Cadmium metabolism by rat liver endothelial and Kupffer cells

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The metabolism of cadmium was investigated in Wistar-rat liver non-parenchymal cells. Kupffer and endothelial cells, the major cell populations lining the sinusoidal tracts, were isolated by collagenase dispersion and purified by centrifugal elutriation. At 20 h after subcutaneous injection of the metal salt (1.5 mg of Cd/kg body weight), endothelial cells accumulated 2-fold higher concentrations of Cd than did Kupffer or parenchymal cells. Most of the Cd in non-parenchymal cells was associated with cytosolic metallothionein (MT), the low- M_r heavy-metal-binding protein(s). When MT was quantified in cytosols from cells isolated from control rats by a ^{203}Hg competitive-binding assay, low levels were found to be present in Kupffer, endothelial and parenchymal cells. Cd injection significantly increased MT levels in all three cell types. The induction of MT synthesis was investigated *in vitro* by using primary monolayer cultures. The incorporation of [^{35}S]cysteine into MT increased 47% over constitutive levels in endothelial-cell cultures after the addition of $0.8\text{ }\mu\text{M}$ - Cd^{2+} to the medium for 10 h. MT synthesis in Kupffer cells was not observed. The lack of MT synthesis by monolayer cultures of Kupffer cells *in vitro* was associated with a decreased capacity of these cells to accumulate heavy metals from the extracellular medium. This apparent decreased ability to transport metals did not reflect a general defect in either cellular function or metabolic activity, since isolated Kupffer cells incorporated [^3H]leucine into protein at rates comparable with those shown by liver parenchymal cells and readily phagocytosed particles.

The central importance of the liver in the metabolism of essential and toxic heavy metals is well documented. Since the liver is composed of morphologically and functionally distinct classes of cells, the characteristics of heavy-metal metabolism by each cell type are of particular interest. Cellular transport and metabolism have been studied in the most detail with liver parenchymal cells, commonly referred to as hepatocytes, since they account for 90% of the liver cell mass (Blouin *et al.*, 1977) and are easy to isolate in high yields. Exposure of primary monolayer and suspension cultures of parenchymal cells to elevated levels of zinc (Failla & Cousins, 1978*a,b*; Karin *et al.*, 1980), copper (Weiner & Cousins, 1980) and cadmium (Hildalgo *et al.*, 1978; Failla *et al.*, 1979; Stacey &

Klaassen, 1980; Gerson & Shaikh, 1982) increases the cellular concentration of these metals and their cysteine-rich binding protein, metallothionein (MT). We have previously reported increased MT concentrations in both rat liver parenchymal and non-parenchymal cells after administration *in vivo* of zinc and cadmium salts (Sciortino *et al.*, 1982). Hepatic non-parenchymal cells line the sinusoidal regions. Consequently, the capacity of these cells to store and detoxify heavy metals delivered from the portal circulation may serve a critical function in hepatic trace-metal homeostasis. In the present work we have isolated the two major classes of hepatic non-parenchymal cells, namely endothelial and Kupffer cells, by centrifugal elutriation, in order to study their accumulation and metabolism of Cd *in vivo* and *in vitro*.

Materials and methods

Animals. Male Wistar rats (250–500 g) from Charles River Breeding Laboratories (Cambridge,

Abbreviations used: MT, metallothionein; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; Tes, 2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]-ethanesulphonic acid.

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MA, U.S.A.) were housed in suspended stainless-steel cages on a 12h light/12h dark cycle. Commercial rodent chow (Ralston Purina 5001; St. Louis, MO, U.S.A.) and deionized distilled water were provided *ad libitum*.

Cell isolation

Liver cells were isolated by a modification of the collagenase-perfusion technique of Berry & Friend (1969). Details of the procedure have been published previously (Failla & Cousins, 1978a) and only additional steps are outlined. After perfusion with collagenase, cells were released from the matrix with a plastic spatula into tissue-culture medium M199 (medium 199; Flow Laboratories, Dublin, VA, U.S.A.). The remaining liver matrix was incubated in medium M199 containing 0.05% collagenase (CLS II; Worthington Enzymes, Fairfield, NJ, U.S.A.) and 0.01% deoxyribonuclease I (Sigma Chemical Co., St. Louis, MO, U.S.A.) for 10 min in a shaker bath at 37°C to increase the yield of non-parenchymal cells. Suspended cells were filtered through four layers of gauze and 60 μ m-pore-size nylon mesh to remove debris and aggregated cells. Parenchymal cells were collected by differential centrifugation (25g, 3 min, 4°C) and the non-parenchymal-cell-enriched supernatants were pooled. Non-parenchymal cells were pelleted (350g, 10 min, 4°C), washed once in M199 and resuspended in 5.0 ml of Gey's balanced salts solution. Residual parenchymal cells, erythrocytes and debris were removed by isopycnic centrifugation with Metrizamide A as described by Knook & Sleyster (1976). Endothelial and Kupffer cells were separated by centrifugal elutriation (JE6B elutriator rotor; Beckman Instruments, Palo Alto, CA, U.S.A.) by the method of Caperna & Garvey (1982). The elutriation buffer consisted of ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-free Gey's balanced salts solution containing 1% (w/v) bovine serum albumin, 0.05 mM-EDTA and 10 mM-Hepes, pH 7.2. The purity of isolated cells was assessed by peroxidative staining activity (Wisse, 1974) and non-specific esterase staining activity with α -naphthyl butyrate (Li *et al.*, 1972). Viability was measured by the ability of cells to exclude 0.1% Trypan Blue.

Cell culture

Purified endothelial cells were maintained on collagen-coated plastic dishes. The number of viable cells added to each plate was either 6×10^6 /35 mm-diameter dish or 24×10^6 /60 mm-diameter plate. Culture medium (M199m) consisted of M199 supplemented with 15 mM-Hepes, 10 mM-Tes, 0.5 mM-sodium pyruvate and either a mixture of penicillin (100 units/ml) plus streptomycin (0.1 mg/ml) or gentamicin (50 μ g/ml). Endothelial cells were incubated overnight in the above

medium containing 30% (v/v) heat-inactivated adult-rat serum. Attached cells were washed with buffered saline (137 mM-NaCl/5 mM-KCl/1 mM- CaCl_2 / 0.63 mM- MgSO_4 / 6 mM-glucose / 15 mM-Hepes/10 mM-Tes, pH 7.4) and exposed to media as indicated in the results section. Serum-free medium, designated A/O, consisted of M199m supplemented with 0.2% (w/v) bovine serum albumin and 15 μ M-oleic acid.

Kupffer cells were added to multi-well plates (either 1.2×10^6 /16 mm or 3×10^6 /35 mm dish) in M199m plus 20% (v/v) foetal-calf serum. Plastic plates were not coated with collagen for Kupffer cells. After overnight incubation, attached Kupffer cells were treated as for endothelial cells.

Cadmium accumulation *in vitro*

After overnight incubation, endothelial and Kupffer cells were washed twice with buffered saline at 37°C. Medium containing 1.6 μ M- $^{109}\text{CdCl}_2$ (0.1–0.2 μ Ci of ^{109}Cd) was added to each dish or well and incubated for periods up to 6.0 h at either 37°C or 4°C. At specified times, cadmium-containing medium was removed from duplicate cultures and cells were washed twice with ice-cold buffer (150 mM-NaCl/10 mM-EDTA/10 mM-Hepes, pH 7.4). A 1 ml portion of aq. 0.2% Triton X-100 was added to each plate. Cellular material was collected and ^{109}Cd content measured (Beckman Biogamma Counter, model 4000). Cell protein was subsequently precipitated from each tube with 2 vol. of 10% (v/v) trichloroacetic acid. The pellet was collected by centrifugation and solubilized in 1 M-NaOH before quantifying protein content by a modified Lowry assay (Nerurkar *et al.*, 1981) using bovine serum albumin as standard. Results are reported as nmol Cd accumulated/mg of cell protein.

MT assay

The caudal lobe of the liver and purified parenchymal, endothelial and Kupffer cells were frozen in liquid N_2 and stored at -70°C until the day of assay. The soluble fractions (cytosol; 125 000g for 60 min; $r_{\text{av}} = 5.9$ cm) were prepared as described by Sciortino *et al.* (1982), except that suspensions of endothelial and Kupffer cells were sonicated in 1.15% KCl for 20 rather than 60 s. The cytosol fraction contained 40, 47 and 50% of the total protein of parenchymal, endothelial and Kupffer cells respectively. MT levels in cytosol were quantitatively determined by the competitive ^{203}Hg binding assay (Piotrowski *et al.*, 1973) as modified by Sciortino *et al.* (1982). Cytosol samples (0.5 ml) from parenchymal cells and caudal lobe were incubated with $^{203}\text{HgCl}_2$ (50–60 nmol of Hg/mg of cytosol protein) for 15 min at 0°C . Hg-binding species of M_r greater than 10 000 were

precipitated with trichloroacetic acid (2%, w/v, final concn.) and removed by centrifugation (3000g, 30min). A portion of the acid-soluble supernatant was applied to columns (0.8cm × 30cm) containing Sephadex G-50 resin, previously treated with NaBH₄ and equilibrated with 0.1M-NaCl/HCl, pH 5.0. Blue dextran, purified Zn-MT from rat liver and K₂Cr₂O₇ were used as standards to determine V_0 , V_{mt} (elution volume of MT) and V_t respectively. The quantity of Hg bound to MT was calculated by determining the percentage of ²⁰³Hg that eluted at $(1.6-2.0) \times V_0$ and is reported as nmol of Hg bound/mg of cytosol protein.

When the cytosol sample contained less than 6mg of protein/ml, which was the case for all samples of endothelial and Kupffer cells, we obtained less than 90% recovery of radioactivity from the Sephadex G-50 columns. We attributed the decreased recovery to non-specific losses and found it necessary to add 'carrier protein' to the cytosol sample. Cytosol from parenchymal cells isolated from control rats was prepared and portions were stored at -70°C. The MT concentration of this carrier cytosol was determined and known amounts were added as needed to cytosols prepared from endothelial and Kupffer cells. The recovery of ²⁰³Hg from such samples exceeded 90%. The quantity of Hg bound to MT of either endothelial or Kupffer cell origin was calculated by using simultaneous equations.

Results and discussion

The yields and viabilities of non-parenchymal rat liver cells isolated in these studies were as follows: $(80.4 \pm 6.8) \times 10^6$ endothelial cells with a mean viability of 92% ($n = 20$), and $(33.6 \pm 1.9) \times 10^6$ Kupffer cells with a mean viability of 91% ($n = 20$). The purity of each cell type after centrifugal elutriation exceeded 90% as evaluated by cytochemical staining characteristics. Contamination of each non-parenchymal cell fraction by parenchymal cells was less than 0.01%. Overnight monolayer cultures of endothelial and Kupffer cells had morphologies similar to that previously described (deLeeuw *et al.*, 1982; Smedsrod *et al.*, 1982; Caperna & Garvey, 1982). Only Kupffer cells phagocytosed 5µm-diameter latex particles. The concentrations of CdCl₂ (0.4–1.6µM) used for studies *in vitro* had no observed effect on non-parenchymal-cell attachment or morphology.

The distribution of cadmium among freshly isolated liver cells 20h after subcutaneous injection of the metal is shown in Table 1. All three cell types accumulated substantial concentrations of Cd. However, parenchymal cells accumulated 8–10-fold more Cd than did either endothelial or

Table 1. Cellular distribution and concentration of Cd in rat liver after subcutaneous injection of CdCl₂

Parenchymal and non-parenchymal cells were isolated from rat liver 20h after injection with CdCl₂ (1.5mg of Cd/kg body wt.) containing 5µCi of ¹⁰⁹CdCl₂. The solution of CdCl₂ was prepared in 0.05M-citrate buffer, pH 4.5. The amount of ¹⁰⁹Cd in each cell isolate was measured. Cell numbers were estimated with a standard counting chamber. Results are means ± S.E.M. ($n = 4$).

Cell type	[Cd]	
	(ng/10 ⁷ cells)	(ng/mg of protein)
Endothelial	87.8 ± 8.6	134.1 ± 5.4
Kupffer	110.6 ± 23.4	70.1 ± 7.6
Parenchymal	825.0 ± 172.9	69.5 ± 6.9

Kupffer cells. This observation agrees with the results of Cain & Skilleter (1980, 1983) and further emphasises the importance of these cells in the accumulation of supra-physiological levels of heavy metals. Since the volume and protein content of parenchymal cells are 10–30 times greater than those of non-parenchymal cells (Blouin *et al.*, 1977; Wisse & Knook, 1979), it may be misleading to compare the efficiency of accumulation on a per-cell basis only. Comparison of the specific activity of cadmium (ng of Cd/mg of cell protein) for each cell population revealed that the concentration of the metal within endothelial cells was about twice that in parenchymal and Kupffer cells. Thus endothelial cells have the highest capacity to accumulate cadmium per mg of cell protein *in vivo* under the conditions employed in the present study. This phenomenon may be related to the extensive membrane surface area associated with the specialized membrane fenestrations of endothelial cells (Pranning-van Dalen *et al.*, 1981).

Almost all (>90%) of the ¹⁰⁹Cd accumulated by endothelial and Kupffer cells isolated from Cd-injected rats was present in the soluble (cytosol) fraction. Analysis of cytosol by Sephadex G-75 gel-filtration chromatography indicated that ¹⁰⁹Cd was eluted as a single peak at 1.7–2.2 times the V_0 (Fig. 1). We previously demonstrated that this material had properties similar to those of purified rat liver metallothionein (Sciortino *et al.*, 1982).

The concentrations of MT in liver-cell types prepared from control and Cd-injected rats were determined by the competitive ²⁰³Hg binding assay (see the Materials and methods section). All three liver cell types isolated from control rats contained low levels of MT (Table 2). The basal concentration of the heavy-metal-binding protein in endothelial and Kupffer cells was approx. 3-fold higher than that in parenchymal cells. The concentration of MT in parenchymal cells and

Table 2. MT concentrations in whole liver and parenchymal and non-parenchymal liver cells isolated from control and Cd²⁺-injected rats

Parenchymal and non-parenchymal cells were isolated from rats injected 20h previously with CdCl₂ (1.5 mg of Cd/kg) and control rats. Metallothionein concentrations in cytosols prepared from intact tissue and purified liver cells were determined by competitive Hg binding assay (see the Materials and methods section). The recovery of ²⁰³Hg from columns (0.8 cm × 30 cm) of Sephadex G-50 was 91.8 ± 1.7%. Results are means ± S.E.M. (n).

Treatment	[Metallothionein] (nmol of Hg ²⁺ /mg of cytosol protein)			
	Whole liver	Parenchymal cells	Endothelial cells	Kupffer cells
Control	0.40 ± 0.04 (4)	0.30 ± 0.01 (3)	0.97 ± 0.18 (4)	0.92 ± 0.09 (5)
+ Cd ²⁺ (1.5 mg/kg)	4.11 ± 0.33 (4)	3.89 ± 0.3 (4)	6.34 ± 0.41 (3)	3.25 ± 0.3 (4)

Fig. 1. Sephadex G-75 gel-filtration chromatography of cytosols prepared from endothelial and Kupffer cells isolated from ¹⁰⁹Cd-injected rats

Non-parenchymal cells were isolated from livers of rats treated 20h previously with CdCl₂ (1.5 mg of Cd²⁺/kg) plus 5 μ Ci of ¹⁰⁹Cd. Cells were immediately sonicated in 1.15% KCl containing 10 mM-Hepes and 1 mM-2-mercaptoethanol at pH 7.6. Cytosols were chromatographically analysed on a column (1.5 cm × 60 cm) of Sephadex G-75 that was equilibrated and subsequently eluted with 10 mM-Tris/acetate buffer, pH 8.6, at 4°C. ●, Endothelial-cell cytosol; ○, Kupffer-cell cytosol.

whole liver were similar, since these cells comprise 90% of the mass of the organ (Blouin *et al.*, 1977). The higher concentrations of MT in control non-parenchymal cells may reflect the importance of these cells in hepatic trace-metal homeostasis. Owing to the strategic location of non-parenchymal cells along the sinusoidal tracts and their highly efficient filtration capacity (Praaning-van Dalen, 1981), they may be exposed to sources (e.g., senescent cells) and molecular forms of the heavy metals different from those to which parenchymal cells are exposed. Distinct characteristics for the transport, intracellular distribution and metabolism of heavy metals for various classes of liver cells are also possible. Indeed, cell-specific differences in the subcellular compartmentation and mobilization of stored iron in rat liver have

recently been described (Düllmann *et al.*, 1982; Hultcrantz *et al.*, 1983).

MT concentrations increased 12-, 6.5- and 3.5-fold in liver parenchymal, endothelial and Kupffer cells respectively after injection with CdCl₂ (Table 2). The absolute concentration of this protein in liver endothelial cells exceeded that in parenchymal and Kupffer by 63 and 95% respectively. Monolayer cultures of endothelial cells also synthesized MT *de novo* when incubated in Cd-free medium (Fig. 2). When such cultures were exposed to 0.8 μ M-CdCl₂ for 10h, the amount of [³⁵S]cysteine that was eluted from Sephadex G-75 as MT was increased by 47% (Fig. 2). Cytosol prepared from endothelial cells exposed to 0.4 μ M-CdCl₂ had 23% more radioactivity eluted as MT than had control samples (results not shown). MT synthesis was not observed in monolayers of Kupffer cells incubated in medium containing from 0 to 1.6 μ M-CdCl₂ for 10h. However, similar levels of [³⁵S]cysteine were incorporated into proteins that were eluted from Sephadex G-75 at or near V₀ in Kupffer and endothelial cells. Analysis (Hg binding assay) of Kupffer cells 20h after plating indicated that the intracellular level of MT was similar to that found in freshly isolated cells, despite the apparent lack of synthesis *de novo* in culture. The characteristics of Cd²⁺ uptake by monolayers of endothelial and Kupffer cells were compared to determine whether the differential ability of Cd²⁺ to increase MT synthesis *in vitro* was related to heavy-metal accumulation.

Monolayer cultures of liver endothelial cells accumulated extracellular Cd²⁺ at a linear rate for up to 6h at 37°C (Fig. 3). Minimal accumulation of the metal was observed when cells were incubated at 4°C. The quantity of cadmium accumulated by endothelial cells was increased by decreasing the serum content of the medium. Endothelial cells incubated in serum-free (A/O) medium accumulated similar amounts of Cd²⁺ as previously reported for liver parenchymal cells (Failla *et al.*, 1979). The inverse relationship between medium serum content and cellular Cd²⁺ accumulation may result from the increased concentrations of

Fig. 2. $[^{35}\text{S}]$ MT synthesis by rat liver endothelial cells *in vitro*

Endothelial cells were seeded on collagen-coated dishes in M199m plus 30% (v/v) rat serum. Non-attached cells were removed after 2h and replaced by cysteine-free Waymouth's MB752/1 medium containing $1.0\mu\text{Ci}$ of $[^{35}\text{S}]$ cysteine (sp. radioactivity 898 Ci/mmol/ml and 20% (v/v) rat serum. CdCl_2 ($0.8\mu\text{M}$) was added to half the dishes after 16h. After 10h, all monolayers were washed twice with ice-cold Puck's saline solution, cells were collected and were sonicated in 1.15% KCl (w/v) containing 10mM-Tris/acetate, 1mM-2-mercaptoethanol, 1mM-phenylmethanesulphonyl fluoride, aprotinin (235 units/ml), soya-bean trypsin inhibitor (1mg/ml) and bovine serum albumin (2mg/ml). Cytosols were analysed as described in the legend to Fig. 1. $\blacktriangle$, Control culture; $\bullet$, Cd^{2+} -exposed culture.

Zn^{2+} and albumin in high-serum media. Cd^{2+} and Zn^{2+} inhibit accumulation of each other by parenchymal cells (Failla & Cousins, 1978a; Failla *et al.*, 1979), whereas albumin is the primary Cd^{2+} binding ligand in serum-containing culture medium (Failla *et al.*, 1979).

Kupffer cells accumulated 6–10 times less Cd^{2+} than endothelial cells in 6h at 37°C (Fig. 3). The quantity of the metal associated with cells incubated at 4°C was similar for both cell types. Kupffer-cell uptake of Cd^{2+} was also influenced by the serum content of the medium. Preincubation with $1\mu\text{M}$ -dexamethasone, a stimulator of parenchymal-cell Zn^{2+} and Cd^{2+} accumulation (Failla & Cousins, 1978b; Karin *et al.*, 1980), failed to increase Kupffer cell uptake of Cd. Kupffer cells also accumulated markedly less Zn^{2+} and Mn^{2+} than did endothelial and parenchymal cells *in vitro* (results not shown). However, decreased heavy-metal uptake was not due to a general impairment of Kupffer-cell metabolism. For 4h these cells

Fig. 3. Cd^{2+} accumulation by monolayers of rat liver endothelial (a) and Kupffer (b) cells

Cells were incubated in Cd^{2+} ($1.6\mu\text{M}$) containing medium with either 25% ($\blacktriangle$), 5% ($\blacksquare$) or 0% ($\bullet$) serum at 37°C and 25% ($\triangle$) and 0% ($\circ$) serum at 4°C . Each point represents the mean result $\pm$ S.E.M. for two to five separate experiments.

incorporated $[^3\text{H}]$ leucine into trichloroacetic acid-insoluble material (d.p.m. of $[^3\text{H}]$ leucine incorporated/mg of cell protein) at a rate similar to that observed for parenchymal cells. They also efficiently engulfed immunoglobulin G-coated sheep red blood cells and latex particles. Activation by incubation with latex particles before exposure of the cells to Cd^{2+} failed to stimulate Cd^{2+} accumulation.

Low levels of Cd^{2+} accumulation by cultured rat liver Kupffer cells (Skilleter & Cain, 1981) and rabbit pulmonary macrophages (Cox & Waters, 1978) have been reported by others. In contrast, murine peritoneal macrophages readily accumulated Cd^{2+} and synthesized MT *in vitro* (Patierno *et*

al., 1983). Several possibilities for the apparent inability of rat liver Kupffer cells to accumulate heavy metals *in vitro* are possible. First, one or more components of the plasma membrane required for transfer of the metals into the cell may be irreversibly lost or altered during the cell-isolation procedure. Second, since Kupffer cells are phagocytic, they may preferentially accumulate metals bound to aggregates and particles. Skilleter & Cain (1981) have found that monolayers of Kupffer cells accumulated markedly more Cd^{2+} when the metal was presented as colloidal Cd^{2+} -dithiol complexes. Similar results were subsequently observed *in vivo* (Cain & Skilleter, 1983). A third possibility is that accumulation of heavy metals by Kupffer cells is dependent on interactions with other liver-cell types. Further work will be required to explain the discrepancy between Kupffer-cell uptake and the metabolism of heavy metals *in vivo* and *in vitro*.

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