Comparison of cadmium-metallothionein synthesis in parenchymal and non-parenchymal rat liver cells

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The time course of cadmium-metallothionein synthesis was studied in non-parenchymal and parenchymal cells, isolated by a cell-separation technique from the livers of rats after the simultaneous injection of CdCl_2 (0.05 mg of Cd/kg) and a 10-fold molar excess of 2,3-dimercaptopropanol. Under these conditions of dosing, in contrast with the injection of CdCl_2 alone, both cell types accumulate similar concentrations of Cd and synthesize equivalent concentrations of metallothionein. It is concluded that both cell types have a similar capacity to synthesize the metalloprotein, and that the limiting factor under normal cadmium exposure is the relatively inefficient metal uptake into the non-parenchymal cells.

Cadmium is a highly toxic metal that accumulates predominantly in the liver and the kidney (for review see Webb, 1979). We have shown previously that, in the livers of Cd-dosed rats, 90% of the hepatic cadmium is located in the parenchymal-cell population bound to the inducible metalloprotein metallothionein (Cain & Skilleter, 1980). Less than 10% of the liver cadmium burden was located in the non-parenchymal-cell population and, although the cell cytosol contained a low concentration of a soluble cadmium-binding protein, we were unable to characterize this unequivocally as a metallothionein (Cain & Skilleter, 1980). Sciortino *et al.* (1982), however, by using a highly sensitive '*in vitro*' ^{109}Cd binding technique, were able to identify the non-parenchymal-cell cytosol protein as metallothionein. The low concentration of this protein in the non-parenchymal cells could be due to a poor capacity of these cells to synthesize metallothionein, or alternatively, a poor ability to take up cadmium. In support of the latter, our recent '*in vitro*' studies with primary cell cultures of non-parenchymal cells have demonstrated that the rate of cadmium uptake in these cells is about one-tenth that observed for parenchymal cells (Skilleter & Cain, 1981). The present work was designed to determine whether or not parenchymal and non-parenchymal cells have similar abilities to synthesize metallothionein under conditions of comparable cadmium loading. These conditions were achieved by the use of a previously described '*in vivo*' technique (Skilleter & Cain, 1981), in which cadmium accumulation in non-

parenchymal cells can be markedly enhanced and the cadmium content of parenchymal cells decreased, by the administration of the cation as a Cd–2,3-dimercaptopropanol complex. The results demonstrate that the capacity for metallothionein synthesis in the different hepatic cell populations is limited predominantly by the efficiency of cadmium uptake.

Materials and methods

Materials

Carrier-free $^{109}\text{CdCl}_2$ (specific radioactivity 21.4 MBq/ml, $1\text{ }\mu\text{g}$ of Cd^{2+} /ml) was obtained from Amersham International, Amersham, Bucks., U.K. All chemicals were obtained from the sources detailed previously (Cain & Skilleter, 1980; Skilleter & Cain, 1981).

Animal injections

Male Wistar LAC/P rats ($190 \pm 5\text{ g}$) were injected intravenously (0.1 ml/100 g via a tail vein) with 0.05 mg of $^{109}\text{CdCl}_2/\text{kg}$ complexed with a 10-fold molar excess of 2,3-dimercaptopropanol ('complexed' means that the metal and dithiol are mixed in the stated ratio; the exact nature of the complex is unknown). Injection solutions were prepared by mixing a solution of $\text{CdCl}_2 \cdot 2\frac{1}{2}\text{ H}_2\text{O}$ (labelled with $^{109}\text{CdCl}_2$, final specific radioactivity approx. 30 000 c.p.m./ng-atom of Cd) in glycerine/formal

(glycerol/dimethoxymethane) (3:1, v/v) with an equal volume of 2,3-dimercaptopropanol dissolved in the same solvent. The injection solution was used immediately after mixing.

Isolation of parenchymal and non-parenchymal cells

At the indicated times after the Cd/dithiol injections, rats were anaesthetized with 7.5 mg of Sagatal (sodium pentobarbital)/100 g body weight, and liver parenchymal and non-parenchymal cells isolated by a collagenase (Sigma Type I) liver perfusion based on a technique reported previously (Skilleter & Price, 1978; Cain & Skilleter, 1980). The method was that described by Seglen (1973) as far as the preparation of the initial liver-cell suspension. At this stage cells were transferred to a 100-ml measuring cylinder and the volume adjusted to 100 ml with the Seglen suspension buffer. The cells were allowed to settle under gravity for 15–20 min, after which the parenchymal cells formed a distinct lower layer. The upper layer, which contained predominantly non-parenchymal cells, was carefully siphoned and given two consecutive centrifugations (50 g for 1 min) to remove contaminating parenchymal cells. The non-parenchymal cells were then sedimented by centrifugation (500 g for 3 min), followed by one further resuspension (60 ml) and centrifugation (500 g for 3 min). The parenchymal-cell fraction was purified by one initial centrifugation (50 g for 1 min), followed by two further resuspensions (40 ml) and centrifugations (50 g for 1 min) as described by Seglen (1973). For the treated rats these procedures gave a final yield of $(80\text{--}140) \times 10^6$ parenchymal cells (85–90% viable) containing only 2–4% non-parenchymal cells and $(30\text{--}60) \times 10^6$ non-parenchymal cells (>95% viable) containing <2% parenchymal cells.

Isolation of metallothionein

Cytosol fractions were prepared from both cell types by resuspension of the cells in 6 ml of 10 mM-ammonium formate buffer, pH 8.0, and sonication at maximum power for 1 min in a Dawe Instruments (London W.3, U.K.) sonicator. The resulting disrupted cell suspension was centrifuged at 40 000 rev./min (100 000 g, r_{av} 5.8 cm) in a Beckman 65 rotor for 1 h. The supernatant solution ('cytosol') was then analysed for metallothionein by Sephadex G-75 filtration as described by Cain & Holt (1979) on a column (80 cm $\times$ 1.5 cm) equilibrated with 10 mM-ammonium formate buffer, pH 8.0, operated at a flow rate of 7–10 ml/h (fraction volume 3 ml). In one experiment the non-parenchymal cells from three livers were pooled and the cytosol prepared as described above. This was then chromatographed on Sephadex G-75 and those fractions corresponding to the metallothionein peak

(V_e/V_0 ratio of 1.8–2.0) pooled and fractionated on a column (30 cm $\times$ 1.5 cm) of DE-52 DEAE-cellulose equilibrated with 10 mM-ammonium formate buffer, pH 8.0. The column was then eluted with a linear gradient of 10–200 mM-ammonium formate buffer, pH 8.0 (400 ml, fraction volume 2.8 ml).

Analytical techniques

^{109}Cd was assayed in a Packard model 5330 Auto Gamma spectrometer. With column fractions, the total volume was counted for radioactivity. Cell viability and numbers were assayed as described previously (Cain & Skilleter, 1980).

Results and discussion

Cadmium is rapidly cleared from the blood after an intravenous injection, and a large proportion of the dose is taken up by the liver (Decker *et al.*, 1957; Perry & Erlanger, 1971). Previously we showed that this hepatic accumulation is selective, and, 30 min after the injection of a wide range of cadmium doses by several routes, the major hepatic cadmium burden is located in the parenchymal cells (Cain & Skilleter, 1980). In the present study, the cadmium dose (0.05 mg of Cd/kg), when administered alone, produced a similar pattern of uptake, and the parenchymal and non-parenchymal cells contained 44 and 7 pg-atoms of Cd/ 10^6 cells respectively. In apparent contrast, Sciortino *et al.* (1982) in similar experiments found that the parenchymal-cell metallothionein content was only 2.5 times that of the non-parenchymal cells. As virtually all of the cadmium burden of the liver is bound to thionein (for review see Webb, 1979) this would indicate that the cadmium content of the parenchymal cell is also only 2.5-fold greater than the non-parenchymal cell, a result which apparently contradicts our findings. However, the results of Sciortino *et al.* (1982) are expressed in terms of cell protein and therefore do not allow for the approx. 10-fold greater protein content of parenchymal cells relative to non-parenchymal cells (Munthe-Kaas *et al.*, 1975; Knook & Sleyster, 1980; Skilleter & Price, 1981). When this and the greater number of parenchymal cells in the liver (Weibel *et al.*, 1969) are taken into account, it can be calculated that, in the studies of Sciortino *et al.* (1982), between 90 and 95% of the hepatic metallothionein pool is located in the parenchymal-cell population, a value that is then in good agreement with our findings and others (Danielson *et al.*, 1982).

The administration of cadmium as a complex with 2,3-dimercaptopropanol significantly alters the hepato-cellular distribution of the cation, probably as a result of the behaviour of the complex as a colloid (Skilleter & Cain, 1981). In our previous studies *in vivo* the complex was injected in the presence of saline, which gave a marked initial

Table 1. ¹⁰⁹Cd content in parenchymal and non-parenchymal cells of the liver at various times after the administration of a ¹⁰⁹Cd-2,3-dimercaptopropanol complex

Rats received 0.05 mg of Cd/kg body weight as a ¹⁰⁹Cd-dimercaptopropanol complex (1:10) dissolved in glycerine/formal. At the indicated times, animals were anaesthetized and liver parenchymal and non-parenchymal cells were isolated and assayed for ¹⁰⁹Cd and cell content.

Time after injection	¹⁰⁹ Cd content of cell fraction (pg-atoms of Cd/10 ⁶ cells)	
	Parenchymal cells	Non-parenchymal cells
30 min	29.0	18.42
1 day	26.5	15.6
2 days	28.56	22.99
10 days	21.75	26.18
16 days	24.69	12.37

increase in the cadmium content of the non-parenchymal cells compared with the injection of cadmium alone. Subsequently, we have shown that this is followed by a sharp decrease of approx. 50% between 1 and 2 days, probably owing to cell death resulting from the cytotoxic action of cadmium (Skilleter & Cain, 1981). Table 1 illustrates the time course of cadmium uptake into parenchymal and non-parenchymal cells when the cadmium-dithiol complex was dissolved in glycerine/formal alone and illustrates a rapid cadmium accumulation by both cell types. Further, the results show that, under these conditions, initial cadmium uptake by the non-parenchymal cells was comparable with that of the parenchymal cells, and no rapid loss of cellular cadmium was seen, indicating the absence of marked cell death in either cell population during the period of study. The difference in the cadmium loading of the non-parenchymal cells under the two different injection procedures is not clearly understood. In the present study, injections in glycerine/formal alone were preferred, since they resulted in approximately equivalent cadmium concentrations in the two cell types, and thus metallothionein accumulation/synthesis could be compared under similar cadmium-loading conditions.

Fig. 1(a) shows Sephadex G-75 elution profiles obtained from the cytosols of parenchymal cells at 30 min, 1 day and 16 days after the injection of the Cd-dithiol complex. At 30 min most of the soluble cadmium was bound to the high-molecular-weight proteins that were eluted in the void volume of the column; the remaining cadmium (10%) was associated with a fraction eluted at a V_e/V_0 ratio of

Fig. 1. Metallothionein accumulation in rat liver parenchymal cells at various times after the administration of 0.05 mg of ¹⁰⁹CdCl₂/kg complexed with a 10-fold molar excess of 2,3-dimercaptopropanol. Cell cytosols were prepared as described in the Materials and methods section from the cell suspensions isolated and described in Table 1. A 6 ml portion of each cytosol (approximately equivalent to 80 × 10⁶ cells) was chromatographed on a column (80 cm × 1.5 cm) of Sephadex G-75 at a flow rate of 7–10 ml/h and with a fraction volume of 3 ml. Each fraction was analysed for ¹⁰⁹Cd in a Packard Auto Gamma spectrometer. Typical elution profiles are shown in (a); the high-molecular-weight proteins eluted in the void volume of the column are indicated as HMW and the metallothionein fractions ($V_e/V_0 = 1.8–2.0$) as MT. The time course of ¹⁰⁹Cd binding to the HMW (▲) and MT (●) fractions is shown in (b).

1.8–2.0 and corresponds to the metallothionein peak (Cain & Holt, 1979; Cain & Skilleter, 1980). The height of this peak increased with time, and 24 h after the injection accounted for nearly 80% of the cytosolic cadmium. Fig. 1(b) shows that the concentration of metallothionein was maximal at 2 days, when approx. 90% of the soluble cadmium was

bound to metallothionein; the residual cytosolic cadmium was eluted with the high-molecular-weight proteins. Thereafter there was a loss of metallothionein-bound cadmium, some of which was excreted from the liver and corresponded to the small decrease in the total metal content of the cells (Table 1). Most of the released cadmium, however, became bound to the high-molecular-weight proteins, and thus by 16 days the cytosolic cadmium was equally distributed between the metallothionein and high-molecular-weight proteins. The increase in metallothionein-bound cadmium in the first 48 h after the injection of the Cd-dithiol complex is consistent with studies on the time course of metallothionein synthesis in the whole liver after CdCl_2 injection. Cempel & Webb (1976), for example, showed that initially there is a lag phase of 3–4 h between cadmium uptake and the onset of induced metallothionein synthesis. During this period the cytosolic cadmium is bound predominantly to the high-molecular-weight proteins, until the initiation of thionein synthesis results in the progressive transfer of the cation to the higher-affinity binding sites of the newly synthesized thionein. The latter can be clearly demonstrated by comparing the ratio of metallothionein to high-molecular-weight-protein-bound cadmium (MT/HMW). In the studies of Cempel & Webb (1976), at 4 h the ratio is 0.3 and at 24 h, when thionein synthesis is near the maximum, the ratio increased almost 30-fold to 8.5. Similar results were found in the present study, in which for parenchymal cells the ratio increased from 0.11 at 30 min to 6.49 at 48 h. This shows that the time course of cadmium-thionein synthesis in the parenchymal cells after an injection of cadmium–2,3-dimercaptopropanol is identical with that observed in the whole liver after CdCl_2 injection. From day 2 to day 16, however, thionein synthesis was not maintained and the MT/HMW ratio rapidly declined to 1.11 at 16 days, presumably owing to thionein degradation. The loss of thionein-bound cadmium was an unexpected finding, as several workers have shown that the cation is liberated by degradation and then rebinds to newly synthesized apo-thionein (Chen *et al.*, 1975; Shaikh & Smith, 1976; Oh *et al.*, 1978; Cain & Holt, 1979). Thus the cadmium-thionein content of the liver normally remains constant even though the protein moiety is continually turning over. In the present study, however, the cell cadmium content was low, and it is probable that the quantity of metal released by metallothionein degradation did not exceed the threshold concentration necessary to initiate further synthesis (Squibb & Cousins, 1974; Webb, 1979).

Fig. 2(a) shows the Sephadex G-75 elution profiles of cytosols isolated from the non-parenchymal cells at 30 min, 1 day and 16 days after the administration of the metal-dithiol complex.

Fig. 2. Metallothionein accumulation in rat liver non-parenchymal cells at various times after the administration of 0.05 mg of $^{109}\text{CdCl}_2/\text{kg}$ complexed with a 10-fold molar excess of 2,3-dimercaptopropanol

Cells cytosols were prepared as described in the Materials and methods section from the cell suspensions isolated and described in Table 1. A 6 ml portion of each cytosol (approximately equivalent to 20×10^6 cells) was chromatographed on a column ($80\text{ cm} \times 1.5\text{ cm}$) of Sephadex G-75 as described in Fig. 1. Typical elution profiles are shown in (a) and the time course of ^{109}Cd binding to the high-molecular-weight-protein (HMW) ($\blacktriangle$) and metallothionein (MT) ($\bullet$) fractions is shown in (b).

After 30 min, 25% of the cellular cadmium was located in the cytosol, and the Sephadex G-75 elution profile showed that most of the metal was bound to the high-molecular-weight proteins (80%). At 24 h, 80% of the non-parenchymal-cell cadmium burden was found in the cytosol, and of this, over 80% was bound to the metallothionein fraction eluted at a V_e/V_0 ratio of 1.8–2.0. Fig. 2(b) shows that the latter reached a maximum at 2 days and thereafter slowly decreased with time. In contrast with the parenchymal cells, the high-molecular-weight-protein-bound cadmium remained constant.

These results demonstrate that the time course of synthesis of the metallothionein-like protein isolated from the non-parenchymal cells is very similar to

Fig. 3. Anion-exchange chromatography of metallothionein isolated from the cytosol of non-parenchymal cells

Three rats were injected with 0.05 mg of $^{109}\text{CdCl}_2/\text{kg}$ complexed with a 10-fold molar excess of 2,3-dimercaptopropanol complex, and 48 h later the liver non-parenchymal cells were isolated as described in Fig. 2. Non-parenchymal cells from the three animals were pooled, and the cell cytosol isolated and chromatographed on Sephadex G-75 as described in Fig. 2. Those fractions corresponding to the metallothionein peak were pooled and chromatographed on a column (30 cm $\times$ 1.5 cm) of DE-52 DEAE-cellulose anion-exchange resin equilibrated in 10 mM-ammonium formate buffer, pH 8.0, which was then eluted with a linear gradient of 10–200 mM-ammonium formate buffer at a flow rate of 30 ml/h (fraction volume 2.8 ml). All fractions were analysed for ^{109}Cd , and the elution profile (▲) is shown above with the broken line (----) indicating the buffer gradient. MT1 and MT2 refer to the isometallothioneins.

that described for metallothionein synthesis in the parenchymal cells. The identification of the non-parenchymal-cell ^{109}Cd -binding peak as metallothionein was confirmed by anion-exchange chromatography. Fig. 3 shows the anion-exchange analysis of the Sephadex-G-75 metallothionein peak prepared from the non-parenchymal-cell cytosol 48 h after the rat had received the cadmium–dithiol injection. Two main ^{109}Cd -binding peaks were detected, corresponding to the isometallothioneins MT1 and MT2. In addition a third peak was detected that was eluted between MT1 and MT2. A similar peak was observed for metallothionein isolated from parenchymal cells (Cain & Skilleter, 1980), although this is not normally seen in metallothionein preparations isolated from the intact liver by conventional methods (see, for example, Cain & Holt, 1979). Furthermore, Fig. 3 shows that all the peaks were less well resolved and more heterogeneous than is normally expected for rat liver metallothionein. In this context, other workers (Sciortino *et al.*, 1982) have also compared the anion-exchange elution profiles of metallothionein preparations isolated from intact rat liver, parenchymal and non-parenchymal cells and ob-

served elution profiles with dispersed peaks on anion-exchange chromatography, incorporating a third peak of cadmium binding. These differences between the isolated cells and intact liver are probably due to artefacts produced by the cell-isolation and -purification procedures.

In conclusion, both parenchymal and non-parenchymal cells have a similar ability to synthesize metallothionein. However, under normal conditions, cadmium uptake in non-parenchymal cells is low and this limits the extent of metallothionein synthesis. A similar situation has been reported for peritoneal macrophages (Cox & Waters, 1978), and it is probable that all cells have a similar capacity for metallothionein synthesis, but not necessarily the same capacity for metal uptake.

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