Uptake and degradation of filamentous actin and vitamin D-binding protein in the rat

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Tissue uptake and degradation of ^{125}I -tyramine-cellobiose-labelled filamentous actin, vitamin D-binding protein (DBP) and actin-DBP complex were studied in the rat. Actin and actin-DBP complex were cleared from plasma at a faster rate than was DBP. About 40% of injected actin was recovered in the liver between 10 and 30 min after administration. Of the total radioactivity recovered in the liver, about 35% and 40% was detected in parenchymal and endothelial cells respectively when labelled actin or DBP-actin complex was injected intravenously. When labelled DBP alone was injected, approx. 55% of the radioactivity recovered in liver was in the Kupffer cells. These results suggest that actin is targeting the DBP-actin complex to the endothelial and parenchymal liver cells. Filamentous actin was also taken up in large amounts and at a rapid rate in parenchymal as well as non-parenchymal liver cells *in vitro*. Our data indicate that the rat has a mechanism to clear actin and the DBP-actin complex from plasma and that both parenchymal and non-parenchymal liver cells are involved in this process.

INTRODUCTION

Actin accounts for a major fraction of the protein in mammalian cells. Although actin is an intracellular protein, it has also been detected in normal human serum [1]. Serum from patients with cardiac injury [2] and chronic hepatitis [3] has been shown to contain antibodies against actin.

Intracellular actin exists in two forms, monomeric (M_r 43 000) and polymeric [4]. During cell lysis both forms may be released into the circulation. Actin will spontaneously polymerize in a solution with an ionic strength similar to that of plasma [5]. Actin-binding proteins in plasma inhibit the formation of polymeric actin and they also depolymerize filamentous actin [6,7]. The two major actin-binding proteins in plasma are gelsolin and vitamin D-binding protein (DBP), also named Gc-globulin [6–10]. The quantitatively most important actin-binding protein in plasma is DBP [6,8,9], which is an α -globulin with an M_r of 52 000–58 000 [11,12]. DBP has, in addition to the binding site for vitamin D metabolites, high-affinity binding sites for actin [13], and a DBP-actin complex is rapidly formed *in vitro* during tissue homogenization [13]. Binding of actin to DBP results in an enhanced clearance of the actin-DBP complex from circulation compared with the clearance of DBP alone [8,9,14].

Plasma from patients with acute fulminant hepatic necrosis [15] and plasma from pregnant women during the third trimester of pregnancy [16] have been reported to contain significant amounts of DBP-actin complex. In contrast with these findings, plasma from normal healthy humans does not contain any significant amounts of the complex [15,16]. We have previously shown that the DBP-actin complex is rapidly removed from the circulation by the liver [14]. This may be the reason why the complex is normally undetectable in the circulation. However, when there is an increased release of actin due to cell lysis or a decreased clearance of the complex due to liver disease, the complex may be detected in the circulation [15,16].

In the present report we have extended the study of the uptake of DBP and DBP-actin complex *in vivo*. No data are available on the mechanism behind the plasma clearance of actin. We therefore

examined the tissue uptake and degradation of filamentous actin, and the different liver cells responsible for uptake and degradation of radiolabelled actin, DBP and DBP-actin complex *in vivo*. We also report data on the uptake and degradation of filamentous actin in isolated rat liver parenchymal and non-parenchymal cells.

MATERIALS AND METHODS

Materials

Carrier-free ^{125}I was purchased from Amersham International, Amersham, Bucks., U.K. Cyanogenbromide-activated Sepharose A, Agarose A, Polybuffer exchanger PBE 94 and Polybuffer 74 for chromatofocusing were obtained from Pharmacia, Uppsala, Sweden. Gel filtration material AcA-44 was obtained from LKB, Stockholm, Sweden. Rabbit muscle actin and BSA were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.

Animals

Adult rabbits (about 3 kg) were used for antiserum production. Male Wistar rats (150–250 g) were fed on an ordinary laboratory chow.

Purification of rat DBP

Rat DBP was prepared as previously described [17]. The amount of DBP was quantified by single radial immunodiffusion [18].

Iodination of purified rat DBP and rabbit actin

The purity of actin was evaluated as previously described in detail [14] by SDS/PAGE and gel filtration. Purified rat DBP and rabbit actin were labelled with ^{125}I -tyramine-cellobiose (^{125}I -TC) as described by Pittman *et al.* [19] with minor adjustments [14]. The purity of ^{125}I -TC-labelled proteins was confirmed by SDS/PAGE (0.1% SDS; 10% polyacrylamide) and gel filtration [14]. Labelled DBP was incubated with actin as previously

Abbreviations used: DBP, binding protein for vitamin D and its metabolites; TC, tyramine-cellobiose; PBS, phosphate-buffered saline.

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described [14] to form a 1:1 molar DBP-actin complex. The labelled DBP-actin complex was isolated by gel filtration on an AcA-44 column [14]. TC-labelled actin was polymerized in phosphate-buffered saline (PBS) to filamentous actin as determined by gel filtration on a AcA-44 column.

Intravenous injection of labelled protein

^{125}I -TC-labelled actin, DBP or DBP-actin complex were injected intravenously into rats through the femoral vein under barbitol anaesthesia. About 100–500 pmol of the different proteins was injected intravenously. Blood samples (20–120 μl) were collected from a tail vein at different time points (1 min to 24 h) after injection. The rats were killed after between 10 min and 24 h, after which blood and different organs were collected. Liver and kidneys were rinsed twice in ice-cold PBS. The amount of radioactivity recovered in blood was calculated on the assumption that blood amounts to 6% of total rat body weight [20]. Radioactivity was counted in a Kontron Automatic Gamma Counter System MR 252.

Isolation of liver cells

Isolated rat liver cells were obtained by collagenase perfusion. Liver parenchymal and non-parenchymal cells, including endothelial and Kupffer cells, were then separated by centrifugal elutriation using a Beckman JE-6 rotor [21]. The viability of parenchymal cells determined by Trypan Blue uptake was $95 \pm 2\%$. Parenchymal, endothelial and Kupffer cells were isolated 1 h after intravenous injection of labelled actin, DBP or DBP-actin complex into rats.

Incubation of isolated liver cells with ^{125}I -labelled actin

Isolated parenchymal and non-parenchymal cells were incubated as previously described [22]. Labelled and unlabelled filamentous actin dissolved in PBS was added to the medium and incubated at 37°C or 4°C for different periods of time. In some experiments 10 μM -monensin (dissolved in 20 μl of ethanol), 5 mM-EGTA, asialofetuin (10 $\mu\text{g}/\text{ml}$), dextran sulphate (5 $\mu\text{g}/\text{ml}$) or mannan (1 mg/ml) was added at the same time as labelled actin.

Trichloroacetic acid precipitation

Radioactive material recovered in blood and different tissues was separated into trichloroacetic acid-precipitable (protein-bound) and trichloroacetic acid-soluble material (degraded products) using a final trichloroacetic acid concentration of 12.5% (w/v) [14].

RESULTS

Rabbit actin, rat DBP or actin-DBP complex labelled with ^{125}I -TC was injected intravenously into rats. At 1 min after administration of radiolabelled actin, $15.4 \pm 1.1\%$ (mean $\pm$ S.D.; $n = 4$) of the injected radioactivity was recovered in the blood. The amount of labelled actin detected in the blood decreased to about 3% by 5 min after injection (Fig. 1). In comparison, when labelled DBP or DBP-actin complex was injected intravenously, $45.2 \pm 11.1\%$ ($n = 4$) and $9.5 \pm 1.5\%$ ($n = 3$) respectively of the radioactivity was recovered in the blood 5 min after injection. The decay curves of labelled DBP-actin complex and labelled DBP in the circulation were parallel (results not shown).

Injected ^{125}I -TC-labelled actin was rapidly taken up by the liver. At 10 min after injection, $38.7 \pm 3.8\%$ ($n = 3$) of the administered dose was recovered in liver. There was no further increase in the hepatic radioactivity between 10 and 30 min ($41.4 \pm 2.9\%$, $n = 3$) after administration of labelled actin. Although the total amount of radioactivity in the liver from 10

Fig. 1. Clearance from the plasma of ^{125}I -TC-labelled filamentous actin

Actin was injected intravenously into rats and blood samples were collected between 1 and 30 min after injection. The calculation of radioactivity in blood was based on the assumption that blood amounts to 6% of rat body weight [20]. The results are from four rats and given as percentages of total injected radioactivity recovered in the blood.

to 30 min after injection was unchanged, the amount of radioactivity recovered as degraded material (trichloroacetic acid-soluble) increased from $13.5 \pm 3.5\%$ to $33.1 \pm 2.3\%$ during this time period. In contrast with actin, only $9.9 \pm 1.1\%$ of labelled DBP was recovered in the liver 10 min after administration. At this time point, hepatic degradation products of ^{125}I -TC-labelled DBP accounted for only 1% of the injected dose. The percentage of injected radioactivity recovered in the liver as trichloroacetic acid-soluble material 10 min after injection was about 15 times higher when labelled actin was injected as compared with labelled DBP. Actin was also taken up by the spleen at a higher rate than was DBP ($2.6 \pm 0.7\%$ and $0.6 \pm 0.1\%$ of injected dose respectively). At 10 min after administration of actin and DBP, about

Fig. 2. Recovery of ^{125}I -TC-labelled DBP, actin and DBP-actin in various liver cell types

Radioactivity was recovered in different types of liver cells 60 min after intravenous injection of ^{125}I -TC-labelled DBP ($\square$), actin ($\square$) or DBP-actin complex ($\blacksquare$). Total radioactivity recovered in the liver was taken as 100%, representing about 100–250 pmol of the different proteins injected. The calculation was based on the percentage of radioactivity recovered in the liver per 10^6 cells. Data are given as means $\pm$ S.D. from 3–4 rats.

Fig. 3. Distribution of total (a) and trichloroacetic acid-soluble (b) radioactivity in liver cell types after injection of ^{125}I -TC-labelled DBP, actin or DBP-actin

The distributions of ^{125}I -TC-labelled DBP (□), actin (▨) and DBP-actin (■) were measured 60 min after injection. The total amounts of radioactivity recovered in liver of the different labelled proteins injected was taken as 100%. Calculation of radioactivity recovered was based on the assumption that rat liver parenchymal, endothelial and Kupffer cells amount to 65, 19 and 10% of the total number of liver cells respectively [35]. Data are given as means $\pm$ S.D. from 3–4 rats.

17% and 7% respectively of the recovered radioactivity in the spleen was trichloroacetic acid-soluble. In contrast with the preferential accumulation of actin in liver and spleen, less than 1% of the injected dose of labelled actin was recovered in the kidneys. In comparison, $1.5 \pm 0.1\%$ and $6.1 \pm 1.8\%$ of injected radioactivity was recovered in the kidneys at 10 and 30 min after injection of labelled DBP.

Hepatic uptake and degradation were further elucidated by studying the different cell types responsible for metabolism *in vivo* of ^{125}I -TC-labelled actin, ^{125}I -TC-labelled DBP and ^{125}I -TC-labelled DBP-actin. Liver cells were isolated 60 min after intravenous injection of labelled proteins. The different TC-labelled proteins were taken up and degraded in all of the different cell types studied (Figs. 2 and 3). On a per cell basis, Kupffer and endothelial cells took up and degraded more of all of the labelled proteins compared with hepatocytes (Fig. 2). When the total number of hepatocytes, endothelial cells and Kupffer cells in the liver was taken into consideration, most of the radioactivity was detected in the Kupffer cells after injection of labelled DBP (Fig. 3a). This cell type contributed $56.6 \pm 4.8\%$ ($n = 4$) and $25.4 \pm 4.9\%$ ($n = 3$) of total radioactivity of labelled DBP and actin respectively recovered in liver (Fig. 3a). When

labelled actin was injected, most of the recovered radioactivity was detected in endothelial cells ($40.1 \pm 4.2\%$, $n = 3$) and parenchymal cells ($34.6 \pm 8.3\%$, $n = 3$) (Fig. 3a). Injection of radiolabelled DBP-actin complex resulted in a distribution of activity similar to that obtained when labelled actin was injected. When the complex was administered, endothelial and parenchymal cells accounted for $46.9 \pm 10.6\%$ ($n = 3$) and $37.0 \pm 9.1\%$ ($n = 3$) respectively of the radioactivity recovered in the liver (Fig. 3a).

The accumulated radioactivity in different liver cells was separated into trichloroacetic acid-soluble and -precipitable material. The cell types containing most of the radioactivity after injection of the different labelled proteins also contained the majority of degraded material (Fig. 3b). The percentage of recovered radioactivity in different liver cells detected as trichloroacetic acid-soluble products varied with the cell type studied and the labelled protein injected. The highest percentage of recovered radioactivity as trichloroacetic acid-soluble material was detected in Kupffer cells, where approx. 70% of radioactivity was trichloroacetic acid-soluble 60 min after actin was injected. In contrast with this finding, only 43% of the radioactivity recovered in hepatocytes after labelled DBP was injected was trichloroacetic acid-soluble material.

Binding, uptake and degradation of filamentous actin was also studied in isolated cell suspensions of rat hepatocytes and non-parenchymal cells. Binding studies were performed at 4°C and the mean value of ^{125}I -TC-labelled actin that was bound to both hepatocytes and non-parenchymal cells increased with incubation time up to 30–60 min (Fig. 4). Isolated hepatocytes and non-parenchymal cells (about 5×10^6 cells/ml) were incubated with increasing concentrations (1–1000 $\mu\text{g}/\text{ml}$) of actin for 30 min at 4°C . The amount of actin that was bound to the hepatocytes, as well as to the non-parenchymal cells, increased linearly with increasing concentration of the ligand up to 1000 $\mu\text{g}/\text{ml}$ (Fig. 5).

Labelled actin was also incubated with different liver cells at 37°C . At this temperature the amount of labelled actin that was associated with hepatocytes and non-parenchymal cells increased during the first 15–30 min (Fig. 6a). At 37°C , trichloroacetic acid-soluble radioactivity was detected in both cell populations after a lag period of about 10 min. The amount of trichloroacetic acid-soluble activity in both cell types increased linearly with time from 10 to 60 min (Fig. 6a).

^{125}I -TC-labelled actin was incubated with parenchymal and

Fig. 4. Time course of binding of ^{125}I -TC-labelled filamentous actin to isolated parenchymal (○) and non-parenchymal (●) rat liver cells

Labelled filamentous actin (3 $\mu\text{g}/\text{ml}$) was added to the cells and incubated at 4°C . Results are presented from one experiment which was representative of five. Data are given as means of duplicate determinations.

Fig. 5. Concentration-dependence of binding of ^{125}I -TC-labelled filamentous actin to parenchymal (○) and non-parenchymal (●) rat liver cells

Parenchymal and non-parenchymal cells were incubated with increasing amounts of filamentous actin for 30 min at 4 °C. Data are given as means $\pm$ S.D. of triplicate determinations from one experiment out of four which gave similar results.

Fig. 6. Cell-associated total (a) and trichloroacetic acid-soluble radioactivity (b) in parenchymal (○) and non-parenchymal (●) cells after incubation with ^{125}I -TC-labelled filamentous actin for different periods of time at 37 °C

Data are given as means $\pm$ S.D. of triplicate determinations from one out of four experiments.

non-parenchymal rat liver cells at 37 °C for 30 min in the presence of 10 μM -monensin, 5 mM-EGTA, asialofetuin (10 $\mu\text{g}/\text{ml}$), dextran sulphate (5 $\mu\text{g}/\text{ml}$) or mannan (1 mg/ml). In experiments with hepatocytes, no change in the amount of cell-associated radioactivity was observed after addition of the different

substances, although monensin decreased the degradation of actin to a mean of 29 % of the control value.

When similar experiments were performed with non-parenchymal cells, dextran sulphate lowered the amount of labelled actin that was cell-associated to a mean of 42 % of the control value. The other substances tested had no effect on the cell-associated radioactivity. Monensin and dextran sulphate decreased the amount of trichloroacetic acid-soluble radioactivity in non-parenchymal cells to 21 and 25 % respectively of that in control incubations. The other substances were without effect on the amount of trichloroacetic acid-soluble activity.

DISCUSSION

In this paper we report data showing that rats have a mechanism for rapidly removing filamentous actin from the circulation. Clearance of actin from the circulation was very rapid (Fig. 1) and markedly faster than clearance of DBP [14]. Furthermore, we have shown that a large amount of the cleared actin accumulates in the liver, and that parenchymal as well as non-parenchymal cells are responsible for the uptake and degradation both *in vivo* and *in vitro* (Figs. 2, 3a, 6a and 6b). Lind *et al.* [8] and Goldschmidt-Clermont *et al.* [9] have shown that, in the rabbit, DBP is quantitatively the most important of the actin-binding proteins present in plasma when considering clearance of filamentous actin from the circulation.

Conflicting results have been published concerning plasma clearance of the DBP-actin complex compared with the clearance of DBP. Lind *et al.* [8] and Goldschmidt-Clermont *et al.* [9] have reported increased plasma clearance of DBP-actin complex compared with DBP in the rabbit. Along these lines, we have previously shown that, in the rat, less of labelled ^{125}I -TC-labelled DBP-actin complex was recovered in plasma at 30 min, 5 h and 24 h after injection compared with when ^{125}I -TC-labelled DBP was injected [14]. This increased removal of labelled DBP-actin complex compared with labelled DBP was due to increased uptake of the complex in the liver [14]. In contrast with these findings, Harper *et al.* [23] reported that the DBP-actin complex and DBP were cleared from rat plasma at similar rates. In all of these studies the plasma decay curves were based on the amount of radioactivity recovered in plasma 2–10 min after the intravenous injection of the labelled proteins, and DBP-actin was labelled in the DBP moiety of the complex [8,9,23]. Plasma contains 6–10 μM -DBP [18,24], or about 50–80 nmol in a 250 g rat. We injected about 0.5 nmol of ^{125}I -TC-labelled DBP as a DBP-actin complex, representing about 1 % of the total plasma DBP. The large excess of unlabelled DBP as compared with labelled DBP-actin might result in the transfer of actin from labelled DBP to unlabelled DBP *in vivo*, resulting in formation of ^{125}I -DBP. This may explain the parallel (results not shown) and identical plasma decay curves observed when labelled DBP and labelled DBP-actin complex were injected [8,9,23].

The distribution of actin among the various liver cells was different from that of DBP. Most of the labelled actin and DBP-actin complex recovered in the liver cells was detected in endothelial and parenchymal cells, whereas the majority of labelled DBP was recovered in the Kupffer cells (Figs. 2 and 3). When filamentous actin is injected intravenously, most of the actin recovered in the circulation is bound to DBP [6–9]. Our results suggest that actin is targeting the DBP-actin complex to endothelial and parenchymal liver cells (Figs. 2 and 3). This targeting results in an increased plasma clearance and in increased hepatic uptake and degradation of the DBP moiety of the DBP-actin complex, as compared with degradation of DBP alone [14].

Only 1–3 % of DBP in the circulation is occupied by vitamin

D metabolites, resulting in a huge molar excess of the binding protein compared with its ligands [18,24]. Despite a worldwide screening, no plasma without DBP has been reported, suggesting that deletion of DBP is a lethal mutation [25]. In contrast, analbuminaemia has been described [26]. This might suggest that DBP has a crucial physiological function, and one important function of DBP may be the ability to form a complex with actin whenever actin is present in plasma, thereby inhibiting potentially harmful filamentous actin from building up in the circulation. It has been reported that filamentous actin will interfere with the coagulation process [27], and this might be prevented by interaction with DBP.

Liver parenchymal cells contain plasma membrane receptors which are involved in uptake of different ligands [28–31], whereas hepatic endothelial cells contain a scavenger receptor known to bind acetylated low-density lipoproteins and formaldehyde-treated serum albumin [32]. This receptor is involved in clearing denatured and modified serum proteins from plasma. A receptor that recognizes proteins with a terminal mannose moiety has been reported on endothelial cells as well as on parenchymal cells of the liver [33].

The distribution of TC-labelled filamentous actin between different liver cells was compared with distributions of proteins known to be cleared by different receptors. Our *in vivo* as well as *in vitro* data indicate that there must be a mechanism involved in hepatic uptake of filamentous actin in addition to the scavenger receptor, for the following reasons. (1) The uptake of actin in different liver cells both *in vivo* and *in vitro* was different from that of proteins known to be cleared by a scavenger receptor. (2) Goldstein *et al.* [34] have shown that uptake of ligands via the scavenger receptor is inhibited by dextran sulphate. Our results show only a partial decrease in the amount of cell-associated and degraded filamentous actin in non-parenchymal cells *in vitro* in the presence of dextran sulphate.

No saturation of binding of filamentous actin to parenchymal and non-parenchymal cells was observed (Fig. 5). An explanation for the lack of saturation of the binding *in vitro* might be that increasing the concentration of actin in the medium will result in an increased length of the filamentous actin chain formed. In this way the same number of receptors may bind an increasing amount of actin molecules, since only the chain length of the filamentous actin is increased and not the number of actin chains. However, our data do not exclude the possibility that some actin may be taken up via a scavenger receptor in non-parenchymal cells, especially at a low concentration of filamentous actin. The decreased catabolism of actin in the presence of monensin and the delay in the formation of trichloroacetic acid-soluble material in liver cells (Fig. 6b) suggest that degradation of actin takes place after internalization of the protein.

In conclusion, filamentous actin is rapidly cleared from the circulation, mainly by the endothelial and parenchymal cells of the liver. When the DBP-actin complex is present in the circulation, actin is responsible for the rapid removal of the complex by targeting the complex to the endothelial and parenchymal liver cells. Although no conclusion can be drawn on whether specific receptors are involved in clearance of filamentous actin from plasma, our data indicate that the uptake of actin by the liver cannot be explained solely by uptake via the scavenger receptor.

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