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The interaction of gadolinium complexes with isolated rat hepatocytes

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The lanthanide metal, gadolinium, is currently used in contrast agents for magnetic resonance imaging. We have performed a study of the interaction between isolated rat hepatocytes and ^{153}Gd complexed to diethylene-triamine pentaacetic acid (DTPA) or to DTPA–albumin conjugates. The study shows that isolated hepatocytes are able to take up both types of ^{153}Gd complexes. The ^{153}Gd–DTPA–albumin complexes are apparently taken up by pinocytosis, and possibly receptor-mediated endocytosis and/or adsorptive endocytosis, whereas the uptake mechanism of ^{153}Gd–DTPA is unknown. The ^{153}Gd–DTPA–albumin complexes, but not the ^{153}Gd–DTPA complex, are degraded by the cell. The degradation is inhibited by ammonium chloride. Gadolinium is slowly released back to the medium after loading of the cells with both complex types. In the experiments reported here no evidence of any adverse effects on the hepatocyte resulting from exposure to the ^{153}Gd-complexes were observed.

Keywords: albumin, DTPA, gadolinium, hepatocytes, metal uptake

Introduction

Gadolinium, in the form of gadopentetate dimeglumine, is increasingly used as a magnetic resonance imaging contrast agent (Goldstein *et al.* 1990). Hence, an increasing number of individuals are exposed to this metal.

Since the gadolinium ion is toxic (Haley 1965), the safety of gadolinium-based contrast agents depends on the stability and the rapid and complete elimination of the intact gadolinium complex from the body (Watson & Rocklage 1992).

The clinical experience with Gd–DTPA shows that the level of acute adverse reactions in humans is low (Niendorf *et al.* 1991). However, the general view that Gd–DTPA is rapidly and completely cleared from the body has been challenged by animal studies demonstrating the retention of gadolinium for extended periods (Wedeking *et al.* 1990, Kasokat & Urich 1992) with accumulation of gadolinium notably in bone, liver and spleen (Kasokat & Urich 1992). Furthermore, at least one human case has been reported where gadolinium was retained up to 8 days within intracerebral masses containing lipid-laden histiocytes (Tien *et al.* 1989). In addition, in the next generation of contrast agents designed for blood pool imaging, gadolinium is complexed to macromolecules in order to prolong its residence time in the plasma. This, however, also increases the possibility of interactions between contrast agent and cell (Rocklage *et al.* 1992).

We have studied the behavior of ^{153}Gd–DTPA and ^{153}Gd–DTPA coupled to albumin in an experimental system with isolated rat hepatocytes. Our aim was to determine if gadolinium is taken up by the hepatocyte, and if so, where and in what form the metal is localized within the cell?

In the present report we show that gadolinium is indeed taken up by the hepatocyte and is only slowly eliminated from the cell. Within the cell ^{153}Gd–DTPA is found as the original complex, whereas the ^{153}Gd–albumin complex is subject to degradation.

Materials and methods

Materials

Bovine serum albumin (BSA; essentially fatty acid free), rat serum albumin and diethylenetriamine pentaacetic acid (DTPA) anhydride were purchased from Sigma (St Louis, MO). $^{153}GdCl_3$ (50 MBq/μg Gd) was obtained from Amersham International (Buckinghamshire, UK). [^{14}C]Carboxyl dextran (31.5 MBq/g) was a product of New England Nuclear (Boston, MA). DTPA was obtained from Fluka (Buchs, Switzerland) and $GdCl_3 \cdot 6H_2O$ was a product of Aldrich (Milwaukee, MI). Instant thin layer

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chromatography silica gel impregnated glass fiber sheets (ITLC™, SG) was from Gelman Sciences (Ann Arbor, MI).

Gadolinium complexes

Radioactive DTPA complexes were prepared by mixing $^{153}GdCl_3$ (in 0.1 M HCl) with DTPA (in water) in the ratio 1:1 (mol/mol), and then adjusting the pH to 6.9–7.1 by careful addition of NaOH. The DTPA–albumin complexes were prepared by adding solid DTPA anhydride to a solution of BSA or rat serum albumin (RSA) in HEPES in the ratio 50:1 (mol/mol) according to the procedure of Unger *et al.* (1985). Free DTPA was removed by dialysis against 50 mM HEPES, pH 7.4.

The number of DTPA molecules coupled to albumin was determined by adding increasing concentrations of $^{153}GdCl_3$ in 0.1 M Na-citrate, pH 5, to aliquots of 1 ml 10 μM DTPA-albumin conjugate. After incubation at room temperature for 30 min, the ^{153}Gd–DTPA–albumin complex was precipitated with 80% ammonium sulfate. Radioactivity in the precipitate and the supernatant was determined. Under the experimental conditions used non-specific precipitation of ^{153}Gd amounted to less than 5% of total binding. The binding data were treated according to Scatchard (Scatchard 1949), utilizing a computer program as described by Osterloh & Aisen (1989). The DTPA–albumin complexes used in the present study contained an average of 15 DTPA molecules per albumin molecule. Labeling of the DTPA–albumin complexes was achieved by adding $^{153}GdCl_3$ (1.1 kBq/nmol Gd) in 6 mM HCl dropwise during continuous stirring to the DTPA–albumin solution. The pH was adjusted to approximately 7.5 by addition of 0.5 M Tris, pH 9.4. The reaction mixture was applied to a Sephadex G-50 column (PD-10, Pharmacia LKB Biotechnology, Uppsala, Sweden) and eluted in 0.5 ml fractions with 0.1 M HEPES, 0.9% NaCl, pH 7.4. The final product contained an average of 8.3 Gd per $DTPA_{15}$–BSA and 9.2 Gd per $DTPA_{15}$–RSA, giving specific activities of 9.1 kBq/nmol BSA and 10.1 kBq/nmol RSA. The complexes will hereafter be referred to as ^{153}Gd–BSA and ^{153}Gd–RSA, respectively.

The complete formation of gadolinium chelates was ascertained by thin layer chromatography utilizing the method described by Tweedle *et al.* (1991). Free or weakly bound gadolinium ions precipitate as solid $GdPO_4$ in the presence of phosphate. In thin layer chromatography the precipitate is held back at the application point. Thus, ^{153}Gd–DTPA, ^{153}Gd–BSA or ^{153}Gd–RSA were mixed with phosphate buffered saline (PBS), pH 7.4, or with water. The mixtures were incubated at room temperature for 10 min and 5 μl were then applied to ITLC strips. The strips were developed with 10% (w/v) ammonium acetate in 50% (v/v) methanol. When the elution front had moved 10 cm the strips were dried in air. Each lane was cut into 10 pieces of 1 cm and the pieces were counted for ^{153}Gd in a 1282 Compugamma (Pharmacia LKB Biotechnology) gamma counter. In this system free $^{153}Gd^{3+}$ or ^{153}Gd–DTPA moves with the elution front, whereas ^{153}Gd-phosphate is held back at the application point. Thus, by comparing the elution profile of two samples of which one has been incubated with PBS, and the other has been incubated with water, the amount of free $^{153}Gd^{3+}$ and/or free ^{153}Gd–DTPA can be determined. Figure 1 shows the results of a quality check on ^{153}Gd-BSA and ^{153}Gd-RSA by this method. It was found that a maximum of 0.04% and 0.28% of total ^{153}Gd could be free $^{153}Gd^{3+}$ and ^{153}Gd–DTPA, respectively. With ^{153}Gd–DTPA no free ^{153}Gd could be detected (data not shown).

Cells

Rat hepatocytes were isolated according to the method of Seglen (1975). Incubation and processing of cells was as previously described (Thorstensen & Romslo 1984). Viability of the cells was assessed by Trypan blue staining. Pinocytosis was determined by incubation of the cell with [^{14}C]carboxyl-dextran (Thorstensen & Romslo 1984).

The distribution of ^{153}Gd between stroma and cytosol after incubation of cells with ^{153}Gd complexes was determined following disruption of the cells by ultra-sonication, centrifugation of the sonicate at 10000 *g* for 10 min and then centrifugation of the supernatant at 100000 *g* for 1 h. The final supernatant was taken as the cytosol fraction whereas the combined pellets were taken as the stroma fraction. Gel filtration of the cytosol was performed on a Sephacryl S-300 column (1.6 × 35 cm) at a flow rate of 1 ml/min in 50 mM Tris, 100 mM NaCl, pH 7.4. All subcellular fractionation procedures were performed at below 5 °C.

Results and discussion

Isolated rat hepatocytes take up gadolinium from both the ^{153}Gd–DTPA and the ^{153}Gd–albumin complexes at 37 °C (Figure 2). The uptake is roughly linear with time and

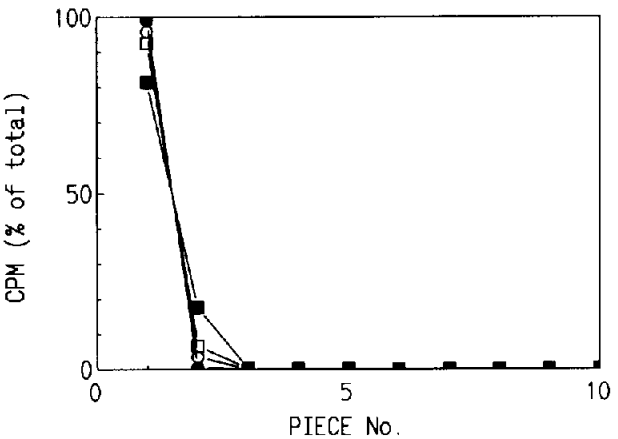

Figure 1. Quality control of ^{153}Gd–albumin complexes by ITLC. To ascertain complete binding of gadolinium to DTPA–albumin, thin layer chromatography was performed after incubation of the complexes in the absence (open symbols) or presence (filled symbols) of phosphate (see Materials and Methods section). (○, ●) ^{153}Gd–BSA and (□, ■)) ^{153}Gd–RSA.

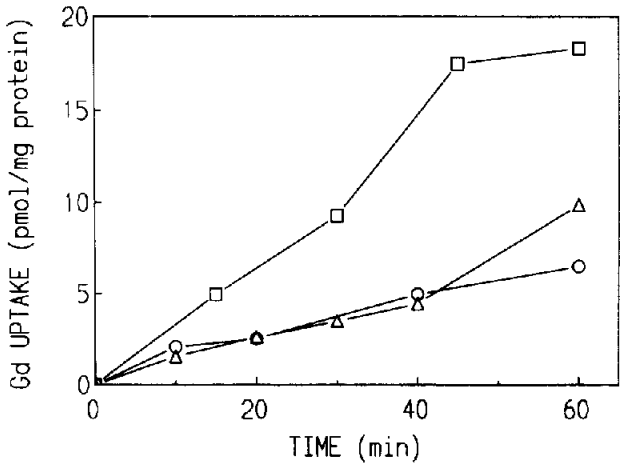

Figure 2. Uptake of gadolinium by isolated hepatocytes. The figure shows the time course of ^{153}Gd uptake at 37 °C by isolated hepatocytes from (□) 20 μM ^{153}Gd–DTPA, (△) 2.5 μM ^{153}Gd–BSA (20.8 μM Gd) and (○) 2.5 μM ^{153}Gd–RSA (23.0 μM Gd).

proceeds at a rate of approximately 180 000 Gd atoms cell^{-1} min^{-1} for the ^{153}Gd–DTPA complex. The uptake rates for the two albumin complexes are similar to each other at approximately 75 000 Gd atoms cell^{-1} min^{-1}. During 1 h, approximately 0.8% of total extracellular ^{153}Gd–DTPA and 0.4% of the total ^{153}Gd–albumin is taken up by the cells. At 4 °C the uptake is virtually zero (data not shown).

Assuming that the ^{153}Gd complexes are taken up by pinocytosis only, the uptake data of Figure 2 may be converted into equivalents of fluid volume internalized by the hepatocyte. For the ^{153}Gd–DTPA complex the equivalent fluid volume is 0.92 μl mg cell protein^{-1} h^{-1} whereas the figures for the two albumin complexes are in the range 0.32–0.36 μl mg^{-1} cell protein h^{-1}. By comparison, the rate of pinocytosis as assessed with the pinocytosis marker [^{14}C]carboxyl-dextran is 0.27 μl mg cell protein^{-1} h^{-1} (data not shown). This is similar to previously reported values obtained with [^{14}C]carboxyl-dextran (Thorstensen & Romslo 1984) as well as with other pinocytosis markers (Sibille *et al.* 1982, Cole & Glass 1983, Blomhoff *et al.* 1989). Thus, the uptake of the ^{153}Gd–albumin complexes is compatible with pinocytosis. However, experiments with a preparation of (^{153}Gd–DTPA)$_4$–BSA showed an uptake larger than what could be accounted for by pinocytosis only (data not shown). These data are compatible with receptor-mediated endocytosis (RME) of albumin bound to approximately 4 × 10^5 low affinity binding sites per hepatocyte, as assessed by a computer simulation program (Bakøy & Thorstensen 1994). This may indicate that the albumin complexes are internalized by RME. Since hepatocytes have been reported to express albumin receptors (Trevisan *et al.* 1982), the possibility therefore exists that a high degree of DTPA coupling to albumin affects the molecule's ability to bind to the albumin receptor. It should be kept in mind, however, that the presence of hepatocyte albumin receptors is controversial (Stremmel *et al.* 1983). Moreover, adsorptive endocytosis where albumin nonspecifically bound to the cell membrane is internalized, may be indistinguishable from the low affinity binding site concept.

During the experiments reported above hepatocytes were exposed to the ^{153}Gd complexes for 1 h at 37 °C at concentrations up to 100 μM Gd without any negative effects on cell viability as assessed by Trypan blue staining (data not shown).

Having established that the isolated hepatocyte is able to take up gadolinium from the two complexes we addressed the question of whether the metal remains inside the cell. As seen in Figure 3, following uptake from ^{153}Gd–DTPA or ^{153}Gd–albumin, gadolinium is slowly released back to the medium. During the first hour the release amounts to 30–40% of total cellular ^{153}Gd. Thus, once taken up the metal may reside within the cell for a considerable length of time. The release of [^{14}C]carboxyl-dextran is apparently slower than the release of the ^{153}Gd complexes, particularly during the first 5–10 min of release (Figure 3). However, all three release curves fit a two-component exponential equation ($Y = Ae^{-BX} + Ce^{-DX} + E$).

The localization of ^{153}Gd within the cell following cellular uptake of the metal is not equal for the two types of gadolinium complexes. This is evident from the distribution of the metal between the stroma and the cytosol fraction. Approximately 70% of the ^{153}Gd taken up from ^{153}Gd–DTPA is found in the cytosolic fraction. By comparison, the cytosol contains only approximately 35% of the ^{153}Gd taken up from the albumin complexes. This suggests the localization of ^{153}Gd–albumin to vesicular fractions.

When the cytosol is subjected to gel filtration the ^{153}Gd taken up from ^{153}Gd–DTPA elutes at the same position as the original complex (Figure 4A). Thus, ^{153}Gd–DTPA appears not to undergo any changes during its interaction with the cells. By contrast, the ^{153}Gd–DTPA–albumin

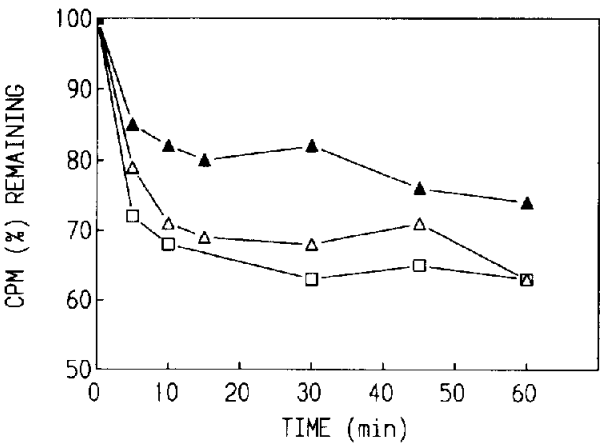

Figure 3. Release of gadolinium and dextran from hepatocytes. Hepatocytes were loaded for 1 h at 37 °C with ^{153}Gd–DTPA, ^{153}Gd–BSA or [^{14}C]carboxyl-dextran. After washing, the cells were reincubated at 37 °C and the release of radioactivity was determined. The figure shows the amount of ^{153}Gd or [^{14}C]-carboxyl-dextran remaining in the cell. (□) ^{153}Gd–DTPA, (△) ^{153}Gd–BSA and (▲) [^{14}C]carboxyl-dextran.

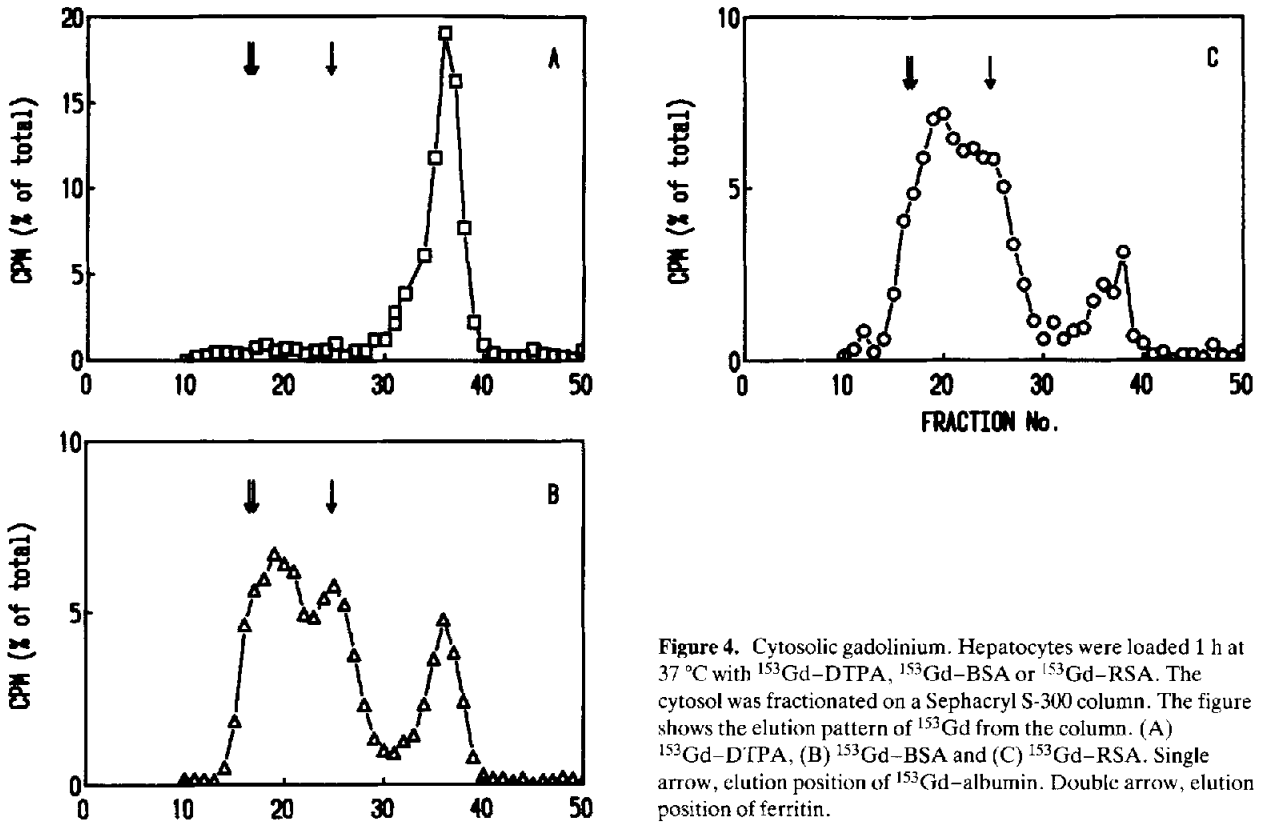

Figure 4. Cytosolic gadolinium. Hepatocytes were loaded 1 h at 37 °C with ^{153}Gd–DTPA, ^{153}Gd–BSA or ^{153}Gd–RSA. The cytosol was fractionated on a Sephacryl S-300 column. The figure shows the elution pattern of ^{153}Gd from the column. (A) ^{153}Gd–DTPA, (B) ^{153}Gd–BSA and (C) ^{153}Gd–RSA. Single arrow, elution position of ^{153}Gd–albumin. Double arrow, elution position of ferritin.

complexes are clearly changing during interaction with the cell. In the experiment shown in Figure 4(B) only approximately 40% of the ^{153}Gd elutes at the same position as the parent compound. The rest elutes at positions corresponding to higher molecular weight (approximately 30%), as well as lower molecular weight (approximately 20%) than ^{153}Gd–albumin. The position of the low molecular weight compound corresponds to the position of ^{153}Gd–DTPA. This may suggest that the Gd–DTPA moiety is split off from the protein part. The identity of the high molecular weight compound is unknown. It does not, however, correspond to the position of ferritin as determined by an ELISA (Figure 4). Furthermore, it is not likely to represent association of Gd–DTPA with cytosol proteins since incubation of Gd–DTPA with cytosol prior to gel filtration does not result in the formation of a high molecular weight compound (data not shown). Possibly, the high molecular weight compound represent association of albumin fragments with cytosol protein or the aggregation of such fragments. As shown in Table 1, the extent of Gd–albumin degradation varied between cell preparations. The

Table 1. Distribution of ^{153}Gd in the cytosol fraction of hepatocytes

Experiment no.	Treatment	Complex type	^{153}Gd (% of total)		
			high MW F10–20	albumin F21–30	low MW F31–50
I	control	RSA	17.9	81.5	0.6
I	control	BSA	20.5	78.9	0.6
I	none	BSA	32.3	40.7	22.7
I	none	RSA	33.1	42.8	15.3
II	none	BSA	30.1	61.9	7.1
II	NH_4Cl	BSA	29.2	65.0	5.9
III	none	BSA	17.2	66.5	16.4
III	NH_4Cl	BSA	19.0	74.8	6.2

Rat hepatocytes were incubated for 1 h at 37 °C with 2.5 μM ^{153}Gd–BSA (20.8 μM Gd) or 2.5 μM ^{153}Gd-RSA (23.0 μM Gd) in the presence or absence of 10 mM NH_4Cl. Cytosol was prepared and run through a Sephacryl S-300 column as described in Materials and methods. The resulting chromatogram (see Figure 4) was split into three parts consisting of fractions 10–20, 21–30 and 31–50. The three parts are designated 'high MW', 'albumin' and 'low MW', respectively. The total amount of ^{153}Gd in each part is shown as % of total ^{153}Gd eluted from the column. In the control experiments ^{153}Gd–albumin was incubated with non-labeled cytosol for 1 h at 4 °C before gel filtration. The table shows the results from three experiments.

observation that the weak base NH_4Cl, which raises the pH of the lysosome (Ohkuma & Poole 1987), inhibits the degradation of the albumin complex, particularly the formation of the low molecular weight compound (Table 1), suggests that the albumin complexes are internalized by pinocytosis and are subsequently degraded in the lysosomes. However, subcellular fractionation studies are required to determine this with any degree of certainty.

In conclusion, the present study shows that isolated hepatocytes are able to take up ^{153}Gd complexes, albeit to a modest degree. The ^{153}Gd–albumin complexes appear to be taken up by pinocytosis and possibly RME and/or adsorptive endocytosis (depending on the degree of DTPA coupling to the protein) whereas the uptake mechanism of ^{153}Gd–DTPA is unknown. The ^{153}Gd–albumin complexes are degraded by the cell. The degradation is inhibited by ammonium chloride, indicating lysosomal involvement. The ^{153}Gd–DTPA complex appears to be stable. Gadolinium is slowly released back to the medium after loading of the cells with both complex types. Finally, in the experiments reported here no evidence of any adverse effects on the hepatocyte resulting from exposure to the ^{153}Gd complexes were observed.

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