoglobin-basic band (7.35), lentil lectin-acidic and (8.15), lentil lectin-middle band (8.45), lentil lectin-basic band (8.65) and trypsionogen (9.30).

at 21 hr. The intestinal activity was less than 9% ID throughout the time period (Fig. 4).

To investigate the effect of pI, we conjugated succinic acid moieties to avidin via the acylation with succinic anhydride. The modified avidins with different pI values were fractionated using a preparative isoelectric focusing device. The pI value of each fraction was also determined by an analytical isoelectric focusing instrument (Fig. 3). We have selected 3 fractions with pI's of 7.0~9.3, 5.5~6.2, and 4.0~4.8 for biodistribution studies.

The major difference in biodistribution was observed in blood and kidney activities. The results indicated that the kidney uptake is proportional to the pI value whereas the initial blood activity is inversely proportional. At 10 min, the kidney activity was 19%, 3.1% and 1.7% and blood activity was 14%, 6% and 30% for the labeled avidin with pI values 7.0~9.3, 5.5~6.2, and 4.0~4.8, respectively. However, the native avidin and the modified avidin were excreted very rapidly so that the activities remaining in blood and kidney were negligible. The decrease of the pI appeared to increase the liver activity upto 1 hr but the effect was not as drastic as the effect shown on the kidney activity. The intestinal activities of the low pI avidins were similar to that of avidin (Fig. 5). The whole body clearance of the modified avidins ($T_{1/2}$: pI 7.0~9.3, 14.4 hr; pI 5.5~6.2, 14.6 hr; pI 4.0~4.8, 13.7 hr) were slightly faster than that ($T_{1/2}$= 16.5 hr) of the native avidin. But the whole body

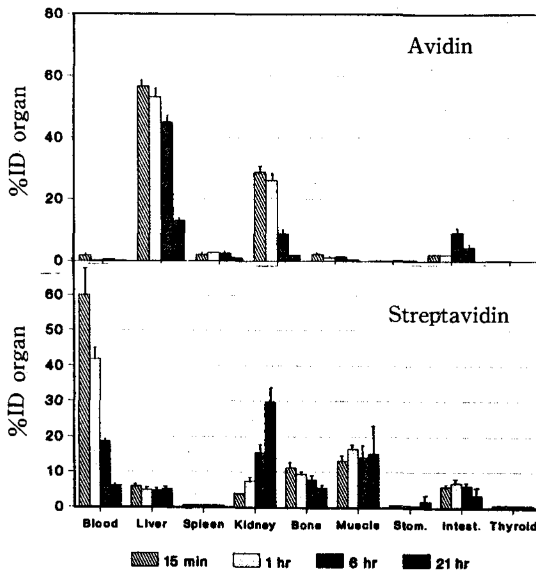

Fig. 4. Comparative biodistribution of ^{99m}Tc-MAG3-biocytin/avidin and ^{99m}Tc-MAG3-biocytin/streptavidin in normal balb/c mice. The animals were sacrificed in a group of five at 10 min up to 21 hr. The results are reported in % ID/organ and 1 SD.

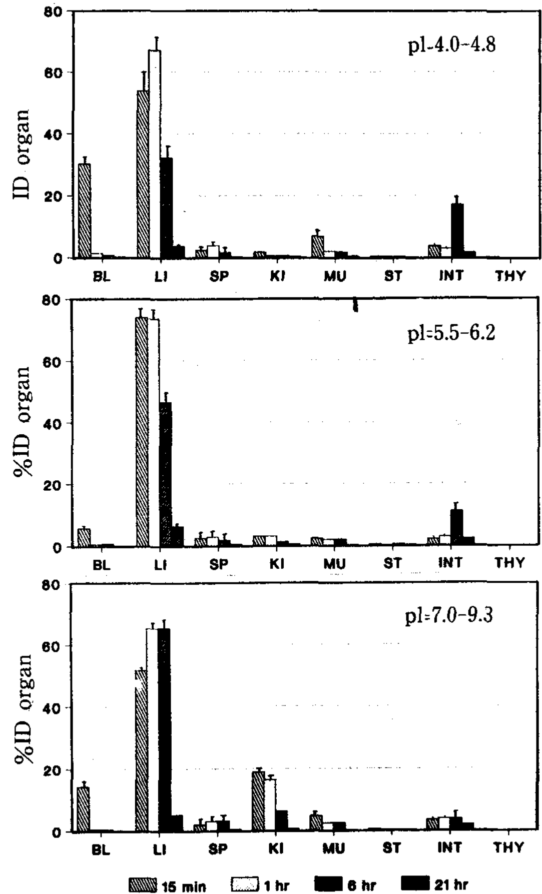

Fig. 5. Comparative biodistribution of ^{99m}Tc-biocytin/avidin preparations with different pI values (pI=4.0−4.8, 5.5−6.2 and 7.0−9.3) in normal balb/c mice. The animals were sacrificed in a group of five at 10 min up to 21 hr. The results reported in % ID/organ and 1 SD. BL, blood; LI, Liver; SP, spleen; KI, kidney; ST, stomach; IN, intestine; THY, thyroid.

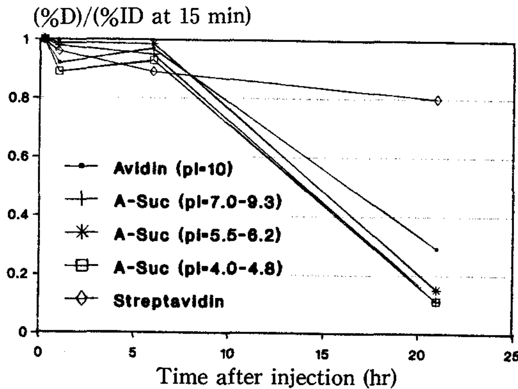

Fig. 6. Whole body retention of ^{99m}Tc activity in normal balb/c mice (n=5) after intravenous injection of ^{99m}Tc-MAG3-biocytin/avidin, modified avidin, or streptavidin.

clearance of streptavidin ($T_{1/2}$=61.5 hr) was much slower than native avidin or modified avidins (Fig. 6).

DISCUSSION

Although the high affinity binding ($K_a=10^{15}$) of biotin to avidin has been applied to amplify the sensitivity of *in vitro* binding assays[11~13], *in vivo* tumor targeting[14,15], and the detection of infection sites[16], this binding system has not been actively pursued in radiolabeling. We have developed a methodology to use this binding system to label avidin with ^{99m}Tc labeled biocytin.

After 1:1 mixing of avidin and ^{99m}Tc labeled biocytin, three binding sites are still available for binding to biotinylated antibodies theoreticaly. This approach to label avidin is advantageous as compared to the labeling of the MAG3-conjugated avidin because the labeling of MAG3 requires either a high temperature or a high pH (> 11) which is detrimental to protein.

The advantage of radiolabeling avidin with ^{99m}Tc through this avidin-biotin binding approach achieved by the fact that the biotin moiety was stable at high temperature, which was required for the hydrolysis of the benzoyl protecting group for the ^{99m}Tc labeling of MAG3, and also the overall ^{99m}Tc labeling yield was almost quantitative.

We have suspected that the high pI value of avidin might be responsible for high liver and kidney activities. Generaly cell membrane is composed of negatively charged phospholipids, but the avidin will exist as positively charged molecule in physiological pH due to its high pI. It has been reported that the high nonspecific binding of avidin can be reduced by modifying with acetic anhydride, phthalic anhydride, or succinic anhydride, *in vitro* solid phase binding assay[17].

The differences of liver uptakes between avidin and streptavidin can be explained by the carbohydrate moieties. Both avidin and streptavidin have similar molecular weights, and low pI avidin shows high liver uptakes. It is reported that liver cell has receptors for galactose[18], mannose/N-acetylglucosamine[19], etc. The structure and composition of carbohydrate moiety of avidin was studied and found that it is composed of mannose and N-acetylglucosamine N-linked to asparagine residue[20,21]. So the native avidin and modified avidin can be taken up quickly by liver.

The results showing higher whole body retention of ^{99m}Tc-streptavidin can be explained by the difference in liver uptake. All the native avidin, modified avidins, and streptavidin will not be excreted directly from kidney because of their high molecular weight. They should be metabolized or the ^{99m}Tc-MAG3-biocytin should be dissociated from avidin before excretion. This explains that higher liver uptake is related to higher metabolism rate and faster excretion. Schechter et al[22] reported that avidin shows lower liver uptake than streptavidin at 6 hr. In the experiment, avidin was labeled with ^{125}I by Bolton-Hunter method[23] and streptavidin was laveled with ^{125}I by chloramine-T method[24], and the earlist time point for checking biodistribution was 6 hr. The differences in labeling method, radioisotope,

and time point can show different results. Generaly ^{125}I is easily dehalogenated and excreted from body.

CONCLUSION

In conclusion, avidin was labeled with ^{99m}Tc through the avidin-biotin binding approach with high labeling yield. The ^{99m}Tc labeled avidin accumulated in liver and kidneys in high concentration. The reduction of the pI of avidin to 6.2 decreased the kidney uptake but not the high liver uptake.

REFERENCES

1) Hnatowich DJ, Virzi F, Ruscowski M: *Investigation of avidin biotin for imaging applications. J Nucl Med 28:1294, 1987*
2) Goodwin DA, Mears CF, McCall MJ, et al: *An avidin-biotin system for imaging tumors. J Nucl Med, 28:722, 1987*
3) Pervez S, Paganelli G, Epenetos AA, Mooi WJ, Evans DJ, Krause T: *Localization of biotinylated monoclonal antibody in nude mice bearing subcutaneous and intraperitoneal human tumor xenografts. Int J Cancer No.3 wuppl, 1988*
4) Paganelli G, Magnani P, Zito F, et al: *Three-step monoclonal antibody tomor targeting in carcinoembryonic antigen-positive patients. Cancer Research 51:5960, 1991*
5) Paganelli G, Belloni C, Magnani P, et al: *Two-step tumour targeting in ovarian cancer patients using biotinylated monoclonal antibodies and radioactive streptavidin. Eur J Nucl Med 19:322, 1992*
6) Kalofonos HP, Rusckowski M, Siebecker DA, et al: *Imaging of tumor in patients with In-111-labeled biotin and streptavidin-conjugated antibodies: Preliminary communication. J Nucl Med 31:1971, 1990*
7) Paganelli G, Perez S, Siccardi AG, et al: *Intraperitoneal radiolocalization of tumors pretargeted by biotinylated monoclonal antibodies. Int J Cancer 45: 1184*
8) Paganelli G, Magnani P, Zito F, et al: *Three step monoclonal antibody tumor targeting in carcinoembryonic antigen-positive patients. Cancer Res 51: 5960, 1991*
9) Jeong JM, Kinuya S, Paik CH, et al: *Synthesis of a ^{99m}Tc-MAG3-biotin as a biliary agent. IXth Intenational Symposium of Radiopharmaceutical Chemistry, 1992*
10) Fritzberg AR, Kasina S, Eshima D, et al: *Synthesis and biological evaluation of Technetium-^{99m} MAG3 as a hippuran replacement. J Nucl Med 27: 111, 1986*
11) Wilcheck M and Bayer EA: *Introduction to avidin-biotin technology. Methods in Enzymology 184:5-13, 1990*
12) Varasteh A, Ewllman M, Artur Y, el al: *An avidin-biotin ELISA for the measurement of mitochondrial aspartate aminotransferase in human serum. J Immunol Meth 128:203, 1990*
13) Kongtawelert P, Ghosh P: *A new sandwich-ELISA method for the determination of keratin sulphate peptide in biological fluids employing a monoclonal antibody and labeled avidin biotin technique. Clin Chim Acta 195:17, 1990*
14) Wang ZF, Stein R, Sharky RM, et al: *Carcinoembryonic antigen and alpha-fetoprotein expression and monoclonal antibody targeting in a human hepatoma/nude mouse molel. Cancer Res 50:869, 1990*
15) Khawli LA, Alauddin MM, Miller GK, et al: *Improved immunotargeting of tumors with biotinylated monoclonal antibodies and radiolabeled streptavidin. Antibody, Immuinoconjugates, and Radiopharmaceuticals 6:13, 1993*
16) Rusckowski M, Fritz B, Hnatowich DJ: *Localization of infection using streptavidin and biotin: An alternative to nonspecific polyclonal immunoglobulin. J Nucl Med 33:1810, 1992*
17) Chiolerio F, Filippini E, Magnani, el al: *Modified avidins in detection systems. Macromolecular Biorecognition, Clifton, NY, Humana Press, 303-7, 1988*
18) DeLange, RJ: *Eff white avidin: I. Amino acid composition: sequence of the amino- and carboxyl- terminal cyanogen bromide peptides. J Biol Chem 245: 907, 1970*
19) Bruch RC, White HB: *Compositional and structural heterogeneity of avidin glycopeptides. Biochemistry 21:5334, 1982*
20) Morell AG, Irvine RA, Sternlieb Um et al: *Physical and chemical studies on ceruloplasmin: V. Metabolic studies on sialic acid-free ceruloplasmin in vivo.*

J Biol Chem 243:155, 1968

21) Stockert RJ, Morell AG, Scheinberg IH: *The existence of a second route for the transfer of certain glycoproteins from the circulation into the liver. Biochem Biophys Res Commun 68:988, 1976*
22) Schechter B, Silberman R, Arnon R: *Tissue distribution of avidin and streptavidin injected to mice. Eur J Biochem 189:327, 1990*
23) Bolton AE, Hunter WM: *The labelling of proteins to high specific radioactivities by conjuigation to a I-125-containing acylating agent. Biochem J 133: 529, 1973*
24) Hunter WM, Greenwood FC: *Prepatation of Iodine-131 labelled human grwth houmone of high specific activity. Nature 194:496, 1962*