Radioactive Metal Complexes with Affinity for Tumors. II.[1] Biodistribution of Radioactivity in Cellular and Subcellular Fractions of Tumor Tissues

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The ^{99m}Tc and ^{57}Co complexes of ethylenediamine-*N*,*N*-diacetic acid (EDDA) are accumulated in tumor tissue. The complexes and related radioactive compounds were administered to experimental animals bearing Ehrlich tumor, and the blood, tumor, abscess, and other tissues were separated, fractionated and analyzed. In blood, the EDDA complexes of ^{99m}Tc and ^{57}Co were in dialyzable forms, whereas other tumor-nonlocalizing compounds were in undialyzable or protein-bound forms. The tumor/blood and tumor/muscle ratios of the radioactivity showed that the complexes had the high affinity for tumor tissues. Density gradient centrifugation analysis of the ascites tumor tissues showed that a significant amount of the radioactivity of the complexes was present in tumor cells. Subcellular fractionation of solid tumor tissue showed that the radioactivity was present in nuclear fraction.

Keywords radioactivity; ethylenediamine-*N*,*N*-diacetic acid; ^{99m}Tc; ^{57}Co; metal complex; Ehrlich tumor; tumor scintigraphy

We have shown that radioactivity was concentrated in tumor tissues of experimental animals a few hours after the administration of the complexes of ethylenediamine-*N*,*N*-diacetic acid (EDDA) with ^{99m}Tc (^{99m}Tc EDDA) and ^{57}Co (^{57}Co EDDA).[1,2] The tumor tissues were clearly visualized in scintigrams. Higher affinity for tumor was observed with μ-oxo ^{57}Co EDDA, a complex prepared by treatment of ^{57}Co EDDA with hydrogen peroxide.[3] The ^{51}Cr, ^{59}Fe, ^{64}Cu and ^{67}Ga complexes of EDDA as well as ^{3}H-labeled EDDA were not concentrated in the tumor.

For further development of tumor scintigraphic agents, knowledge of the mechanism of localization of the radioactive metal complexes is required. To elucidate the mechanism, the localization and the physicochemical behavior of the radioactivity in blood and tissues were studied. The EDDA complexes with tumor-localizing activity and related radioactive compounds were administered to experimental animals. The biodistribution of the radioactivity in blood, tumor, abscess, and other tissues were studied. The blood and tumor were fractionated and the distribution of the radioactivity in the fractions was measured. The present paper describes the results of the study.

Results

Biodistribution in Blood The EDDA complexes and related radioactive compounds were administered to rats and the radioactivity in the blood was analyzed by means of density gradient centrifugation and dialysis. The radioactive compounds studied were $^{99m}TcO_4^-$, ^{99m}Tc EDDA, the ^{99m}Tc complex of *N*-acetylethylenediamine-*N'*,*N'*-diacetic acid (AcEDDA), $^{57}CoCl_2$, μ-oxo ^{57}Co EDDA, and ^{57}Co AcEDDA. Tumor tissues were not clearly visualized with the complexes of AcEDDA.[2] A saline solution of the compounds was injected through the tail vain in the rat. Blood was collected into a heparinized syringe at 1 h after the injection.

The results of the density gradient centrifugation analysis are summarized in Table I. Most of the radioactivity was present in blood plasma and the cellular fractions contained only about 10% of the radioactivity in the blood. There was no marked difference in distribution between the tumor-localizing complexes (^{99m}Tc EDDA and μ-oxo ^{57}Co EDDA) and the nonlocalizing compounds ($^{99m}TcO_4^-$, $^{57}CoCl_2$, ^{99m}Tc AcEDDA, and ^{57}Co AcEDDA). The radioactivity was more concentrated in the erythrocyte fraction with $^{99m}TcO_4^-$ and ^{99m}Tc AcEDDA than with the other compounds.

TABLE I. Distribution of Radioactivity in Blood[a]

	A	B	C	D	E	F %
$^{99m}TcO_4^-$	73.75	3.09	3.81	6.19	10.64	2.53
^{99m}Tc EDDA	89.91	2.92	3.26	0.38	3.13	0.40
^{99m}Tc AcEDDA	81.79	2.41	1.78	3.01	9.96	1.05
$^{57}CoCl_2$	92.54	1.71	2.98	0.70	1.67	0.40
μ-Oxo ^{57}Co EDDA	89.44	5.93	3.31	0.50	0.40	0.42
^{57}Co AcEDDA	91.56	3.01	4.16	0.49	0.40	0.38

a) The blood samples taken 1 h after the administration of radioactive metal complexes were analyzed by density gradient centrifugation. A, blood plasma; B, mixture of blood plasma and lymphocytes; C, lymphocytes; D, mixture of lymphocytes, granulocytes and erythrocytes; E, erythrocytes; F, residue.

TABLE II. Dialyses of Blood[a]

	Time (h)	Residual radioactivity (%)
$^{99m}TcO_4^-$	1	40.4
	3	9.6
	24	6.4
^{99m}Tc EDDA	1	62.5
	3	38.3
	24	18.0
^{99m}Tc AcEDDA	1	85.5
	3	78.7
	24	76.2
$^{57}CoCl_2$	1	77.6
	24	64.5
μ-Oxo ^{57}Co EDDA	1	44.9
	24	8.1
^{57}Co AcEDDA	1	87.8
	24	55.5

a) The blood samples (1 ml) taken 1 h after the administration of radioactive metal complexes were analyzed by dialysis against 1 l of saline.

The blood (1 ml) was dialyzed against 1 l of saline at 4 °C and the dialyzed radioactivity was measured. Table II summarizes the results. Most of the radioactivity was dialyzable in the blood of the $^{99m}TcO_4^-$ injected rat. More than 80% of the radioactivity was dialyzed in 24 h in the samples of the tumor-localizing EDDA complexes (^{99m}Tc

EDDA and μ-oxo ^{57}Co EDDA). In the cases of ^{99m}Tc AcEDDA, $^{57}CoCl_2$, and ^{57}Co AcEDDA, more than 55% of the radioactivity remained undialyzed in 24 h. The results showed that $^{99m}TcO_4^-$ and ^{99m}Tc EDDA and μ-oxo ^{57}Co EDDA were present in unbound or dissociable forms in blood, whereas the other radioactive compounds were firmly bound to plasma proteins.

Biodistribution between Tissues A number of radioactive compounds were administered intravenously to mice bearing Ehrlich tumor or abscess. The compounds studied were ^{99m}Tc-bovine serum albumin (BSA), ^{99m}Tc-polyvinylpyrrolidone (PVP), ^{125}I-BSA, $Na^{125}I$, $^{99m}TcO_4^-$, ^{99m}Tc EDDA, $^{57}CoCl_2$, ^{57}Co EDDA, and μ-oxo ^{57}Co EDDA. The animals were killed 3 h after administration and the blood and tissues were separated. Table III lists the ratios of the radioactivity in tumor/blood, tumor/muscle, and abscess/blood.

The tumor/muscle ratios of radiolabeled BSA and PVP were about 2. The values in abscess were three times those in the tumor tissues. BSA and PVP do not normally penetrate into tissues from blood vessels and the distributions of the radiolabeled BSA and PVP may reflect those of the normal serum components. The high radioactivity in the tumor and abscess should indicate the extravasation of serum components due to the vascularities in these tissues. The radioactivity was not significantly concentrated in tumors in the cases of $Na^{125}I$, $^{99m}TcO_4^-$, and $^{57}CoCl_2$. The large tumor/blood and tumor/muscle ratios for the EDDA complexes of ^{99m}Tc and ^{57}Co indicate high affinity for the tumor. Extravasation from blood can not account for such large values.

Table III. Biodistribution of Radioactivity in Mice[a)]

	Tumor/blood	Tumor/muscle	Abscess/blood
^{99m}Tc BSA[b)]	0.22	2.17	0.68
^{99m}Tc PVP[c)]	0.21	1.95	0.65
^{125}I BSA[b)]	0.11	2.19	0.37
$^{99m}TcO_4^-$	0.46	2.52	0.61
$Na^{125}I$	0.69	3.41	—
$^{57}CoCl_2$	0.87	3.98	—
^{99m}Tc EDDA	3.32	7.96	1.45
^{57}Co EDDA	8.92	14.51	—
μ-Oxo ^{57}Co EDDA	13.84	26.44	38.95

a) The ratios of radioactivity 3 h after the administration of radioactive metal complexes. *b*) BSA = bovine serum albumin. *c*) PVP = polyvinylpyrrolidone.

Cellular Distribution in Tumor Solutions of ^{99m}Tc EDDA, μ-oxo ^{57}Co EDDA and ^{67}Ga citrate were administered intravenously to mice bearing Ehrlich ascites tumor. Ascites fluid was collected at a selected time. The cells in the ascites fluid were separated by density gradient centrifugation and the radioactivity of the fractions was measured.

Figure 1 shows the results. Though high radioactivity was present in both the ascites and tumor cell fractions, that in the ascites fraction decreased rapidly with time after the administration. In μ-oxo ^{57}Co EDDA and ^{67}Ga citrate administered mice, the radioactivity in the tumor cells increased 5 and 48 h after the administration, respectively. In the ^{99m}Tc EDDA administered mice, the radioactivity in the tumor was almost constant for a few hours.

The results confirmed that the radioactivity was concentrated in tumor cells after the administration of ^{99m}Tc EDDA, μ-oxo ^{57}Co EDDA and ^{67}Ga citrate. ^{67}Ga citrate is widely used as a clinical tumor scintigraphic agent.

Subcellular Distribution in Tumor Mice bearing Ehrlich solid tumor were intravenously given a solution of $^{99m}TcO_4^-$, ^{99m}Tc EDDA, ^{99m}Tc AcEDDA, $^{57}CoCl_2$, μ-oxo ^{57}Co EDDA, or ^{57}Co AcEDDA. The tumor tissues were

Table IV. Subcellular Distribution of Radioactivity in Ehrlich Solid Tumor

	Time (h)	A	B	C	D	E %
$^{99m}TcO_4^-$	1	15.3	3.0	6.0	2.7	76.0
	3	10.7	4.0	8.3	2.6	78.4
^{99m}Tc EDDA	1	13.1	6.5	5.1	3.4	78.4
	3	25.4	18.1	17.8	4.1	52.7
^{99m}Tc AcEDDA	1	9.9	6.8	11.2	4.4	74.5
	3	13.0	9.1	12.2	3.6	71.2
$^{57}CoCl_2$	1	12.6	9.9	18.4	9.8	59.2
	3	40.1	31.8	20.3	6.6	33.0
μ-Oxo ^{57}Co EDDA	1	22.6	19.5	28.1	7.7	41.6
	3	33.1	30.3	33.0	10.9	23.0
	3[a)]	56.8	54.2	28.1	6.4	8.7
	24	21.9	18.8	35.6	14.7	27.8
	24[a)]	46.5	42.6	25.9	11.0	16.6
	336	43.9	41.7	37.6	8.1	10.4
^{57}Co AcEDDA	1	19.8	16.6	26.8	4.8	48.6
	3	26.3	22.0	25.2	12.3	36.2

A, nuclear fraction; B, purified nuclear fraction; C, mitochondrial fraction; D, microsomal fraction; E, cytosol fraction. *a*) Indicates tumor tissues obtained from metastatic foci in the liver.

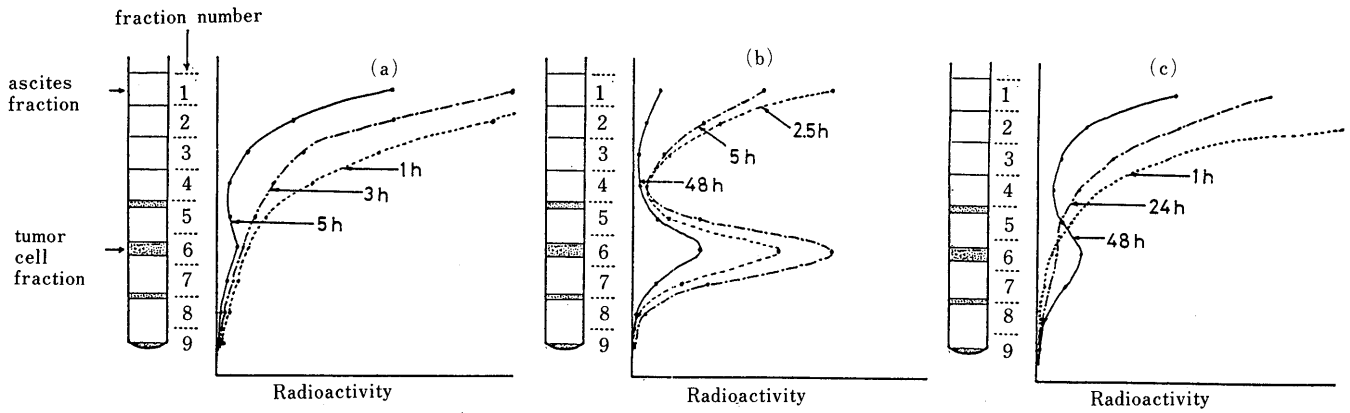

Fig. 1. Density Gradient Centrifugation Analysis of Ascites Cells of Mice Administered Radioactive Metal Complexes

Ascites fluids were taken at the indicated times after the intravenous administration of a) ^{99m}Tc EDDA, b) μ-oxo ^{57}Co EDDA and c) ^{67}Ga citrate.

removed at a selected time, homogenized and separated into the nuclear, mitochondrial, microsomal, and cytosol fractions by centrifugation.

The distribution of radioactivity in the fractions is summarized in Table IV. Most of the ^{99m}Tc radioactivity was present in the cytosol fraction 1 h after the administration. The radioactivity in the nuclear fraction increased significantly 3 h after the administration of ^{99m}Tc EDDA. On the other hand, a considerable part of the ^{57}Co radioactivity was present in the nuclear and mitochondrial fractions.

The distribution patterns of the ^{57}Co radioactivity in the fractions were different between the tissues of Ehrlich solid tumor and metastasized tumors. This may show that the uptake by tumor cells is affected by the nature and size of the tumor and by the development of its blood supply.

Discussion

Our previous study[1] showed that there are two kinds of EDDA complexes, tumor-localizing and nonlocalizing ones. The present study on the radioactivity in the blood gave a clue to understand the difference. In the blood of the animals given the tumor-localizing complexes, those with ^{99m}Tc and ^{57}Co, the radioactivity was not incorporated into the cellular fraction and was present in plasma in dialyzable forms. Binding to plasma proteins or incorporation into cells may prevent transfer to tumor and other tissues. The ^{57}Co radioactivity was bound to plasma proteins when administered in the form of $^{57}CoCl_2$ and the radioactivity in blood obscured the scintigraphic images of tumors.

The EDDA complexes of ^{99m}Tc and ^{57}Co are inert to ligand exchange reactions[1] and may not rapidly dissociate or decompose *in vivo*. The chemical species should remain unchanged in blood. Hence they were rapidly transferred into tumor tissues and excreted from the kidneys. The rapid clearance from the blood and the relatively high radioactivity in tumor tissues should give clear scintigrams of the tumor tissues.

The results of the biodistribution between tissues showed that extravasation was not the main mechanism of transfer of the EDDA complexes to the tumor tissues. The mechanism of the rapid transport is not fully understood at present. The cellular distribution study indicated that the radioactivity in the tumor tissues accumulated in the tumor cells.

The subcellular distribution study showed that a large part of the radioactivity was present in the cytosol fraction at the initial stage and was transferred gradually to the nuclear fraction. Concentration in the nuclear fraction seems faster in the case of ^{57}Co than ^{99m}Tc complexes. In contrast, ^{67}Ga radioactivity was reported to be incorporated into the cytosol and lysosome fractions.[4]

Experimental

Materials EDDA[3] and AcEDDA[5] were prepared by the reported methods. Other chemicals were of analytical grade. The chemicals and radioactive materials were obtained from commercial sources. Preparations of radioactive metal complexes and details of experimental animals bearing tumors and abscess were described previously.[1,2] A group of three animals received 0.1—0.2 ml each of a saline solution of the radioactive complex (50—100 μCi). The data shown in the tables are the average values of those obtained from the three animals. Cellulose tubing (size, 8/32; diameter, 0.6 cm; half-width, 1.0 cm) made by Visking Co. was used for the dialysis of blood.

Density Gradient Centrifugal Analysis of Blood Heparinized blood (3 ml) was mixed slowly with 6 ml of saline. The mixture was layered on the top of 3 ml of Conray 400-Ficoll solution and centrifuged at $400 \times g$ (1550 rpm) for 30 min. Each fraction was separated and its radioactivity was measured with an autogamma scintillation spectrometer.

Density Gradient Centrifugal Separation of Ascites The solution of ascites (0.2 ml) was mixed with a solution having a density of 1.010. The mixture was layered on the top of Percol-PBS density gradient solution (1.080, 1.070, 1.065, 1.060, 1.055, 1.050). The solution was centrifuged at 3000 rpm for 15 min. Each fraction was separated and its radioactivity was measured.

Fractionation of Solid Tumor Tissue The Ehrlich solid tumor tissues were homogenized in 0.25 M sucrose–0.01 M Tris–HCl buffer solution (pH, 7.6). The mixture was passed through two layers of gauze and centrifuged at $700 \times g$ for 10 min in a refrigerated centrifuge. The crude nuclear fraction separated was suspended in 2.1 M sucrose and purified by centrifugation at $105000 \times g$ for 60 min. After the separation of the crude nuclear fraction, the supernatant fluid was centrifuged at $6000 \times g$ for 15 min and the mitochondrial fraction was separated. The supernatant was further centrifuged at $105000 \times g$ for 60 min in a refrigerated centrifuge and the microsomal fraction was separated. The $105000 \times g$ supernatant was regarded as the cytosol fraction. The radioactivity of each fraction was measured. Tissues of metastasized tumors were fractionated analogously.

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