Galactosylated Liposomes as Carriers for Targeting Meso-2,3-dimercaptosuccinic Acid to Cadmium Storage Sites in CadmiumExposed Mice

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Received September 30, 1999 and accepted July 17, 2000

Abstract: In this study an attempt has been made to examine the efficacy of meso-2,3-dimercaptosuccinic acid (DMSA) using galactosylated liposomes as carriers for mobilization of cadmium from the body of mice preexposed to cadmium chloride (0.005 mmoles/kg intraperitoneally daily for 4 days). Cadmium-exposed mice after a rest period of 8 weeks were administered DMSA intravenously, two injections 15 µmoles/kg with an interval of 48 h, as free form of DMSA, or DMSA encapsulated in liposomes composed of phosphatidyl choline, cholesterol and phosphatidyl ethanolamine (7:2:1; PC-lip-DMSA) or in liposomes to which p-aminophenyl galactoside had been anchored (Gal-lip-DMSA). Excretion of cadmium through urine and feces was monitored for 5 days. Thereafter animals were sacrificed, liver, kidneys, spleen and isolated hepatocytes were analysed for cadmium, copper and zinc concentration. Efficacy for cadmium mobilization from the body was found to be in the order Gal-lip-DMSA>PC-lip-DMSA>DMSA. These results show that liposomes can be used as targeted carrier system for chelating agents safely and efficiently as compared to administration of free chelating agent.

Key words: Cadmium, Detoxification, Liposomes, Targeting, Dimercaptosuccinic acid

Cadmium is a well recognised nephrotoxic agent and induces the synthesis of metallothionein (MT) to which it gets sequestered mainly in liver1–3). Handling of cadmium poisoning therefore, requires decorporation of the metal from its intracellular storage sites in liver and the kidneys, the target organs. Efficacy of a variety of drugs have been improved when they were administered after encapsulation in liposomes4, 5). Liposome encapsulated diethylenetriaminepentaacetic acid (DTPA) administered to mice loaded with cadmium has been shown to improve the efficacy for removal of cadmium from the body organs and its excretion from the body6). Investigations on the role of size and lipid composition of liposomes revealed that triethylenetetraminehexaaacetic acid (TTHA) and dimercaptosuccinic acid (DMSA) showed better efficacy towards cadmium removal when encapsulated in small unilamellar vesicles7, 8) rather than larger vesicles. Further TTHA when encapsulated in liposomes composed of sphingomyelin and cholesterol showed better efficacy for cadmium mobilisation from the body of exposed animals compared to the material encapsulated in phosphatidylcholine and cholesterol liposomes9). Cadmium is mainly stored in parenchymal cells of liver10), where receptor for free galactose are known to exist. Evidence exists that galactoside residues or mannoside residues present on the surface of liposomes can bring about selective uptake of liposomes of parenchymal cells or non parenchymal cells respectively of rat liver11, 12). With this genesis we investigated the role of targetable liposomes as carriers of DMSA to see if better efficacy could be achieved using such liposomes as carriers of DMSA.

Egg phosphatidyl choline (PC) was isolated from egg yolk according to the method of Singleton et al.13). Cadmium chloride meso-2,3-dimercaptosuccinic acid (DMSA),
cholesterol, 5,5’-dithiobis-2-nitro-benzoic acid (DTNB), phosphatidyl ethanolamine (PE), glutaraldehyde etc. were obtained from Sigma Chemicals Co (St. Louis, MO USA), p-aminophenyl- -D-galactopyranoside was prepared by reduction of p-nitrophenyl- -D-galactopyranoside. The method described by Bloch and Burger was used:

\[ \text{p-nitrophenyl glycoside (100 mg)} \text{ was dissolved in 50 ml of 0.5 M sodium biocarbonate containing 0.1 M sodium dithionite separately and stirred vigorously for 3 h at room temperature. It was then dried under vacuum and extracted three times with 50 ml methanol each time. The total methanol extract was dried under vacuum. The reduction of NO}_2 \text{ group to the amino group was ascertained by measurement of amino group and neutral sugar.} \]

Liposomes encapsulating DMSA were prepared by taking Egg PC, cholesterol and PE (7:2:1) in chloroform in a round bottom flask. A thin film of lipids was prepared under nitrogen at 37°C and hydrated with 50 µ moles DMSA in water (neutralised with sodium bicarbonate). It was mixed by hand shaking and vortexing. The suspension was bath sonicated followed by sonication in a Vibronics probe sonicator for 3 min and 1 min rest for 10 cycles keeping the material submerged in ice cold water. It was then centrifuged (1500 rpm) to remove titanium particles coming from the metallic probe used for sonication. The liposomal suspension was loaded on a Sephadex G-75 column, preequilibrated with phosphate buffered saline and 3 ml fractions were collected in the same buffer to separate liposomes from the unencapsulated material. The concentration of DMSA in the fractions was determined by measuring the intensity of yellow colour developed with DTNB at 412 nm using a Bausch and Lomb spectrophotometer. Triton X-100 was added in liposome fractions for rupturing the vesicles before estimation. The small unilamellar vesicles so produced encapsulated 5% DMSA. For preparation of glycosylated liposomes containing DMSA liposomes encapsulating DMSA were prepared as described above. After purification these liposomes were incubated at 20°C for 20 min with 15 mM concentration of glutaraldehyde and 20 mg p-aminophenyl- -D-galactopyranoside. After incubation these liposomes were dialysed against phosphate buffered saline (PBS) to separate the uncoupled product. The amount of DMSA entrapped in these liposomes was found to be 2–3% in these vesicles.

Male albino mice of ITRC colony (3–4 months old, 25–30 g body wt.) fed on pellet diet (Liptons India Ltd) and water ad libitum were used in the study. Twenty eight animals were administered cadmium 0.005 m moles/kg (dissolved in 10 ml of 0.9% NaCl) daily for four days intraperitoneally along with 7 normal controls given saline. After a period of 8 weeks rest to allow stable binding to cadmium in the body. Cadmium loaded animals were administered two injections i.v., 15 µ moles/kg of DMSA, either free or encapsulated in PC.chol-PE (7:2:1) liposomes or in liposomes to which p-aminophenyl-galactose had been anchored. DMSA or liposomal suspensions were administered in phosphate buffered saline. The injections were administered at a gap of 48 h. Immediately after the first injection 5 animals from each group were kept in metabolic cages for collection of 24 hr urine and feces. Five animals from each group were sacrificed under ether anaesthesia, along with 5 control animals. Liver, kidneys and spleen were collected, washed and weighed. All the tissues, hepatocyte suspension (isolated as described below), urine and feces were digested with acid mixture (HNO₃, HClO₄, H₂SO₄-6:1:1) and after appropriate dilution cadmium, copper and zinc analysis was carried out by Atomic Absorption Spectrophotometry.

For isolation of hepatocytes, two animals from each of cadmium treated groups and two normal controls were used for isolation of hepatocytes. Four types of buffers were prepared. Buffer A was modified Hanks buffer containing 0.5 mM EGTA and 2% albumin; Buffer B was modified Hanks buffer containing 0.12% collagenase type IV and 4 mM CaCl₂; Buffer C was Krebs Hanseleit buffer containing 2% albumin and Buffer D was Krebs Hanseleit buffer containing 0.39% Hepes but not albumin. All these solutions were bubbled with carbogen gas and preincubated to 37°C. Mice were anaesthetised and placed on the perfusion platform. After making vertical cut, the abdomen was opened and portal vein was canulated. Inferior venacava was ligated above renal veins. The chest was opened and thoracic portion of inferior venacava was perfused with buffer A for 5 min followed by buffer B for 10 min. At the end of perfusion liver appeared swollen and pale with no blebs on surface. Thereafter liver was immersed in buffer C, capsule was cut and cells were dispersed with gentle pressing. Suspension was filtered through mesh to remove connective clumped cells and then centrifuged at 1000 x g for 2 min. The cells were suspended in buffer C, and Viability of cells was checked by trypan blue exclusion test and found to be 98%. When DMSA was administered in either of the three forms to cadmium exposed animals the efficacy for removal of cadmium from the body organs was found to be in the order Gal-lip-DMSA>PC-lip-DMSA>DMSA, while the level of cadmium in kidneys was lowered maximum by gal-lip-DMSA treatment. This is also elucidated in the mobilization pattern of cadmium from hepatocytes, the chief storage site of cadmium (Table 1). Results on the analysis of copper
however indicate some mobilization of copper from the kidneys by treatment with Gal-lip-DMSA encapsulated in liposomes while lowering of copper in hepatocytes and spleen was observed by both the liposomal chelator treatments. Similarly the levels of zinc enhanced by cadmium exposure were lowered by treatment with Gal-lip-DMSA in liver, kidneys, as well as spleen (Table 2). The lowering of zinc in hepatocytes was however observed when free DMSA treatment was given, while the other two liposomal forms of DMSA did not show any remarkable effect. The excretion of cadmium in urine and feces is shown in Fig. 1. Urinary excretion was enhanced by administration of DMSA encapsulated in glycosylated liposomes throughout the treatment in comparison to the other three groups. Significant enhancement in fecal elimination of cadmium was observed by both the liposome encapsulated DMSA treated groups on day 4 and 5 compared to saline or free DMSA treatments. DMSA encapsulated in galactosylated liposomes however eliminated relatively higher amount of cadmium via feces. Since there is selective storage of cadmium in hepatocytes, biliary excretion of the metal constitutes an important route for its elimination from the body.\(^{18}\) Even though DMSA is lipophilic compared to polyaminocarboxylic acids, the presence of carboxyl groups in the molecule renders it hydrophilic enough and makes it non-penetrable across the cell membrane.\(^{18}\) The enhancement in the fecal elimination of cadmium as a result of treatment with liposome encapsulated DMSA shows that the availability of DMSA for interaction with cadmium is enhanced.

Polyaminocarboxylic acids specially DTPA have been found to be effective against cadmium intoxication only when given soon after cadmium intoxication, since after metallothionein

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**Table 1. Mobilization of cadmium from the body organs and hepatocytes of mice exposed to cadmium followed by administration of DMSA in free form, or encapsulated in liposomes**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Liver (µg/g)</th>
<th>Kidney (µg/g)</th>
<th>Spleen (µg/g)</th>
<th>Hepatocytes (µg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cd-saline</td>
<td>24.67 ± 1.20</td>
<td>19.07 ± 1.50</td>
<td>3.26 ± 0.25</td>
<td>0.87 ± 0.06</td>
</tr>
<tr>
<td>Cd-DMSA</td>
<td>21.50 ± 0.86</td>
<td>14.37 ± 0.54</td>
<td>1.77 ± 0.18</td>
<td>0.57 ± 0.07</td>
</tr>
<tr>
<td>Cd-lip-DMSA</td>
<td>19.37 ± 0.87</td>
<td>14.05 ± 0.82</td>
<td>1.36 ± 0.10</td>
<td>0.43 ± 0.10</td>
</tr>
<tr>
<td>Cd-Gal-Lip-DMSA</td>
<td>17.41 ± 0.67</td>
<td>8.33 ± 0.95</td>
<td>0.74 ± 0.20</td>
<td>0.34 ± 0.03</td>
</tr>
</tbody>
</table>

Values are expressed as µg/g tissue representing mean ± SE for five animals, while that of hepatocytes for two animals derived from 1 g liver analysed in duplicate (expressed as mean ± SE of four values). Comparison was made by one way analysis of variance (ANOVA). a, p<0.05 when compared to Cd-saline; b, p<0.05 when compared to Cd-DMSA group.

**Table 2. Effect of DMSA encapsulated in liposomes on the levels of copper and zinc (µg/g) in body organs and hepatocytes of cadmium exposed animals**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Copper (µg/g)</th>
<th>Zinc (µg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-saline</td>
<td>6.02 ± 0.26</td>
<td>30.84 ± 3.10</td>
</tr>
<tr>
<td>Cd-saline</td>
<td>6.92 ± 0.22</td>
<td>65.75 ± 0.39a</td>
</tr>
<tr>
<td>Cd-DMSA</td>
<td>6.02 ± 0.38</td>
<td>56.25 ± 4.35a</td>
</tr>
<tr>
<td>Cd-lip-DMSA</td>
<td>6.42 ± 0.25</td>
<td>55.30 ± 1.95ab</td>
</tr>
<tr>
<td>Cd-Gal-lip-DMSA</td>
<td>6.02 ± 0.17</td>
<td>51.84 ± 3.63ab</td>
</tr>
</tbody>
</table>

Values are expressed as µg/g tissue representing mean ± SE for five animals, while that of hepatocytes for two animals derived from 1 g liver analysed in duplicate (expressed as mean ± SE of four values). Comparison was made by one way analysis of variance (ANOVA). a, p<0.05 when compared to n-saline; b, p<0.05 when compared to Cd-saline group.
induction, cadmium gets sequestered into a deeper pool to become less accessible for interaction with the chelator\textsuperscript{19}. Esters of DMSA were also used for improvement in the efficacy as they possess enhanced lipophilicity and have been shown to interact with metallothionein-bound cadmium \textit{in vitro}. Their active transport in renal and hepatic cells was also demonstrated \textit{in vivo}\textsuperscript{20, 21}. However, these esters may undergo hydrolysis \textit{in vivo} and may also result in toxic organic moieties in the body. The interaction of liposomal DMSA with cadmium results in improvement in mobilization/removal of cadmium from the body organs specifically liver and spleen. Further, the removal of cadmium from the body is also expected to be in a fashion so as to result in a slow and sustained release of the metal chelator complex, thereby presenting lower concentration of the metal complex to the kidneys for excretion.

The results of the present study show that modulation of lipid composition incorporating appropriate galactoside residue on the surface of liposomes used as carriers of chelating agents may thus accelerate the delivery of the chelator to the cadmium storage site and render efficient removal of cadmium from its stored deposits in the body and thus may serve as a safe and sustained carrier system for targeted delivery of chelators in cadmium intoxication.

Liposomes have been used for drug targeting in a variety of disease conditions and several different approaches have been successfully used to achieve this goal\textsuperscript{22}. Increased liver uptake of liposomes and targeting efficacy has been shown in rodents by labeling with asialofetuin\textsuperscript{23}. Targeting of doxorubicin-loaded liposomes to T cells via transferrin receptor has also been reported\textsuperscript{24}. Banerjee \textit{et al.}\textsuperscript{25} have developed a novel polypeptide graftable liposomal system targeted to macrophages. Further, the use of liposomes is also reported to protect tissue against local toxicity of the drugs by adopting proper route of administration\textsuperscript{26}. It is interesting to note that the targeting of chelating agents to hepatocytes, the storage site of cadmium could also reduce kidney burden when galactosylated liposomes were used as carriers. Further investigations on such targeted delivery by other methods are needed to confirm these results. Our results also showed reversal of imbalance in trace metal levels caused by cadmium exposure. Treatment with DMSA has also been shown to result in alteration in the level of trace elements in our earlier studies\textsuperscript{27}. Homoeostasis of the trace metals in body is an important factor for normal physiological functions of the body and also for response to the treatment against diseases. We thus conclude that targeted carrier system for chelating drugs can be used in the management of heavy metal poisoning while avoiding the side effects of chelation therapy.

**Acknowledgements**

Authors are grateful to Dr. P.K. Seth, Director, ITRC for his encouragement and support of the work. Financial assistance from Indian Council of Medical Research for carrying out the work is gratefully acknowledged. Help in the assay of viability of hepatocytes by Dr. RKS Dogra and statistical evaluation of data by Mr. N. Mathur is gratefully acknowledged. Thanks are due to Sri Ram Chander for providing technical assistance in the work. Thanks are also due to Miss Navita Verma and Mrs. Balbir Kaur for typing the manuscript.

**References**

2) Fowler BA, Nordberg GF (1978) The renal toxicity of cadmium metallothionein. Morphometric and X-ray