[Chem. Pharm. Bull. 36(7)2570—2575(1988)]

Mechanism of Tumor Transport of ^{99m}Tc-DL-Homocysteine, a Possible Tumor-Imaging Agent

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(Received December 25, 1987)

The mechanism of transport of ^{99m}Tc-DL-homocysteine (^{99m}Tc-Hcy), a possible tumor-imaging agent, was studied. In the blood of mice at 10 min after intravenous injection of ^{99m}Tc-Hcy, most of the radioactivity was distributed in the plasma in both the protein-bound and free forms. The protein-bound form was not appreciably dissociated in physiological saline. Further, when the protein-bound form was injected into tumor-bearing mice, the tumor-distributed radioactivity was about twice that of free ^{99m}Tc-Hcy. The binding protein was considered to be albumin from the result of high performance liquid chromatography analysis. An *in vitro* experiment showed that the tumor uptake of ^{131}I-albumin was much less than that of ^{99m}Tc-Hcy and ^{99m}Tc-Hcy-albumin. On the other hand, the radioactivity in the tumor cells of ^{99m}Tc-Hcy-injected mice was found to be mostly that of free ^{99m}Tc-Hcy. These results suggest that a portion of the ^{99m}Tc-Hcy injected was transported to the tumor tissue as an albumin complex in the blood, then released from the albumin and taken up by the cells.

Keywords——^{99m}Tc-DL-homocysteine; plasma protein; albumin; tumor cell; transport mechanism; tumor-imaging agent

Introduction

Technetium-99 m(^{99m}Tc)-labeled radiopharmaceuticals have been widely used clinically as diagnostic imaging agents for a variety of organs. Among many types of ligands for ^{99m}Tc, sulfhydryl amino acids constitute an advantageous group due to the high ability for ^{99m}Tc-chelate formation and the organ specificity of each complex formed. For example, ^{99m}Tc-penicillamine[1] is employed as a cholescintigraphic agent and ^{99m}Tc-cysteine[2] as a kidney-imaging agent based on their properties of high biliary excretion and urinary excretion, respectively.

We have reported that ^{99m}Tc-DL-homocysteine (^{99m}Tc-Hcy), which was found to form an oligomeric complex in physiological saline,[3] accumulated strongly in several experimental tumors *in vivo*.[4] Clarification of the *in vivo* behavior of this oligomeric complex is desirable for elucidation of its mechanism of tumor accumulation and also for the development of improved tumor diagnosis agents. In this paper, the behavior of ^{99m}Tc-Hcy in the blood and tumor tissue is analyzed, and a major role for blood albumin in the transport of this complex to the tumor cells is indicated.

Materials and Methods

Chemicals——The ^{99}Mo–^{99m}Tc generator and ^{131}I-human serum albumin (^{131}I-HSA) were products of Daiichi Radioisotope Laboratories Ltd., Tokyo. DL-Homocysteine was purchased from Sigma Chemical Co., St. Louis. Other chemicals used were of guaranteed grade.

Tumor-Bearing Mice——Male mice of ddY strain (16—18 g) were purchased from Shizuoka Agricultural Cooperative Association for Laboratory Animals, Hamamatsu. Mice bearing Ehrlich solid tumors or ascites tumors

were obtained as described previously.[5]

Preparation of ^{99m}Tc-Hcy and ^{99m}Tc-Hcy-Albumin——^{99m}Tc-Hcy was prepared according to the procedure described previously.[4] An aqueous solution of ^{99m}Tc-Hcy (0.1 ml, *ca.* 1 mCi/ml) was incubated with 1 ml of bovine serum albumin solution (50 mg/ml in 0.9% NaCl+5 mM Tris–HCl buffer, pH 7.5) at 37 °C for 30 min. The reaction mixture was chromatographed on Sephadex G-50 and the fraction eluted at the void volume was used as ^{99m}Tc-Hcy-albumin.

Distribution of ^{99m}Tc-Hcy in Blood——Heparinized blood was obtained from mice at a given time after intravenous (i.v.) injection of ^{99m}Tc-Hcy and centrifuged at $700 \times g$ for 20 min. The blood cells were washed once with physiological saline. The radioactivity levels in the blood cells and plasma which was combined with the above washing were counted in a gamma counter (Beckman 5500).

Dialysis of Protein-Bound ^{99m}Tc-Hcy——Protein-bound ^{99m}Tc-Hcy in physiological saline (2 ml) was dialyzed against 500 ml of physiological saline at 4 °C for 24 h. An aliquot of the outer solution was counted for radioactivity in a gamma counter at a given time. As a control, ^{99m}Tc-Hcy and $^{99m}TcO_4^-$ were dialyzed in a similar manner.

Intracellular Distribution of ^{99m}Tc-Hcy in Tumor Cells——Solid tumors were excised from mice at a given time after i.v. injection of ^{99m}Tc-Hcy and homogenized with a 9-fold volume of 0.9% NaCl+5 mM Tris–HCl buffer (pH 7.5). The homogenates were centrifuged and fractionated into 700, 7000, and $105000 \times g$ precipitates and $105000 \times g$ supernatant. The radioactivity of each fraction was counted in a gamma counter.

***In Vivo* Tumor Accumulation**——Four mice bearing Ehrlich solid tumors (approximately 0.5 cm in diameter) were injected i.v. with 0.2 ml of physiological saline solution containing the protein-bound or free form of ^{99m}Tc-Hcy (0.2—1.0 μCi). Mice were sacrificed 3 h later by bleeding under ether inhalation. The solid tumor and organs were excised and weighed. The radioactivity was counted in a gamma counter.

***In Vitro* Uptake by Tumor Cells**——Ehrlich ascites tumor cells harvested at 7 d after transplantation into mice were suspended in Eagle's MEM (pH 7.2). An aliquot (1 ml) of the cell suspension (2×10^7 cells/ml) was incubated with 1 ml of ^{99m}Tc-Hcy, ^{99m}Tc-Hcy-albumin, or ^{131}I-HSA in the same medium at 37°C for a given time. After addition of a 2-fold volume of ice-cold Eagle's MEM, the mixture was immediately centrifuged at $700 \times g$ for 1 min and the precipitated cells were washed twice with the medium. The radioactivity taken up by the cells was counted in a gamma counter.

Results

Behavior of ^{99m}Tc-Hcy in Blood

At 10 min after i.v. injection of ^{99m}Tc-Hcy, the radioactivity retained in the blood of mice was *ca.* 11% dose/g blood, which corresponded to approximately 25% dose/whole blood. More than 90% of this radioactivity was found in the plasma. The radioactivity decreased to about 1/3 at 60 min. However, the distribution ratio between the blood cells and plasma was unchanged (Table I). Gel filtration analysis of the plasma at 10 min after injection revealed two radioactivity peaks. The first peak eluted at the void volume showed absorption at 280 nm. When ethanol was added to this fraction, almost all of the radioactivity was found in the precipitate. This peak was therefore considered to represent the protein-bound form. The position of the second peak agreed with that of ^{99m}Tc-Hcy, suggesting that this peak represented free ^{99m}Tc-Hcy. The radioactivity in the plasma decreased rapidly and only the protein-bound form was detected at 3 h after the injection (Fig. 1).

The dialyzability of the protein-bound form was compared with those of ^{99m}Tc-Hcy and $^{99m}TcO_4^-$ (Table II). More than 80% of the radioactivity of ^{99m}Tc-Hcy and $^{99m}TcO_4^-$ was

TABLE I. Distribution of ^{99m}Tc-Hcy in Blood

Time after injection (min)	% dose/g wet weight	^{99m}Tc distribution (%)	
		Blood cell	Plasma
10	11.4±1.1	6.8	93.2
60	3.9±1.2	5.4	94.6

Heparinized blood obtained from mice at a given time after i.v. injection of ^{99m}Tc-Hcy was centrifuged at $700 \times g$ for 20 min. The blood cells were washed once with physiological saline.

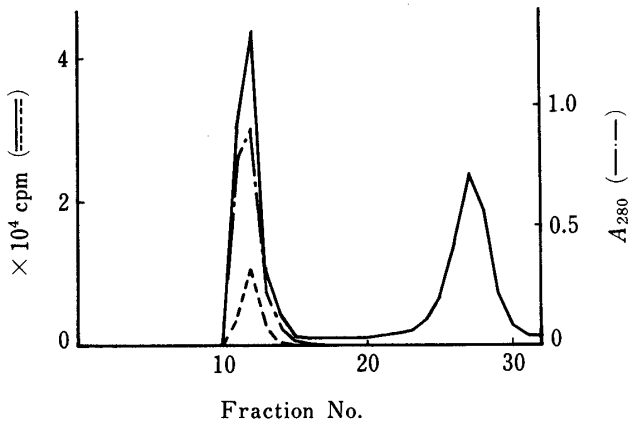

Fig. 1. Sephadex G-50 Elution Profile of Plasma of Mice Injected with ^{99m}Tc-Hcy

—— and ----, radioactivity at 10 min and 3 h, respectively, after injection; —·—, absorbance at 280 nm at 10 min after injection. Column, 1.2 × 56 cm; eluent, 0.9% NaCl.

TABLE II. Dialyzability of Protein-Bound ^{99m}Tc-Hcy

Time of dialysis (h)	Radioactivity dialyzed (%)		
	Protein-bound ^{99m}Tc-Hcy	^{99m}Tc-Hcy	$^{99m}TcO_4^-$
1	1.8	36.9	81.0
2	2.0	47.8	89.3
12	3.2	77.3	94.8
24	4.2	80.7	98.3

The plasma of mice at 10 min after i.v. injection of ^{99m}Tc-Hcy was chromatographed on Sephadex G-50, and the fraction eluted at the void volume was used as protein-bound ^{99m}Tc-Hcy. Each sample was dialyzed in physiological saline at 4 °C for 24 h. An aliquot of the outer solution was counted for its radioactivity in a gamma counter at the given times.

TABLE III. Distribution of Intracellular Radioactivity of ^{99m}Tc-Hcy in Ehrlich Solid Tumor

Fraction	^{99m}Tc distribution (%)		
	10	30	60 (min)
700 × *g* ppt.	14.1	15.2	13.5
7000 × *g* ppt.	5.9	5.3	5.3
105000 × *g* ppt.	5.8	5.7	5.7
105000 × *g* sup.	74.3	73.9	75.7

Tumor cells obtained from mice at a given time after i.v. injection were homogenized and centrifuged in a 9-fold volume of 0.9% NaCl + 5 mM Tris–HCl buffer (pH 7.5).

released to the outer physiological saline by 24 h dialysis. On the other hand, that of the protein-bound form was hardly released from the dialysis bag. It was found that the protein-bound form was not appreciably dissociable in physiological saline, suggesting that this protein complex was stable under such conditions.

In Vivo Tumor Accumulation of Protein-Bound ^{99m}Tc-Hcy

The *in vivo* tumor accumulation of the radioactivity of protein-bound ^{99m}Tc-Hcy was compared with that of free ^{99m}Tc-Hcy. The radioactivity of the former in tumor was as much as about twice that of the latter. A similar tendency was also observed in other tissues (Fig.2).

Intracellular Distribution of ^{99m}Tc-Hcy in Tumor Cells

The intracellular distribution of the radioactivity in Ehrlich solid tumor cells was

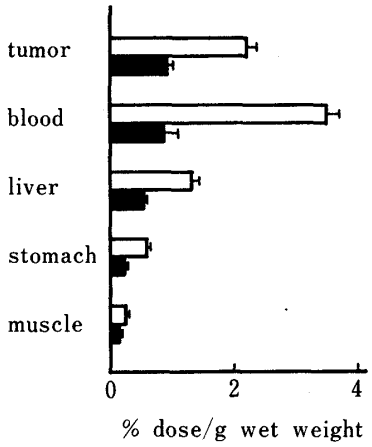

Fig. 2. *In Vivo* Tumor Accumulation of Protein-Bound ^{99m}Tc-Hcy at 3 h after Injection

The protein-bound ^{99m}Tc-Hcy (void volume fraction) and free ^{99m}Tc-Hcy (fraction No. 27) shown in Fig. 1 were used. Four mice were intravenously injected with each sample (0.2—1.0 μCi) in saline and then treated as described in the text.

□, protein-bound ^{99m}Tc-Hcy; ■, free ^{99m}Tc-Hcy. Each bar and line indicate the mean ± S.D. ($n=4$).

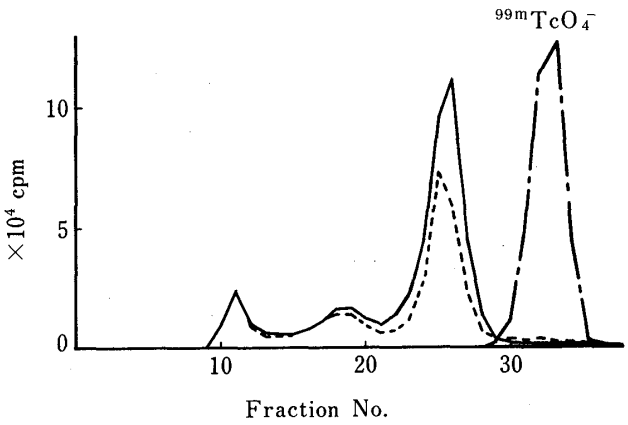

Fig. 3. Sephadex G-50 Elution Profile of 105000 × *g* Supernatant of Tumor of ^{99m}Tc-Hcy-Injected Mice

The 105000 × *g* supernatant fraction shown in Table III was used. The elution position of free $^{99m}TcO_4^-$ is also shown for reference (— · —).

——, 10 min after injection; – – – –, 60 min after injection. Column, 1.2 × 56 cm; eluent, 0.9% NaCl.

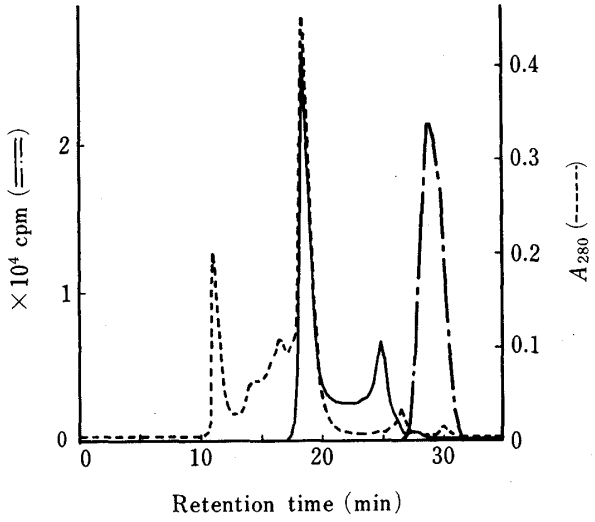

Fig. 4. HPLC Profile of Plasma of ^{99m}Tc-Hcy-Injected Mice

The plasma of mice at 10 min after i.v. injection was analyzed. Column, TSK-G3000SW, 0.75 × 60 cm; eluent, 0.1 M sodium sulfate/0.05 M sodium acetate (pH 5.0); flow rate, 1 ml/min.

—— and – – – –, radioactivity and absorbance at 280 nm, respectively, of plasma injected with ^{99m}Tc-Hcy; — · —, radioactivity of plasma injected with $^{99m}TcO_4^-$ as a reference.

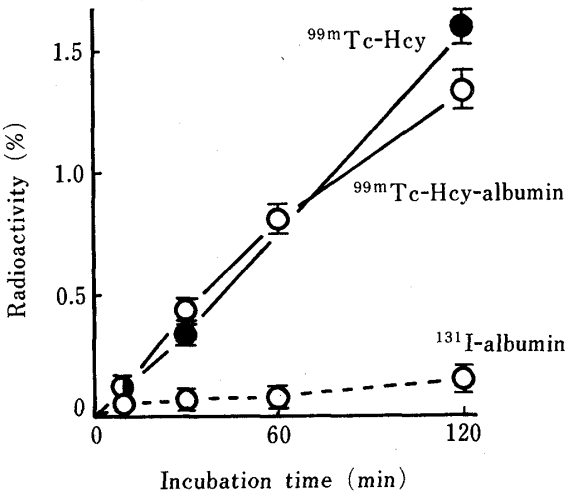

Fig. 5. *In Vitro* Tumor Uptake of ^{99m}Tc-Hcy-Albumin

A suspension (2 ml) containing Ehrlich ascites tumor cells (2 × 10^7 cells) and a radioactive compound was incubated at 37 °C for a given time and then treated as described in the text. Each point and line indicate the mean ± S.D. ($n=3$).

investigated at a given time after i.v. injection of ^{99m}Tc-Hcy (Table III). More than 70% of the radioactivity in the cells was detected in the 105000 × *g* supernatant at any time tested. The radioactivity in the supernatant was mostly eluted at the position of free ^{99m}Tc-Hcy, not of $^{99m}TcO_4^-$, on Sephadex G-50 (Fig. 3).

Identification of the Protein in Protein-Bound ^{99m}Tc-Hcy

The protein bound to ^{99m}Tc-Hcy in the blood was analyzed by high performance liquid chromatography (HPLC). As shown in Fig. 4, a major peak of radioactivity appeared at the retention time of albumin. Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis also demonstrated that most of the radioactivity migrated to the position of bovine serum albumin (data not shown). These findings suggest that the plasma protein bound to ^{99m}Tc-Hcy was albumin. Further, the HPLC analysis showed that $^{99m}TcO_4^-$ in the blood was not protein-bound but free, indicating that the radioactivity bound to albumin was that of ^{99m}Tc-Hcy.

In Vitro Uptake of ^{99m}Tc-Hcy-Albumin by Tumor Cells

In order to elucidate the role of albumin in the tumor accumulation of ^{99m}Tc-Hcy, ^{99m}Tc-Hcy-albumin was prepared as mentioned under Materials and Methods. Since albumin complex was not formed when $^{99m}TcO_4^-$ was used instead of ^{99m}Tc-Hcy, the complex formed in this preparation was ^{99m}Tc-Hcy-albumin. The uptake of ^{99m}Tc-Hcy-albumin by Ehrlich ascites tumor cells was compared with those of ^{99m}Tc-Hcy and ^{131}I-albumin. The radioactivity from ^{99m}Tc-Hcy-albumin was taken up by the cells to almost the same extent as that from ^{99m}Tc-Hcy, while the uptake of ^{131}I-albumin was much less (Fig. 5).

Discussion

^{67}Ga-citrate is employed clinically as a tumor-imaging agent.[6] Although the chemical form of this complex has not yet been fully clarified,[7] several investigators have reported that it was transported to tumor tissues by forming a transferrin complex in blood.[8] A high tumor uptake has also been found for several ^{99m}Tc-labeled compounds such as ^{99m}Tc-dimercaptosuccinic acid[9] and ^{99m}Tc-ethylenediamine-*N*,*N*-diacetic acid.[10] However, these ^{99m}Tc-labeled compounds remain undefined as regards not only their chemical form but also their mechanism of tumor transport owing to the complexity of Tc chemistry.

We have reported that ^{99m}Tc-Hcy was strongly accumulated in several experimental tumors *in vivo*.[4] Its chemical properties and mechanism of tumor accumulation need to be elucidated in order to develop improved imaging agents. Recently, we showed that the form of the tumor-tropic ^{99m}Tc-Hcy was an oligomeric complex in physiological saline and that excess Hcy was required to keep this complex stable.[3]

The mechanism of transport was investigated in the present study. A portion of the ^{99m}Tc-Hcy injected was transported to the tumor tissue by forming a complex with plasma protein in the blood. HPLC analysis demonstrated that the binding protein was albumin. Since the *in vivo* tumor accumulation of this plasma protein-complex was about twice that of free ^{99m}Tc-Hcy, the former form is considered significant for tumor accumulation. On the other hand, the radioactivity in tumors of ^{99m}Tc-Hcy-injected mice was mostly that of free ^{99m}Tc-Hcy. The role of albumin was examined in an *in vitro* uptake experiment, which showed that the tumor uptake of the radioactivity of ^{99m}Tc-Hcy-albumin was much higher than that of ^{131}I-albumin. These results indicated that ^{99m}Tc-Hcy bound to albumin in the blood was released from this protein on being taken up by tumor cells. Although it is not known why this albumin-complex is preferentially transported to the tumor tissue, it is of interest that albumin should play a major role in the transport of ^{99m}Tc-Hcy to tumor cells.

Acknowledgment The authors wish to thank Mr. T. Aomatsu and Miss Y. Suzuki for their technical assistance.

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