Biodistribution and Hepatic Metabolism of Galactosylated ^{111}In-Antibody-Chelator Conjugates: Comparison with ^{111}In-Antibody-Chelator Conjugates

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Abstract

Purpose : To evaluate the use of monoclonal antibody (MoAb) as a carrier of the receptor-binding ligand, the receptor mediated uptake into liver and subsequent metabolism of ^{111}In-labeled galactosylated MoAb-chelator conjugates were investigated and compared with those of ^{111}In labeled MoAb. **Materials and Methods** : T101 MoAb, IgG_2 against human lymphocytic leukemic cell, conjugated with cyclic DTPA dianhydride (DTPA) or 2-p-isothiocyanatobenzyl-6-methyl-DTPA (1B4M) was galactosylated with 2-imino-2-methoxyethyl-1-thio-β-D-galactose and then radiolabeled with ^{111}In. Biodistribution and metabolism study was performed with two ^{111}In-conjugates in mice and rats. **Results** : ^{111}In-labeled T101 and its galactosylated conjugates were taken to the liver by the time, mostly within 10 min. However DTPA conjugate was retained longer in the liver than the 1B4M conjugate (55% vs 20% of injected dose at 44 hr). During this time, the radiometabolite of DTPA conjugate was excreted similarly into urine (24%) and feces (17%). The radiometabolite of 1B4M was excreted primarily into feces (68%) rather than urine (8%). Size exclusion HPLC analysis of the bile and supernatant of liver homogenate showed two peaks, the first (35%) with the retention time (Rt) identical to IgG and the second (65%) with Rt similar to free ^{111}In at 3 hr post-injection for the 1B4M conjugate, indicating that the metabolite is rapidly excreted through the biliary system. In contrast to DTPA conjugate, the small ^{111}In-DTPA-like metabolite was the major radioindium component (90%) in the liver homogenate as early as 3 hour post-injection, but the cumulative radioindium activity in feces was only 17% at 44 hour, indicating that the metabolite from DTPA conjugate does not clear readily through the biliary tract. **Conclusion** : The galactosylation of the MoAb conjugates resulted in higher hepatocyte uptake and enhanced metabolism, compared to those without galactosylation. Metabolism of the MoAb-conjugates is different between compounds radiolabled with different chelators due to different characteristics of radiometabolites generated in the liver.

Key Words: Biodistribution, Metabolism, Galactosylated antibody, ^{111}In-labeled antibody

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Introduction

One of the problems associated with tumor targeting using monoclonal antibody (MoAb) labeled with radiometals is the substantial uptake of radiolabels by non-targeting organs, particularly liver and bone.[1-3] Such a high uptake in normal liver is especially prominent, and approximately 15-20% of the injected dose of ^{111}In-labeled antibody accumulate in the liver of animals or patients.[4,5] High liver uptake may not only result in poor detection of small tumors in close to the liver very difficult, but also reduce the amount of radiolabeled antibody available for tumor targeting.[6] The high radiation exposure to the liver may be significant when therapeutic radionuclides are used. Several bifunctional chelating agents[7,8] and metabolizable linkages between the metal chelate and antibody[9,10] have been introduced to lower ^{111}In accumulation in the liver, in the tumor detection and therapy.

Although the mechanisms of ^{111}In accumulation in the liver are not completely known, the ^{111}In in the liver is associated with intact immunoglobulin at early times after antibody infusion, whereas at later times, the radioactivity is associated with small molecular weight metabolic products mainly located in the hepatocyte.[11,12] A comprehensive understanding of the hepatic uptake, metabolism and sequestration mechanism of the ^{111} In-labeled MoAb could provide alternative methods which may circumvent some of these problems. Intrahepatic degradation and excretion mechanisms of the radiolabeled glycoprotein, which is taken by hepatic cells via asialoglycoprotein receptor mediated endocytosis, have been studied to understand the intracellular metabolism of radiolabeled proteins externally injected.[13-15] They demonstrated that these proteins are degraded in the lysosomes both in vitro and in vivo, and that low molecular weight metabolites containing ^{111}In were formed. The metabolite was identified as ^{111}In-chelator-lysine. The metabolites remain in the lysosomes and are responsible for high liver radioactivity accumulation.

Asialoglycoprotein receptor in hepatic cell has been known for selective uptake of galactose-terminated proteins into the liver, such as toxins, enzymes, hormones and antibodies.[16-18] Galactosyl -neoglycoalbumin is mainly taken up by hepatic parenchymal cell, whereas mannosyl neoglycoalbumin by Kupffer cells and endothelial cell. The rapid clearance of injected galactosylated glycoproteins from the blood of mammals occurs, exclusively to the liver and is mediated by a carbohydrate recognition system present mainly in hepatocytes. The glycoprotein bound to membrane galactose receptor is rapidly internalized, transported within membrane bound structure to lysosomes and subsequently degraded.[16-18] Such an experimental system would be useful to minimize the transchelation and redistribution of radiolabels generated outside liver.[14] Still there are main questions to be answered before generalizing these results to radioimmunodetection with ^{111}In-labeled MoAb. The hepatic metabolism and excretory pathway of ^{111}In-labeled galactosylated MoAb-chelator conjugates can not be the same with that of ^{111}In-nongalactosylated MoAb.

We have investigated the immunoreactivity, hepatic uptake and subsequent metabolism of ^{111}In-galactosylated MoAb-chelator conjugates, and compared with those of corresponding nongalactosylated ones. We have tried to identify the liver cells which are responsible for the uptake of ^{111}In following adminstration of both MoAb conjugates with and without galactosylation.

Materials and Methods

Monoclonal antibody

T101, an IgG2a murine monoclonal antibody that recognizes CD5 (a human pan T-cell antigen), was used for the entire experiment. The production, purification and characterization of this antibody have been previously described.[19]

Preparation of T101-chelator Conjugates

Two bifunctional chelating agents, cyclic DTPA dianhydride (DTPA) and 2-*p*-isothiocyanatobenzyl-6-methyl-DTPA (1B4M) were used in the present study. The chelating agents were first conjugated with MoAb and then galactosylated and radiolabeled with ^{111}In. Cyanomethyl-2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-galactopyranose (Aldrich, St. Louis, Mo.) was added to 6.0 mL of 0.01 M of $NaOCH_3$ in absolute methanol and kept for 2 days. This solution was used as a stock solution of 0.1 M 2-imino-2-methoxyethyl-1-thio-β-D-galactose (A) and used for conjugation with T101. The number of galactose molecule per antibody was determined by TNBS method as described previously.[20-22] T101 was conjugated with 1B4M, a backbone substituted DTPA. Briefly, 20 μl (200 μg) of 1B4M was added to a solution of 320 μl of T101 (15.5 mg/mL, 5.0mg) and 35 μl of 1.0 M of Na_2CO_3-$NaHCO_3$ (pH 9.5). The mixture was then incubated for 3 days at room temperature. To decide the conjugation ratio, 5 μl of the solution and 40 μl of 0.2 M NaOAc (pH 4.2) was added to 10 μl (80 μCi) of $InCl_3$ (NEN DuPont) and incubated for 30 min. The sample was concentrated and rinsed three times with PBS buffer using Amicon 30, resulting 360 μl of T101-1B4M. DTPA was conjugated to T101 using the cyclic DTPA dianhydride. Briefly, 27 μl (132 μg, 4.9 mg/mL DMSO, 0.38 μmol) of cyclic DTPA was added to a solution of 320 μl of T101 (5.0 mg, 15.7 mg/mL, 0.033 μmol) and 2.15 mL of 0.1 M $NaHCO_3$ (pH 8.4). The solution mixture was incubated for 1 hour at room temperature. To determine the conjugation ratio, 50 μl of the mixture solution was added to 40μl of 0.2 M NaOAc (pH4.2) and 10 μl (80 μCi) of ^{111}In-Cl_3. The sample was concentrated and rinsed three times with PBS using Amicon 30, resulting 520 μl of T101-DTPA.[22]

Galactosylation of the Antibody-chelator Conjugate

To the 241 mg (0.60 mmol) of cyanomethyl -2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-galactopyranose, 6.0 ml of 0.01 M of $NaOCH_3$ in absolute methanol was added and stood for 2 days for using as a stock solution of 0.1 M 2-imino-2-methoxyethyl-1-thio-β-D-galactose (A). A 0.04 mL of stock solution (A), which had been vacuum dried, was added to 260 μl of T101-DTPA or 180 μl of T101-1B4M and incubated overnight at room temperature. The reaction rate was determined by the galactosylation of HSA. The reaction was completed in one hour at room temperature.

Radiolabeling of T101-conjugates with ^{111}In

A 40 μl of gal-T101-1B4M was added to 60 μl of buffer (0.2M NaOAc-0.02M NaCitrate, pH 5.0) and 12 μl (2.3 mCi) of $InCl_3$, and incubated for 1 hour. After then, 50 μl of 0.001 M of DTPA was added to the solution and incubated another 10 min. The MoAb-chelates was purified using HPLC with size exclusion column (TSK 3000+2000 SW, 0.02M NaH_2PO_4-Na_2HPO_4, 0.1M Na_2SO_4, pH 6.8, 1 mL/min). Same procedure was used for other nongalatosylated conjugates. The quality control was done using ITLC (eluent: Umezawa, acidic Umezawa) and paper chromatography which was pretreated with 5% human serum albumin (eluent: saline).

Invitro Serum Stability

Twenty μl of the labeled T101 conjugates (20-40 μCi) was mixed with 300 μl of normal mouse serum containing 0.1% sodium azide, 3 μl of 20% NaN_3, and 30 μl of HEPES (pH 6.9), resulting the final concentration of the conjugates 15-20 ug/mL. The mixture was incubated at 37°C for 2 days in a humidified incubator maintained with 5% CO_2/95% air. Twenty μl of aliquot was taken daily, and the stability of conjugates was determined by size exclusion HPLC using a TSK-3000 and a TSK-2000 column connected in series. The columns were diluted with 0.02M sodium phosphate and 0.1M sodium sulfate at pH 6.7 (1 ml/min).

Measurement of Immunoreactivity

The immunoreactivities of the radiolabeled T101 conjugates were determined using CCRF-CEM cells, a CD5 positive leukemic cell line. Briefly, 5 ng of labeled T101 conjugates were incubated with 0.5× 10^6 cells in a total incubation volume of 200 μl at 4°C for 1 hour. Non-specific binding was determined by adding 25 μg of unlabeled T101 (5,000 fold excess) in the assay solution. The maximum cell-bound counts (corrected for non-specific binding) expressed as a percentage of the total counts added were taken as the immunoreactivity.

Liver Cell Separation

The procedure for the separation of parenchymal and nonparenchymal liver cells were adopted from elsewhere,[23,24] with a modification. Fifty μCi of either radiolabeled galactosylated or nongalactosylated antibodies were injected into male rats (Harlane Sprague Dawley, 250-300g, two for each compound) intravenously via tail vein. The portal vein in anesthetized normal adult rats were cannulated with a 16-gauge cannula, and perfused with oxygenated HBSS without calcium and magnesium at a flow rate of 30 ml/min at one hour after injection. After 5 min, 0.05% collagenase (Sigma Chemical Co, St. Louis, MO) in serum free medium was started to recirculate through the liver at a 15 ml/min. At the end of 7 min the collagenase perfusion was washed out by perfusing the liver for 2 min with HBSS and a crude cell suspension was obtained by gentle agitating of the liver. A single cell suspension was produced by filtering the crude cell suspension through 100 mesh stainless steel screen using 30 ml of PBS. Parenchymal and nonparenchymal cells were isolated by differential centrifugation of the cell suspension. Briefly, the cell suspension was pelleted at 500×g in a centrifuge (Beckman J2-21, JA20, USA) and resuspended in HBSS, and recentrifuged 3 times at 50×g force for 5 min. This pellet was used as a source of parenchymal hepatocyte. The first supernatant was re-centrifuged three times at 50×g for 3 min, and final supernatant was considered as a source of nonparenchymal cells. Different cell types were distinguished according to morphology and size. The resulting parenchymal and nonparenchymal cells were resuspended in PBS and counted using a hemocytometer, and the radioactivity in the cells was determined with gamma counter.

Biodistribution Studies

Balb/c mice (Harlan Sprague Dawley Inc, female, 5-8 weeks) were injected intravenously via a tail vein with HPLC purified T101-conjugates with or without galactosylation (5 μCi/μg). The animals were sacrificed by exsanguination at 10 min, 1, 3, 6, 24, and 44hour after injection (n=3-5). Organs were excised, blotted with gauze, and weighed. The radioactivity was measured with a gamma counter (Packard Auto-Gamma 5650). Organ uptakes were expressed as percent injected dosed per organ, or percent injected dose per gram tissue (%ID/g), and were normalized to a 20 g body weight. Urine and feces were collected selectively and counted for

radioactivity. Whole body retention was determined by counting whole carcass including all of the organs. Biodistribution, urinary and fecal excretion, and whole body retention were obtained from the same mouse.

Metabolism Study

For the analysis of the radioactive products generated *in vivo*, 50 μCi/10-20 μg of ^{111}In labeled conjugates were injected intravenously into normal Balb/c mice, and then the serum, urine, bile, feces, and liver supernatant were obtained at 3, 6, 24, and 44hour after injection. To prepare liver supernatant, liver samples were soaked in 50 ml of ice-cold saline for 30 min, then frozen and thawed three times. These samples were homogenized with 0.02 M cold phosphate buffer saline at 1:1 v/w ratio using a motor-driven glass homogenizer. The homogenates were spun at 20,000 rpm for 60 min at 4°C (Beckman J2-21, JA20). In order to extract as much radioactivity into the supernatant as possible, the pellet was homogenized and spun again, and then the supernatants from two centrifugations were mixed for analysis. Feces were ground with mechanical homogenizer and washed with 0.02 M cold phosphate buffer saline. The supernatants and pellet obtained from liver and feces homogentes were counted in a dose calibrator (Squibb, CRC-6A) to assess the percentage of extracted radioactivity into the supernatant. The supernatant was filtered through a 0.2 μm filter (Millex-GV) before analysis. Radioactivity of the filtrate and the filter was measured by a dose calibrator. Bile was obtained by puncturing the gall bladder with a 30-G needle, and diluted with 100 μl of chilled saline.

Characterzation of Radiometabolites in Tissue Specimen

Tissue samples were analyzed by size exclusion HPLC using a TSK-3000 and a TSK-2000 column connected in series (0.02M sodium phosphate and 0.1M sodium sulfate, pH 6.7, 1 ml/min), paper chromatography (Whatman No.1) using normal saline as a solvent, and thin layer chromatography (silica gel, Macherey-Nagel) with a solvent mixture of 10% ammonium acetate : methanol : 0.5 M citric acid (2:2:1). Specimens were also analyzed by reverse phase HPLC (Vydac 201HS104 cartridge connected to Z-module, 5% 99:100 mmol NaOAc:methanol at pH 5.5 for DTPA conjugates and 75:25 5 mmol KH_2PO_4:CH_3CN 2.5 mmol octylamine at pH 7.4 for 1B4M conjugate, flow rate: 1 ml/min). Fecal and liver supernatants were treated with ultrafiltration (10 KD cut-off) and the filtrate was injected into reverse phase HPLC column.

Results

Preparation of ^{111}In-Galactosylated and Nongalactosylated T101 Conjugates

Total four radioconjugates were finally prepared for biodistribution and metabolism studies, those are ^{111}In-DTPA-T101 (DTPA-Ab), ^{111}In-1B4M-T101 (1B4M-Ab), ^{111}In-DTPA-Galactosylated T101 (Gal-DTPA-Ab) and ^{111}In-1B4M-Galactosylated T101 (Gal-1B4M-Ab)(Fig 1).

T101 MoAb was conjugated with 1 DTPA and 35 galactose molecules, and with 0.7 1B4M and 35 galactose molecules per antibody. The same amount of the DTPA or 1B4M chelate was conjugated for nongalactosylated antibody. Radiochemical yields of labeling DTPA- and 1B4M-galactosylated T101 MoAb with ^{111}In were 72% and 69%, respectively. Specific activity of the radiolabeled conjugates ranged between 2.5 to 5 μCi/μg of antibody. Immunoreactivity of monomeric ^{111}In-DTPA-T101 conjugates was 78% and it was 65% with galactosylation, whereas 81% and 63% for corresponding 1B4M- conjugates. The radiolabeled

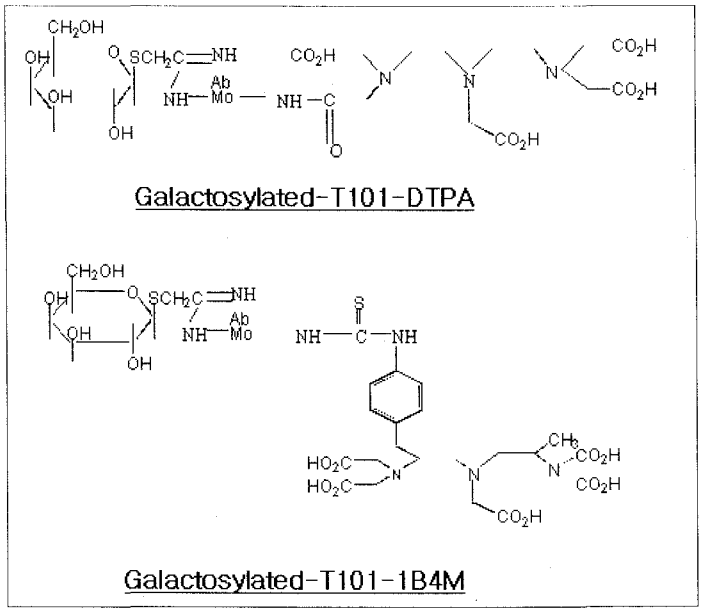

Fig 1. Structure of galactosylated T101 monoclonal antibody conjugates either with DTPA or 1B4M, as a chelator.

antibody conjugates were stable in vitro serum at 37°C for 2 days. No transferrin-^{111}In complex formation was observed with size exclusion HPLC.

Cellular Distribution of Radioactivity in the Liver

Radioactivity remained in the liver cells after collagenase treatment was more than 80% of the injected dose for both Gal-DTPA- and Gal-1B4M-Ab at 1 hour. Seventy four percentage and 76% of the liver activity remained in hepatocyte fraction, whereas 8% and 9% remained in non-parenchymal fraction for Gal-DTPA- and Gal-1B4M-Ab, respectively. The residues remaining after collagenase digestion retained 15% of the liver activity for both conjugates, which contains mostly connective tissue. The distribution of DTPA-Ab and 1B4M-Ab conjugate was similar to those with corresponding galactosylated conjugates.

Biodistribution and Whole Body Retention

Blood clearance of both Gal-DTPA- and Gal-1B4M-Ab was fast, less than 1% ID/g of the activity remained in the blood at 10 min. More than 90% of the injected dose was taken up by the liver for both Gal-DTPA- and Gal-1B4M-Ab at 10 min after injection. The Gal-1B4M-Ab cleared from the liver much faster than Gal-DTPA-Ab, which resulted in less than 20% of injected dose retained in the liver for Gal-1B4M-Ab and more than 40% for Gal-DTPA-Ab at 44 hour. During this time, the radioactivity excretion was similar between urine (23% ID) and stool (18% ID) for Gal-DTPA-Ab. For Gal-1B4M-Ab, the radioactivity was mainly excreted into feces (68% ID) rather than urine (8%). The intestinal uptake was also different between two galactosylated Abs, especially at 24 hour, which representing higher fecal excretion of Gal-1B4M-Ab (7.4% ID for DTPA and 22.6% ID for 1B4M,

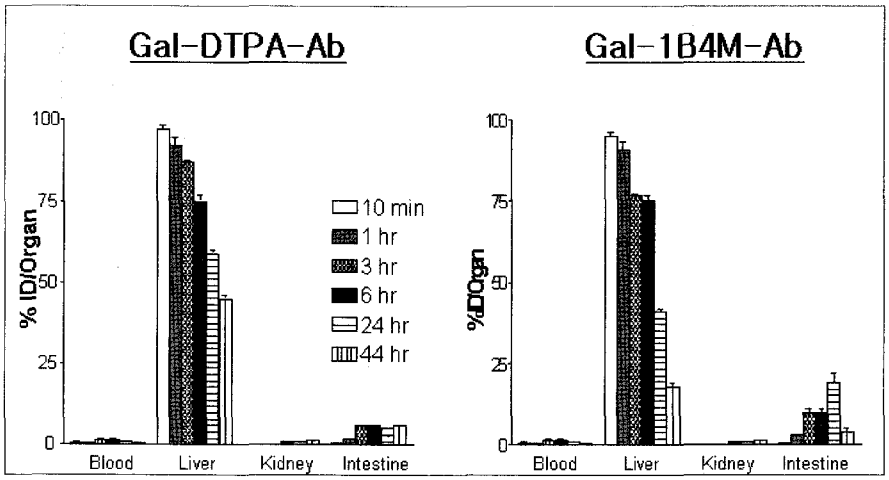

Fig 2. Comparative biodistribution of ^{111}In-labeled galactosylated T101 with DTPA (Gal-DTPA-Ab) and ^{111}In-labeled galactosylated T101 with 1B4M (Gal-1B4M-Ab). Although initial hepatic uptake was similar, clearance of radioactivity was much faster with Gal-1B4M-Ab.

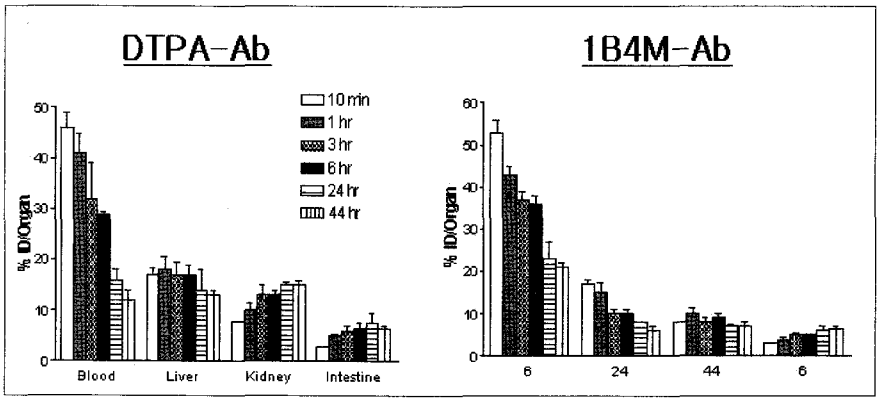

Fig 3. Comparative biodistribution of ^{111}In-labeled T101-conjugates with DTPA (DTPA-Ab) and ^{111}In-labeled T101 with 1B4M (1B4M-Ab). Similar pattern is observed with galactosylated antibody. Initial hepatic uptake was similar, but clearance was also much faster with 1B4M-Ab.

respectively)(Fig 2).

In contrast to galactosylated conjugates, the maximal liver uptake was about 15% ID/g for both DTPA-Ab and 1B4M-Ab (12-13% of liver activity). The differences between two conjugates were also observed in blood, liver and kidney uptakes and whole body clearance. The DTPA-Ab cleared faster from the blood but remained in liver and kidney longer than 1B4M-Ab(Fig 3).

Whole body clearances of the four conjugates were also different. The galactosylated conjugates were cleared from the body faster than native ones. When compared two galctosylated ones, Gal-1B4M-Ab cleared much faster from the body than Gal-DTPA-Ab. In contrast, DTPA-Ab and 1B4M-Ab showed similar whole body clearance. (Fig. 4) Gal-1B4M-Ab was excreted primarily by feces through intestine. The cumulative fecal activity

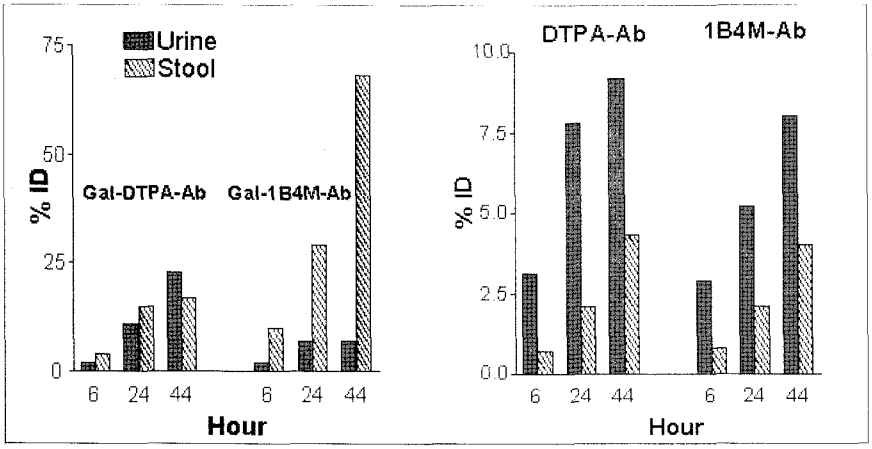

Fig 4. Comparison of urinary and fecal excretion rates among four ^{111}In-conjugates. *^{111}In-labeled galactosylated T101 with DTPA (Gal-DTPA-Ab), ^{111}In-labeled galactosylated T101 with 1B4M (Gal-1B4M-Ab), ^{111}In-labeled T101 with DTPA (DTPA-Ab), ^{111}In-labeled T101 with 1B4M (1B4M-Ab).

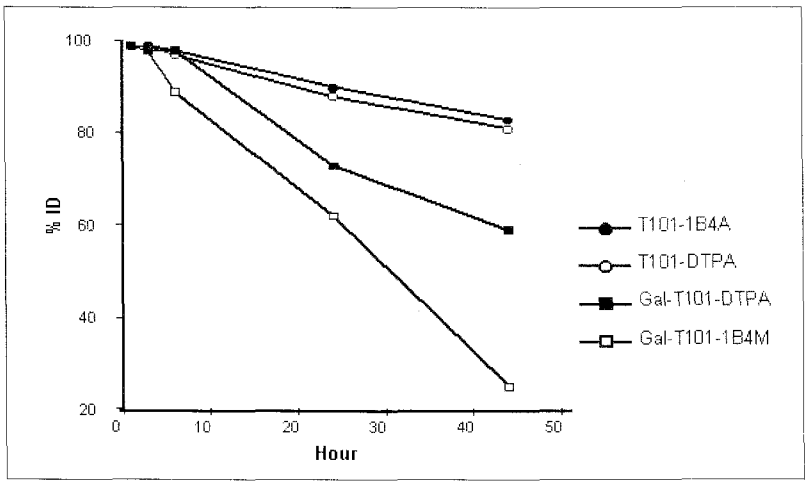

Fig 5. Whole body retention rate of four ^{111}In-conjugates. See Fig 4 for abbreviations. Gal-1B4M-Ab showed fastest clearance from the body, whereas DTPA-Ab retained most at 44 hours after injection.

was 68% and the corresponding urinary activity was less than 10% ID. Whereas, Gal-DTPA-Ab was excreted much slowly than Gal-1B4M-Ab. The combined urinary and fecal activity of Gal-DTPA-Ab was a half of the corresponding Gal-1B4M-Ab. Both Gal-DTPA- and Gal-1B4M-Ab showed similar whole body clearance and combined urinary and fecal activity, except a little more activity excreted into urine compared to feces(Fig 5).

Comparison of Hepatic Metabolism

Size exclusion HPLC of the mice serum showed that the radioactivities of DTPA-Ab and 1B4M-Ab were solely associated with intact IgG peak (Rt=15.5 min) for initial 2 days. The serum obtained from mice injected with galactosylated conjugates also showed similar single intact IgG peak in the size exclusion HPLC at 10 min post-injection, but we

Table 1. Comparison of the percentage of small metabolite remaining in the liver measured with size exclusion HPLC. Approximate molecular weight of radiometabolite was 1000 Da, compatible with the size of ^{111}In-chelate-lysine. The portion other than small radiometabolite was intact monoclonal antibody conjugates. *Gal : galactosylated

Time	Gal-T101-DTPA	Gal-T101-1B4M	T101-DTPA	T101-1B4M
3-hour	95%	95%	60%	35%
6-hour	95%	90%	70%	35%
24-hour	95%	85%	90%	40%

were not able to seperate radioactivity peak due to rapid blood clearance. Both galactosylated conjugates were metabolized rapidly in the liver. Size exclusion HPLC of liver supernatant showed two radioactivity peaks, estimating molecular sizes of 150,000 and 1,000. Almost 95% of the total activity was bound to small metabolites for both Gal-DTPA- and Gal-1B4M-Ab at 3 hour. The concentration of the small metabolite was decreased with regards to time for Gal-1B4M-Ab, reflecting a decreased hepatic activity in biodistribution data. Whereas there was no decreases in the concentration of small metabolite fraction in HPLC for the Gal-DTPA-Ab.

Two galactosylated conjugates were metabolized faster from the liver than those without galactosylation. When comparing two non-galactosylated conjugates, DTPA-Ab was metabolize faster than corresponding 1B4M-conjugates. Size exclusion HPLC of liver supernatant showed that the concentration of the small metabolte was 60% and 35% at 3 hour after injection for DTPA- and 1B4M-Ab, respectively. The metabolite concentration in liver homogenate was steady for two 1B4M conjugates, whereas there was substantial increases for corresponding DTPA conjugate by time. At 24 hour after injection, 90% of the radioactivity retained with small metabolites for DTPA and less than 30% remained for 1B4M conjugates. Size exclusion HPLC of the bile showed 5% of intact IgG peak and 95% of small metabolite for the both galactosylated conjugates at 3 hour after injection. The intact IgG peak was decreased to less than 5% at 6 hour. The total radioactivity of small metabolite was decreased by time for 1B4M, but constant for DTPA. In contrast, bile HPLC showed around 65% of activity remained in intact IgG and 35% in small metabolite peak for both nongalactosylated conjugates at 3 hour. But sampling was unsuccessful after then, because of low radioactivity in the bile (Table 1).

The radioactivity in the urine and stool for the all preparations was identical to small metabolite recovered from liver by size exclusion HPLC. Molecular size of all the small metabolites was around 1000. The reverse phase HPLC showed that the retention time of small metabolite was identical to that of lysine adduct of the corresponding ^{111}In-chelator for both DTPA-conjugates, either with or without galactosylation. The main product for 1B4M conjugates was also lysine adducts of corresponding ^{111}In-chelator, but there were one or two fractions of unidentified small peak in liver homogenate.

Discussion

Radiolabeled galactosylated albumin was developed to study hepatic glycoprotein receptor and imaging of the hepatic parenchymal cell function.[25,26] Galactose-conjugated antibody was also investigated as a potential approach to enhance regional tumor

targeting, and to decrease systemic toxicity of radioisotope or chemotherapeutic drugs conjugated with antibody.[27,28] Recently, ^{111}In-chelator galactosylated albumin was investigated as a receptor binding radioligand to study the mechanism of hepatic radioindium retention in tumor diagnosis.[14,15] Galactosylation did not decrease the antigen bindability, complement dependent cellular cytotoxicity and antibody dependent cellular cytotoxicity of the antibody,[29] as observed in our study.

To have better understanding of the receptor mediated uptake into liver and subsequent metabolism, biodistribution and metabolism of ^{111}In-galactosylated-MoAb conjugates were compared to those of MoAb-conjugates commonly used in our study. Our data showed that galactosylation of the antibody results in higher hepatic uptake. Most of activities for two galactosylated conjugates were taken up by the liver within 10 min. However DTPA-conjugates were retained longer in the liver than 1B4M-conjugates. The radiometabolite was excreted similarly into urine (23%) and feces (17%) for the DTPA conjugate. For the 1B4M conjugate, the radiometabolite was excreted primarily into feces (68%) rather than urine (8%). Size exclusion HPLC of the liver homogenate showed two peaks, the first (40%) corresponding intact IgG and the second (60%) similar to free ^{111}In-1B4M at 3 hour after injection for the 1B4M-conjugate. In contrast, the small metabolite was the major (90%) radioindium component in the liver homogenate for DTPA-conjugate. The concentration of the small metabolite fraction was decreased for 1B4M, but no decreases for DTPA conjugate by time. The radiometabolites for both MoAbs and galactosylated-MoAbs were identified as a lysine adduct of corresponding ^{111}In-chelator (DTPA and 1B4M). Thus, ^{111}In-DTPA-lysine was not cleared rapidly from the liver, whereas ^{111}In-1B4M-lysine was readily excreted through the biliary system.

These differences in biodistribution and metabolism are resulted from different handling of two compounds, conjugated either with DTPA or 1B4M. Diffusion of metabolites out of cells after intra-lysosomal degradation requires passage through both the lysosomal and cell membrane. Since hepatocyte is connected with two distinct surface domains, a bile cannalicular membrane to the biliary tree, and a sinusoidal membrane to the vascular surface, these differences in the radioindium excretory pathway represent different characteristics of the radiometabolites.[30] Diffusion across these lipid bilayer membrane is slow for ^{111}In-DTPA-lysine due to the ionic character of the chelate attached with amino acid, but faster for ^{111}In-1B4M-lysine, which is same with nongalactosylated MoAb-conjugate. Reverse phase HPLC exhibited that the ^{111}In-DTPA is more polar than ^{111}In-1B4M, furthermore ^{111}In-DTPA-lysine adduct was more polar than ^{111}In-DTPA. Previous studies[13,14] have reported the same characteristics of metabolite from neogalactosyl-albumin conjugated with ^{111}In-DTPA or ^{111}In-isothiocyanatobenzyl EDTA. More hydrophobic and less charged metabolites are known to be readily released from the liver.[7,8] Insertion of the hydrocarbon bond between chelator and MoAb exhibited rapid clearance of hepatic radioactivity in mice injected with ^{111}In-chelate MoAb conjugates.[9,10] The low concentration of small metabolite peak for 1B4M compared to DPTA could be explained by the excretion of the metabolite generated in the liver. But high concentration of the intact IgG peak for 1B4M conjugates provide different speculation that the high bond strength between 1B4M and MoAb may resist from digestion by endosomal or lysosomal enzymes more likely, and it caused delayed accumulation of metabolites.[7] Despite that the same metabolite was identified in the liver and excreta, both Gal-DTPA- and Gal-1B4M-Ab exhibit similar whole body clearance and combined urinary and fecal activity. This could be explained by

different biodistribution data. ^{111}In-galactosylated conjugate accumulates exclusively into the liver. Whereas 85% of the injected intact MoAb- conjugate was distributed in organs other than liver. Since liver processes most of the galactosylated conjugates injected, whole body clearance is mainly represented by hepatic clearance of radiolabels, that ^{111}In-1B4M-lysine cleared faster into biliary system compared to DTPA-lysine. In contrast, hepatic clearance consists only parts of whole body elimination of the MoAb-conjugate. This might obscure differences in the biliary excretion of metabolite between DTPA- and 1B4M-conjugates. In case of nongalactosylated conjugates, a little more activity was recovered from urine. Plasma immunoglobulin catabolism occurs diffusely in tissues through the body.[31] Henderson et al.[32] have reported that liver and spleen were more active site in IgG catabolism, on a weight basis, than peripheral tissues, but peripheral tissues such as hide and muscle account for the highest proportion (40-50%) of catabolized dose in the body. In the liver, the radiolabeled metabolite was generated after lysosomal and/or possible endosomal proteolysis of ^{111}In-labeled MoAb in both parenchymal and nonparenchymal cells. After then the radiometabolites are gradually excreted via hepatobiliary excretion, or backward diffusion into blood which finally go through urine. Gore et al.[33] have shown that only small amounts of radioactivity reappeared in the perfusate while much larger amounts were secreted into bile after following ^{99m}Tc-neogalctosylated albumin pulsing into isolated perfused rat liver. It is well known that radiolabels taken by nonparenchymal cells are retained for a longer time and gradually eliminated via urinary excretion.[14,15]

The differences in the hepatic clearance of the radioactivity between four ^{111}In-labeled MoAb conjugate are not solely due to different cellular distribution of these conjugates in the liver. Both galatosylated and nongalactosylated T101 conjugates were mainly located in hepatocyte and the ratio of hepatocyte over nonhepatocyte uptake was similar among four conjugates. Our results are concordant to those results that the parenchymal cells contribute 80-90% of the cellular uptake of radiolabeled MoAb in the liver.[34,35] But galactosylation resulted in not only higher hepatic uptake but also accelerated metabolism. Size exclusion HPLC of the liver homogenate showed that the percentage of the small metabolite fraction was higher in galactosylated conjugates than in nongalactosylated conjugates for both DPTA and 1B4M chelator at 3 hour. But these differences became less obvious at 6 hour, and were disappeared at 24 hour. These observation could be interpretated by following explanations. For internalization and sorting of macromolecules in the cell, two types of endocytosis, which are receptor mediated endocytosis via clathrin-coated pit pathway and nonclathrin-coated pit pathways (non-selective pinocytosis, fluid phase endocytosis), have been described.[36] Macromolecules such as insulin, glucagon, growth hormone, EGF, glycoproteins, immunoglobulin, some peptides are taken by receptor mediated endocytosis via clathrin-coated pit pathway, whereas horse radish peroxidase is known to be taken by non-selective pinocytosis. Classical receptor mediated hepatic uptake of macromolecules is very rapid with plasma half life of the orders of minutes.[37] An electromicroscopic study showed that asialoglycoprotein locates in plasma membrane at 5 min post-injection, and most of activity reaches lysosome at 13 min.[38] Galactosylated MoAb is internalized into clathrin-coated vesicles via receptor mediated endocytosis and undergoes rapid degradation in the liver.[39] Wall et al.[40] have reported evidences against that glycoproteins are internalized via fluid phase endocytosis in studies using two electron microscopic tracers. Non-coated pit internalization appears more amorphorously

shaped, smooth invagination of approximately 150-300 nm diameter. The half time for opening and closing is about 30 min for non-coated pit internalization, and 20 min for coated pit internalization.[36] Slow internalization from the surface may have delayed endosomal and lysosomal digestion of the MoAb without galactosylation. Simutaneous uptake of non-receptor mediated uptake with receptor mediated uptake is common in the liver, such as albumin, certain antigen-antibody complex, parasite and some glycoproteins.[41,42] In this study, we have used non-tumor bearing mice to rule out possible contribution to hepatic uptake rising from formation and hepatic accumulation of immune complexes. Carbohydrate receptors and Fc receptors were suggested as a binding site for immonoglobulin on both parenchymal and nonparenchymal cells of the liver.[3,43,44] The possibility that hepatic uptake was via Fc or galactose receptors were investigated using competitive studies with unlabeled mouse IgG or asialofetuin.[45] When an excess of these material were injected before or with labeled antibody, no effect on either global liver accretion or intrahepatic distribution was observed. This suggests that Fc receptors or a carbohydrate mediated mechanism via galactose residue do not play a significant role in the uptake of this radiolabeled antibody. The uptake of ^{111}In-labeled MoAb by the liver is quite rapid but rather slow process in the hepatocyte.[46] Our data, together with other reports, are suggestive that the ^{111}In-chelator-MoAb conjugates are taken by the liver not solely depends on Fc or glycoprotein receptor mediation rather than fluid phase endocytosis. Furthermore, endocytosis of the radiolabeled antibody is slower and saturable at lower ligand concentration than the binding of asialoglycoprotein to its receptor.[36] Relative slow kinetics also suggest that other than receptor mediated uptake is responsible for the hepatic uptake of ^{111}In-chelator MoAb conjugates.[46] Non-targeted macromolecules have been identified in hepatic lysosomes and most likely internalized by fluid phase endocutosis . Slow internalization and delivery to the endosome and lysosome may delay the digestion of the nongalactosylated conjugates. The fate of the endocytosed proteins is quite variable in liver. These include intracellular degradation, direct trancellular transport into bile, and backward diffusion into blood.[41,42] Endocytotic vesicle containing the endocytosed macromolecule fuse with other organelles such as lysosome to form secondary lysosome. The majority of the endocytosed macromolecule is delivered to the lysosome for degradation by protease and glycosidase. Some endocytotic vesicle containing the macromolecules fuse with the bile canalicular membrane and release their contents directly into bile by exocytosis, while some returned intact to circulation. Circulating macromolecules may utilize both the nonlysosomal and lysosomal pathway to reach the bile. Bile HPLC for both nongalactosylated conjugates showed large intact IgG peak at early time, but it was decreased by time. For the galactosylated conjugates, bile HPLC showed the concentration of the IgG fraction is much smaller even at early time. There are two possible explanations for appearance of intact IgG in bile. First, since transcellular passage without lysosomal degradation would be responsible for intact IgG in bile, more nongalactosylated conjugates bypasses lysosome, compared to the galactosylated ones. The proteins entering bile transport and degradation pathways are recognizes by separate receptors and sorted at the cell surface before reaching lysosomes.[47,48] The galactosylated conjugates taken by receptor mediated endocytosis are more likely delivered to lysosme. Marked differences in the rate and extent of hepatic clearance has been reported in a variety of desialylated glycoproteins.[49] Second, high concentration of injected radiolabels acts as a

reservoir for transcellular trasport for nongalactosylated conjugates, but there is no available IgG in blood for the galactosylated even after 10 min. Most of antibody bound to cell remained on the tumor cell surface until it was gradually internalized and degraded over a period of 2-3 days.[50,51] Whereas a small fraction of the bound antibody was released apparantly intact and it was almost completed within 4 hour. The time required for biliary transport of protein is shorter for nonlysosomal pathway compared to lysosomal pathway.[37]

In summary, we have observed several differences in biodistribution and metabolism between galactosylated and nongalactosylated ^{111}In-chelator conjugates. Even though the same kind of metabolite was generated, galactosylation of antibody results in increased hepatic uptake and accelerated degradation. The fate of the galactosylated MoAb conjugates taken by glycoprotein receptor represents the hepatic handling of the conjugates, while metabolism of the nongalactosylated conjugates occurs through the whole body including nonspecific fluid phase endocytosis into the liver. The hepatic clearance of injected radiolabels depends on the chemical nature of the metabolites. The less polar ^{111}In-1B4M-lysine is readily excreted into biliary tract. The metabolite was mainly excreted into urinary tract with ^{111}In-chelator-MoAb. These observations are especially important in the designing radiolabeled protein and peptide for targeting tumors, infections, and lesions expressing specific receptors.

요 약

목적 : 종양의 진단과 치료에 널리 이용되고 있는 단클론항체를 수용체에 결합하는 수송체로 이용할 수 있는지에 대한 가능성 여부를 평가하기 위하여, 간의 asialoglycoprotein 수용체에 결합할 수 있는 갈락토즈접합 단클론항체를 ^{111}In로 표지하여 체내에서의 분포와 간을 중심으로 한 체내대사를 분석하였고, 그 결과를 갈락토즈를 접합하지 않은 ^{111}In 표지 항체와 비교하였다. **재료 및 방법** : 인체 림프구성백혈병 세포에 대한 T101 단일클론항체를 cyclic DTPA dianhydrate(DTPA) 나 2-*p*-isothiocyanatobenzyl-6-methyl-DTPA(1B4M) 로 접합하고 갈락토즈를 붙인후 ^{111}In으로 표지하였다. 생쥐와 흰쥐에서 갈락토즈를 접합한 화합물과 접합하지 않은 화합물의 체내분포와 간대사를 비교분석하였다. **결과** : ^{111}In 표지 T101항체와 갈락토즈 접합체는 투여량의 대부분이 10분 이내에 간에 섭취되었다. DTPA 접합자를 사용한 경우 1B4M 접합자를 사용한 경우보다 간에 오랫동안 저류되어 주사 후 44시간 간 섭취율이 각각 55%와 20% 였다. 이 기간동안의 DTPA 화합물의 방사성 대사산물은 24%가 소변으로 17%가 대변으로 배설되어 유사하였으나 1B4M 화합물은 68%가 대변으로 8%가 소변으로 배설되어 배설경로에 차이가 있었다. 1B4M 화합물을 주사후 3시간의 담즙과 간 현탁액을 HPLC로 분석한 결과 IgG와 저류시간(Rt)이 같은 첫 절정에 35%, 유리 ^{111}In과 유사한 절정의 Rt에 65%가 관찰되어 대사산물이 빠르게 담즙으로 배출됨을 알 수 있었고, DTPA 화합물 주사후 3시간 대사산물은 90%가 ^{111}In-DTPA와 유사한 Rt의 절정을 보였다. 그러나 대변의 ^{111}In의 축적량은 낮아 DTPA 접합화합물은 담도를 통한 빠른 배설이 일어나지 않음을 알 수 있었다. **결론** : 단일클론항체에 갈락토즈를 접합한 경우 보통의 항체에 비하여 간 섭취가 많고, 간에서의 대사가 촉진된다. 이 경우 사용되는 접합자의 선택에 따라서 대사산물의 성분이 달라지고 간에서의 제거도 차이가 있다. 이러한 대사의 차이점은 향후 종양세포나 조직의 탐색에 이용할 방사능 표지 항체의 제조에 응용될 수 있을 것이다.

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