《原　著》

Relation of Heparan Sulfate Content and ^{67}Ga Uptake in Various Tissues of Rats

Yukie HAMA*, Shuji KOJIMA* and Akiko KUBODERA**

*Department of Radiopharmacy, School of Pharmaceutical Sciences, Teikyo University, Kanagawa
**Department of Radiopharmacy, School of Pharmaceutical Sciences, Science University of Tokyo, Tokyo

Abstract The distribution of ^{67}Ga citrate in various tissues of rats at 24 hrs after i.v. injection was investigated in relation to the heparan sulfate (HS) content of the tissues. The order of ^{67}Ga % dose per g tissue in normal rats was spleen>liver>kidney>stomach>small intestine>heart>lung>muscle>brain. This order is in good accord with that of HS content in these tissues. A comparison of the ^{67}Ga binding affinities with mucopolysaccharides showed that HS has a characteristically high affinity for ^{67}Ga. The binding percent (radioactivity in the precipitate as a percentage of total radioactivity in the incubation mixture) of HS with ^{67}Ga was 95%, but other mucopolysaccharides gave values of about 20% or less. HS appears to play an important role in the mechanism of ^{67}Ga accumulation.

Introduction

Recently ^{67}Ga citrate has been increasingly used as a diagnostic agent for imaging varieties of human cancers and for the detection of inflammatory lesions. However, the accumulation mechanism of ^{67}Ga in these tissues is not yet clearly understood. Our previous studies on ^{67}Ga accumulation during hepatocarcinogenesis induced by 3'-methyl-4-dimethylaminoazobenzene[1] and on uptake of ^{67}Ga in rats with liver damage induced by CCl$_4$[2] indicated that heparan sulfate (HS) might be an acceptor for ^{67}Ga and play an important role in its accumulation in tumor cells and inflammatory lesions. To obtain more information on the relationship between ^{67}Ga accumulation and HS, we investigated the distribution of ^{67}Ga in various tissues of rats in relation to HS content.

Materials and Methods

1)　Animals and Diets

Male Donryu rats, weighing about 170 g and 6 weeks of age, were obtained from Nihon Rat Co., Urawa, Japan. The animals were maintained on a basal diet EC-2 (CLEA Japan Inc., Tokyo, Japan) and water *ad libitum* for 2 weeks before use.

2)　Levels of ^{67}Ga Radioactivity in Blood and Liver

Carrier-free ^{67}Ga was supplied by Nihon mediphysics Co. Ltd., Takarazuka, Japan, and diluted with 0.08 M sodium citrate to 50 μCi/ml. The rats were given an i.v. injection of ^{67}Ga citrate (10 μCi) into the tail vein under pentobarbital sodium anesthesia. The rats were killed by a blow on the head at 1, 2, 3, 6, 12, 24, and 48 hrs following injection. The blood and the livers were obtained and counted in a well-type scintillation counter (Aloka TDC-501). The livers were minced and one gram of tissue was subjected to measurement of the radioactivity. The percentages of the administered radioactivity per gram of liver and per ml of blood in the 5 rats of each group were averaged for each time interval.

3) Distribution of [67]Ga Radioactivity in Various Tissues of Rats

The administration of ^{67}Ga citrate was performed in the same manner as described above. At 24 hrs after the injection, the rats were killed and the tissues were removed, wiped well with filter paper, weighed and counted for radioactivity. The percentages of the administered radioactivity per gram of tissues in the 5 rats were averaged.

4) Extraction and Separation of Mucopolysaccharides

The tissues were minced in 5 volumes of acetone in a Waring blender, defatted with ether, and dried under a vacuum to constant weight in a desiccator. Extraction and separation of mucopolysaccharides (MPS) were carried out by the method of Schiller[3] with a slight modification. Namely, dry defatted tissues were extracted with 0.1N NaOH at 4° for 24 hrs, followed by neutralization with 0.1N HCl. The solution was digested with 20 mg of Pronase-P (Kaken Chemicals Co., Tokyo, Japan) per gram of dry defatted tissues at 37° for 24 hrs. The solution was then cooled to 4°, and 100% trichloroacetic acid (TCA) was added to give 10% final concentration. The solution was allowed to stand overnight at 4°, and the TCA-insoluble fraction was discarded by centrifugation at 3,000 rpm for 20 minutes. This supernatant was dialyzed against running tap water for 24 hrs, and then against distilled water for 24 hrs. The MPS was precipitated completely by adding one-tenth volume of 0.4M NaCl-1% cetylpyridinium chloride (CPC, Wako Fine Chemicals Co., Tokyo, Japan) solution to the dialyzed solution. The precipitate was incubated at 37° for 1 hr, then approximately 1 g of heavy Celite per gram of dry tissues was added. The mixture was stirred thoroughly with a glass rod, then centrifuged for 30 minutes at 3,000 rpm, and the supernatant was decanted into another tube. The precipitate was washed once by stirring with a solution of 0.03M NaCl-0.1% CPC, then centrifuged for 30 minutes, and the supernatant was discarded. Then the MPS was extracted with a solution of 2.1M NaCl-0.1% CPC by incubation at 37° for 1 hr followed by centrifugation.

5) Determination of Uronate

The determination of uronate in the 2.1M NaCl-soluble fraction was carried out by means of the carbazole reaction as modified by Bitter and Muir.[4] Glucuronic acid (Wako Fine Chemicals Co., Tokyo, Japan) was used as a standard.

6) Identification and Composition of MPS of Various Tissues

MPS of various tissues from rats was identified by cellulose acetate strip electrophoresis. MPS was collected by centrifugation after precipitation with 3 volumes of ethanol from 2.1M NaCl-0.1% CPC solution. This MPS was dissolved in a small volume of distilled water, and subjected to cellulose acetate strip electrophoresis (Separax, Jōkō Sangō Co., Tokyo, Japan). Electrophoresis was performed under a constant current (0.6 mA/cm) in 0.2M calcium acetate (pH 7.6). After electrophoresis, strips were stained with 0.5% toluidine blue according to the procedure of Seno[5]. Standards of MPS were chondroitin sulfate A, B, and C (Nakarai Chemicals Co., Kyoto, Japan), heparan sulfate (Seikagaku Kogyo Co., Tokyo, Japan) and hyaluronic acid (Sigma Chemicals Company, USA). Composition of MPS was determined by comparison of the color density of toluidine blue in each band. Namely, each stained band was cut into small pieces, and toluidine blue was extracted from MPS by incubation at 50° for 3 hrs 1 ml of 1N NaOH. The density of this NaOH solution was measured spectrophotometrically at 594 nm.
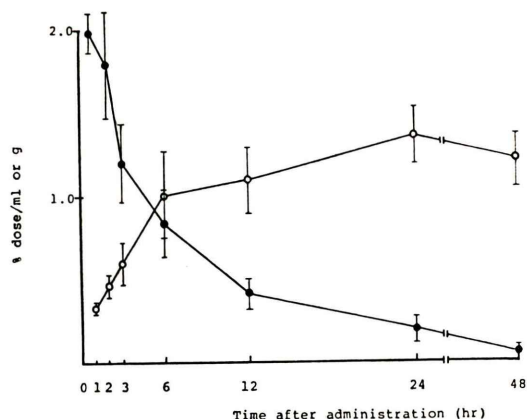

Fig. 1 Levels of ^{67}Ga radioactivity in the blood and liver after intravenous administration. [67]Ga citrate was given intravenously at a dose of 10 μCi to each rat. Vertical lines indicate the standard deviation (n=5). Shaded circles, blood; open circles, liver.

7) Identification of ^{67}Ga-binding MPS

^{67}Ga-binding MPS was identified by acetate strip electrophoresis. Kidney MPS or brain MPS was incubated at 37° for 1 hr with ^{67}Ga citrate (50 μCi/ml) in a mixture adjusted to pH 4.0. The MPS was collected by centrifugation after precipitation with ethanol. Precipitated MPS was washed 3 times with ethanol, and subjected to cellulose acetate strip electrophoresis by the procedure described above. Radioactivity of ^{67}Ga was scanned with a Packard radiochromatogram scanner (Model 7230).

8) ^{67}Ga Binding with MPS

Each standard MPS was incubated with ^{67}Ga citrate (1.0 μCi) at 37° for 1 hr. The MPS was collected by centrifugation after precipitation with ethanol and washed 3 times. The binding percent was calculated from the ratio of the radioactivity

in the precipitate to the total activity.

Results

1) Levels of ^{67}Ga Radioactivity in the Blood and Liver

Levels of ^{67}Ga radioactivity in the blood and liver after intravenous administration are shown in Fig. 1. At 1 hr after administration the percentage of administered radioactivity per ml of blood was 2.0%, and this fell below 0.1% at 48 hrs after administration. On the other hand, in the liver the percentage increased sharply to 1.0% by 6 hrs after administration and reached 1.4% at 24 hrs after administration. Thereafter, the percentage decreased gradually.

2) Distribution of ^{67}Ga Radioactivity in Various Tissues of Rats

The distribution of ^{67}Ga radioactivity in the

**Table 1** Distribution of ^{67}Ga radioactivity and heparan sulfate content in various tissues

Tissue	% dose/g		% of MPS			Content of HS/g tissue
	tissue (^{67}Ga)	Uronate*	Ch A	Ch B	HS	
Brain	0.05±0.01	103.2±15.2	78.7		21.3	22
Muscle	0.20±0.03	77.2±17.8		60.5	39.5	30
Lung	0.25±0.01	89.5±15.0	13.3	25.1	61.7	55
Heart	0.47±0.07	217.0±42.2	18.5	32.2	49.3	107
s-Intestine	0.50±0.02	198.2±18.6	7.4	31.9	60.7	120
Stomach	0.70±0.20	249.0±16.8	15.5	34.8	49.7	124
Kidney	0.89±0.06	188.0±32.4		24.3	75.7	142
Liver	1.39±0.12	205.0±53.0	1.5	11.3	87.2	179
Spleen	2.07±0.38	120.0±22.1	10.3	41.0	48.7	58

Rats were sacrificed at 24 hrs after i.v. injection with ^{67}Ga citrate (10 μCi/animal).
*μg of glucuronic acid per g of tissue
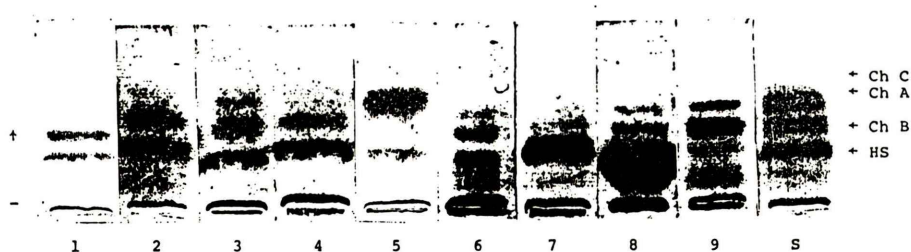
Fig. 2 Cellulose acetate electrophoresis of sulfated mucopolysaccharides obtained from different tissues. Aliquots of 5 μl were applied to 2×6 cm cellulose acetate strips at 1.5 cm from the negative electrode. S, mixture of the standards. Sulfated mucopolysaccharide extracts from: 1, muscle; 2, lung; 3, stomach; 4, heart; 5, brain; 6, small intestine; 7, kidney; 8, liver; 9, spleen.
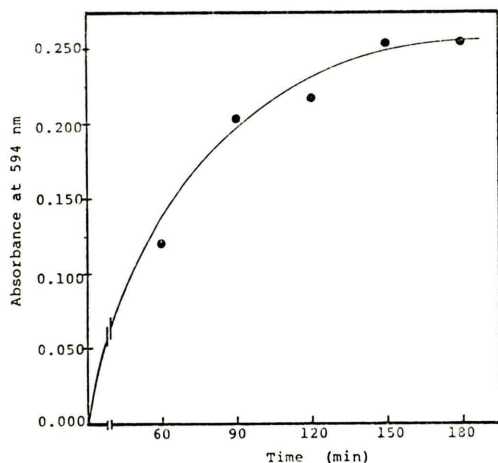
Fig. 3 Plot of absorbance of the extract versus extraction time of Ch A-toluidine blue complexes. 5 ng of chondroitin sulfate A was applied to a cellulose acetate strip. After electrophoresis, the strip was stained with 0.5% toluidine blue, and the dye from each band was extracted into 1N NaOH. Each point represents the average value of duplicate experiments.
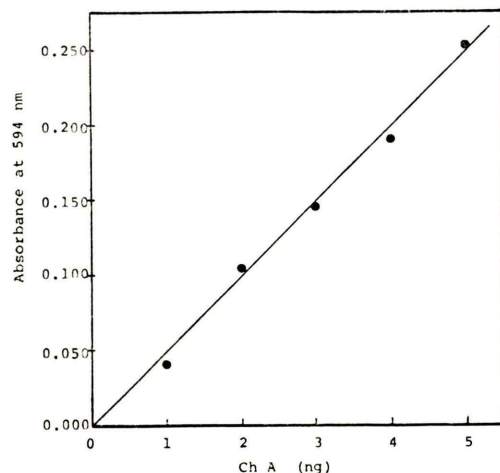
Fig. 4 Plot of absorbance of the extract versus the amount of Ch A subjected to electrophoresis. Various concentrations of standard Ch A were subjected to electrophoresis under the conditions described in Materials and Methods. Each point represent the average value of duplicate runs.
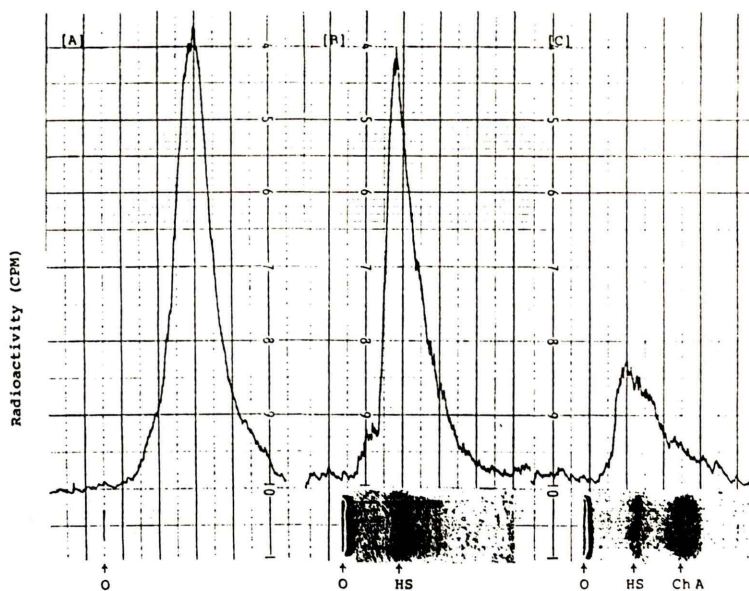
Fig. 5 Results of cellulose acetate electrophoresis of mucopolysaccharides from rat kidney and brain. [A] [67]Ga citrate only, [B] [67]Ga citrate+kidney mucopolysaccharides, and [C] [67]Ga citrate+brain mucopolysaccharides. O, origin; HS, heparan sulfate; Ch A, chondroitin sulfate A.

various tissues of rats at 24 hrs after administration is shown in Table 1. The percentage of administered radioactivity per gram of tissue was 0.25% or less in brain, muscle, and lung, but it was from 0.9% to 2.0% in kidney, liver, and spleen.

3) Identification and Composition of MPS in Various Tissues of Rats

Electrophoretograms of MPS extracted from the brain, muscle, lung, heart, small intestine, stomach, kidney, liver, and spleen of rats are shown in Fig. 2. The composition ratio was determined as described in Materials and Methods; Fig. 3 shows the data which led to the selection of a 3 hrs extraction time for the quantitation of MPS after separation by electrophoresis. The calibration curve (Fig. 4) obtained for standard chondroitin sulfate A was linear. Each tissue had a characteristic MPS composition differing from others in the relative amount and type of MPS. The composition ratio of MPS in each tissue is shown in Table 1. HS was a minor component in brain, muscle, and lung, but a major component in kidney and liver.

4) Identification of ^{67}Ga-binding MPS

Electrophoretograms of MPS and radio-chromatograms in the kidney and brain are shown in Fig. 5. Chondroitin sulfate A was the major MPS in the brain, while HS was predominant in the kidney. In both tissues, radiochromatography indicated that ^{67}Ga was bound only with HS.

The ^{67}Ga radioactivity at the position of the HS band was very much higher in the kidney than in the brain.

5) ^{67}Ga binding percent with MPS

Values of ^{67}Ga binding percent with standard MPS and bovine serum albumin are shown in Table 2. The binding percent of HS with ^{67}Ga was 95%, whereas the values for other MPS were 20% or less. That of bovine serum albumin was very small.

Discussion

Our previous observation of ^{67}Ga accumulation in the rat liver during chemical hepatocarcinogenesis induced by 3′-methyl-4-dimethylaminoazobenzene suggested that HS might be an acceptor for ^{67}Ga in vivo and play an important role in its accumulation.[1] A subsequent investigation, in which we studied the uptake of ^{67}Ga in rats with liver damage induced by CCl$_4$, provided further support for this view.[2] Later, we ascertained that the pattern of ^{67}Ga uptake in the liver during chemical hepatocarcinogenesis induced by 2-aminoacetylfluorene and the pattern of ^{67}Ga uptake in the liver during the recovery from liver injury induced by CCl$_4$ paralleled the change of HS content in the liver. In this study, on the basis of reports that MPS contents and compositions vary greatly in mammalian tissues,[6~7] we investigated ^{67}Ga accumulation in various tissues of rats in relation to HS content. The results of

Table 2 ^{67}Ga binding percent with MPS in relation to functional groups of MPS

MPS	Binding percent (%)*	Functional groups			
		$-NHCOCH_3$	$-COOH$	$-OSO_3H$	$-NHSO_3H$
Chondroitin sulfate A	17	+	+	+	—
Chondroitin sulfate B	22	+	+	+	—
Chondroitin sulfate C	10	+	+	+	—
Hyaluronic acid	6	+	+	—	—
Heparin	1>	—	+	+	+
Heparan sulfate	95	+	+	+	+
Bovine serum albumin	1>				

* Each standard MPS was incubated with ^{67}Ga citrate (1.0 μCi/ml) at 37° for 1 hr. They were collected by centrifugation after precipitation with ethanol. The precipitate was washed three times with ethanol, then the radioactivity was counted. The binding percentage was calculated as the percent of the radioactivity in the precipitate with respect to total radioactivity in the incubation mixture.

+ : containing the indicated functional group

— : not containing the indicated functional group

this study indicate that the order of the percentages of administered ^{67}Ga radioactivities per gram of tissues was in good accord with that of HS content except for the spleen.

It has been reported that there is a direct relationship between neoplastic growth and the presence of MPS.[8] Gersh et al.[9] suggested that the physical state of the MPS and the degree of polymerization are important factors affecting the invasiveness of tumor cells. Though various tumors differ in MPS content and composition, HS has been identified as major component of MPS isolated from normal liver and various tumors.[10~15] HS appears to be localized on the cell membranes,[16~17] basement membrane,[18] arterial wall,[19] and cell adhesive sites.[20] The exact biological roles of HS have not been identified yet. However, several functions such as cell-to-cell and cell-substrate adhesion,[21,22] growth control,[23,24] masking of cell surface receptors,[25] and modulation of cation levels, e.g. Ca^{++}[26] have been suggested. Recent work has shown that HS is bound to the cell surface in two separate and distinct modes.[27] One mechanism entails binding of the protein core of the HS proteoglycan directly to the cell membrane, presumably by intercalation of hydrophobic region directly into the lipid of the membrane. The other mechanism involves ionic binding of the polysaccharide moiety to ionic sites on the cell membrane. It is possible that ^{67}Ga might interact with HS involved in ionic binding. Our study of ^{67}Ga binding with various MPS indicated that HS showed an especially high affinity for ^{67}Ga. HS bound to ionic sites on the cell membrane would be high-sulfated HS.[28] HS has $-NHCOCH_3$, $-COOH$, $-OSO_3H$, and $-NHSO_3H$ functional groups, but other types of MPS lack one or two of them. Thus, this high affinity of ^{67}Ga for HS might be due to the functional groups $-NHCOCH_3$ and $-NHSO_3H$ in HS.

Recently ^{67}Ga citrate has been increasingly used as a diagnostic agent for imaging varieties of human cancers and for the detection of inflammatory lesions.[29~30] These target tissues may thus contain HS as a major component of MPS.

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要　旨

ラットの種々の臓器における [67]Ga の取り込みと Heparan Sulfate 量

浜　　幸江*　　小島　周二*　　久保寺昭子**

*帝京大学薬学部放射薬品学教室　**東京理科大学薬学部放射薬品学教室

　ラットに ^{67}Ga citrate を尾静脈内注入して24時間後の種々な臓器における放射活性の分布を臓器の Heparan Sulfate (HS) の量と関連付け検討した. 正常ラットの各種臓器 1g 当りに取り込まれる [67]Ga の量は，脾＞肝＞腎＞胃＞小腸＞心臓＞肺＞筋肉＞脳の順であった. この順序は，これら臓器の HS の量とよく一致した. また，種々のムコ多糖類と [67]Ga の親和性の差を比較すると，HS が [67]Ga に対して特異的に高い親和性を示した. 以上の結果より，[67]Ga の集積機序において HS は重要な役割を果していることが示唆された.

Key words:　Gallium 67, Heparan sulfate, Tissue distribution.