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EXCRETION PROCESS OF COPPER FROM COPPER PRE-LOADED RAT LIVER PARENCHYMAL CELLS

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Abstract—Excretion of copper from copper pre-loaded rat liver parenchymal cells was investigated. The copper pre-loaded cells were isolated from the liver of copper-administered rats and were maintained for 4 days in modified Williams medium E; sodium pyruvate was increased while calcium was reduced in concentration, and 10% fetal bovine serum and insulin were added. For the first 30 hr after plating, loss of copper was slow, with a half-life of more than 100 hr: subsequently the rate of loss was faster, with a half-life of 51 ± 9 hr. Copper pre-loaded in the cells was mostly bound to metallothionein and the amount of copper–metallothionein decreased with time of culture. Furthermore, the relative ratio of the two iso-metallothioneins changed with length of culture.

Excess copper administered in the body is known to accumulate in the liver and the accumulated copper is mostly chelated to metallothionein, an inducible ubiquitous heavy metal binding protein [1]. The copper accumulated in the liver is gradually excreted from the liver and discharged from the body into urine and feces. Little is known of the mechanism except for some *in vivo* studies using a whole animal [2]. We have examined the excretion process of copper from liver parenchymal cells which are the main storage cells for copper in the liver.

Our previous report showed that copper content in rat liver attained the highest level at 18 hr after a single injection of copper and decreased to the control level on the 4th day after the injection [3]. The present experiment was performed to examine the excretion mechanism of copper from the liver using primary cultured rat liver parenchymal cells. The parenchymal cells were isolated from the copper-administered rat liver at the time when the copper concentration in the liver was at the highest level.

MATERIALS AND METHODS

Isolation of rat liver parenchymal cells. Male Wistar strain rats, weighing about 150 *g* and maintained on a laboratory chow, were used. The copper pre-loaded liver parenchymal cells were isolated from copper-administered rats. The rat was injected s.c. with $CuSO_4$ at a dose of 3 mg of Cu^{2+}/kg body weight. Twenty four hours after the injection liver parenchymal cells were isolated from the liver perfused *in situ* with a collagenase solution according to Seglen [4]. Viability of isolated cells was determined by a trypan blue exclusion test; a 200 μl of the cell suspension was added to a 600 μl of an isotonic 0.6% trypan blue solution. Liver parenchymal cells were counted on a hemocytometer and cells staining blue within 5 min were judged nonviable. Viability of cells was over 90%.

Primary culture. The culture condition was based on the method of Tanaka *et al.* [5]. Two types of the culture media were used. Medium I was Williams medium E [6] without $CaCl_2$, $CuSO_4$, $Fe(NO_3)_3$, $MnCl_2$ and $ZnSO_4$ and supplemented with 10^{-6} M insulin, 10% fetal bovine serum, penicillin 100 U/ml, streptomycin 100 μg/ml and fungizone 0.25 μg/ml. The metals mentioned above were omitted because sufficient amounts of these metals seemed to be supplied to the medium by addition of fetal bovine serum. Medium II was Williams medium E without the same minerals as Medium I and supplemented with 5% fetal bovine serum, 10 mM sodium pyruvate, 10^{-6} M insulin and the same antibiotics as Medium I.

2×10^6 liver parenchymal cells in 4 ml of Medium I were plated in a plastic dish (φ 60 mm, Falcon, Oxnard) as monolayers and cultured in a humidified incubator at 37° under 5% CO_2 in air, the medium being changed to Medium II at 4 hr after plating to remove non-adhered cells. The medium (Medium II) was changed daily. Control liver parenchymal cells were prepared from saline-injected rats and cultured under the same condition as the copper pre-loaded liver parenchymal cells.

Determination of copper and DNA contents in cells. Cells attached on dishes were harvested by rubber policeman after washing three times with Hanks solution and stored at −20° until use. The cells harvested from 5 dishes were suspended in 1.5 ml of 0.1 M Tris/HCl buffer, pH 7.4 (25°) containing 0.25 M glucose and homogenized well with a glass–Teflon homogenizer. A 1.0 ml of the homogenate was digested with mixed acid (HNO_3 : $HClO_4$ = 5 : 1 (v/v)). Amount of copper in the digest was determined by atomic absorption spectrophotometry (Hitachi 170-50A). The copper content in the cells was expressed as μg copper per mg DNA. DNA content in the homogenates was determined by fluorometry according to Thomas and Farquhar [7].

Assay of metallothionein. Contents of copper–

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metallothionein were determined by high performance liquid chromatography (HLC-803A, Toyo Soda Manuf. Co., Ltd., Tokyo) with an atomic absorption spectrophotometer (HPLC-AAS method) [8]. The cells from 25 dishes were homogenized in 1.1 ml of 0.1 M Tris/HCl buffer, pH 7.4 (25°) containing 0.25 M glucose with a glass–Teflon homogenizer in an atmosphere of nitrogen under ice water cooling. The homogenate was centrifuged in a Beckman SW 50.1 rotor at 170,000 *g* for 60 min at 4°. A 100 μl portion of the supernatant (the 170,000 *g* supernatant) was applied on an SW 3000 column (TSK Gel, Toyo Soda Manuf. Co., Ltd. 7.5 × 600 mm with a guard column of 7.5 × 75 mm) and the column was eluted with 50 mM Tris/HCl buffer, pH 8.0 (25°) containing 0.1% NaN_3 at a flow rate of 1.0 ml/min. Copper concentration in the eluate was monitored continuously on an atomic absorption spectrophotometer.

Chemicals. The following reagents were used: collagenase (Wako Pure Chemical Industries, Osaka); fetal bovine serum (M.A. Bioproducts, Walkersulle); insulin (Sigma, St. Louis); fungizone (Sankyo, Tokyo); penicillin and streptomycin (Meiji Seika, Tokyo); Hanks solution (Nissui Seiyaku, Tokyo); as components of Williams medium E, amino acids (Wako and Kyowa Hakko, Tokyo), methyl linoleate (Fluka, Buchs), menadione sodium bisulfate (Sigma), inorganic salts, glucose, phenol red, sodium pyruvate, ascorbic acid, biotin, choline chloride, ergocalciferol, vitamin A acetate and vitamin B_{12} (Wako), other vitamins (supplements as minimum essential medium, Flow, Mcleau). α-Tocopherol phosphate was a gift from Mr. I. Hishinuma.

RESULTS

Under the present condition, liver parenchymal cells were maintained for 4 days (Fig. 1). Amount of cells on dishes was estimated by amount of DNA in cells. About 30% of the initially attached cells were detached from dishes within 30 hr after plating. However, the residual cells adhered on dishes were maintained afterwards and about 60% of the initially attached cells were present attached on dishes at 105 hr after plating. The cells on dishes formed monolayers with pavement-like epithelial morphology, but they showed no indication of cell division (Fig. 2A).

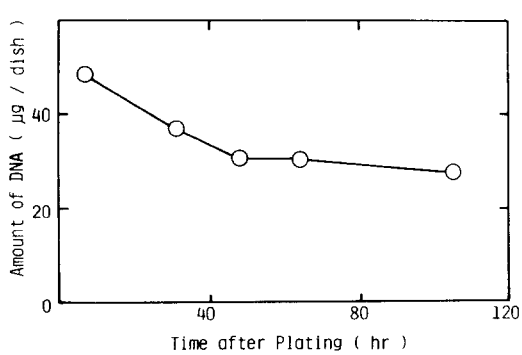

Fig. 1. Maintenance of isolated liver parenchymal cells in primary culture. Amount of cells on a dish was estimated by amount of DNA in the cells. Cells harvested from 5 dishes were homogenized in a 1.5 ml solution of 0.1 M Tris/HCl buffer pH 7.4 (25°) containing 0.25 M glucose. The amount of DNA in the homogenate was determined by fluorometry.

Although addition of glucocorticoid was reported to be important to maintain cultures for a long period [5], the hormone is known to induce metallothionein and probably alters the metabolism of heavy metals in the cells [9–12]. Therefore, glucocorticoid was not added in the present study. Increase of sodium pyruvate and reduction of calcium concentration in the medium were shown effective for maintenance of culture (Fig. 2A) as reported previously [13–15]. However, when the cells were cultured in the same medium as Medium II except for the conventional low level of sodium pyruvate (0.23 mM), the cells became aggregated and were detached from dishes on the 3rd day of culture (Fig. 2B).

The change of copper content in the liver parenchymal cells during a culture period was examined. A typical datum was shown in Fig. 3A. Copper content in the pre-loaded cells was about 10 times higher than that in the control cells at 6 hr after plating. The disappearance-rate of copper was slow at the beginning of the culture and enhanced thereafter. At 105 hr after plating copper in the pre-loaded cells was almost at the control level. On the other hand, copper content in the control liver parenchymal cells was constant throughout. A semilogarithmic plot of the data for the elimination of copper from the pre-loaded liver parenchymal cells showed that the pre-loaded copper disappeared from the liver parenchymal cells with two phases (Fig. 3B). Half-lives were more than 100 hr and 40 hr at the beginning and after 30 hr of culture period, respectively. The latter half-life value obtained from 3 determinations was 51 ± 9 hr (mean ± S.D.).

Copper pre-loaded in liver parenchymal cells was mainly chelated to metallothionein as shown in Fig. 4A–D. The two iso-metallothioneins, MT I and MT II were eluted at retention times of 22.0 and 20.9 min respectively. Copper peaks eluted at retention times of 11.5 min and 19.6 min corresponded to the void volume of the column and superoxide dismutase, respectively. Metallothionein was not detected in the 170,000 *g* supernatant prepared from the control liver parenchymal cells (Fig. 4E). The amounts of copper-MT I and copper-MT II decreased gradually with time after plating. The relative ratio of the two iso-forms changes with time. MT II was the major iso-form at the beginning of culture (Fig. 4A). However, MT II disappeared more rapidly than MT I and MT I turned out to be the major isoform at 95 hr after plating (Fig. 4D).

DISCUSSION

Copper pre-loaded in liver parenchymal cells was excreted at a half-life of 51 ± 9 hr, though excretion of copper was depressed within 30 hr after plating. This depression might be caused by impaired cellular functions within an early time of culture [5]. Previously we reported that copper pre-loaded in rat liver disappeared at a half-life of 40–50 hr [3]. Therefore, excretion rate of the pre-loaded copper from the liver seems to be determined mainly by the

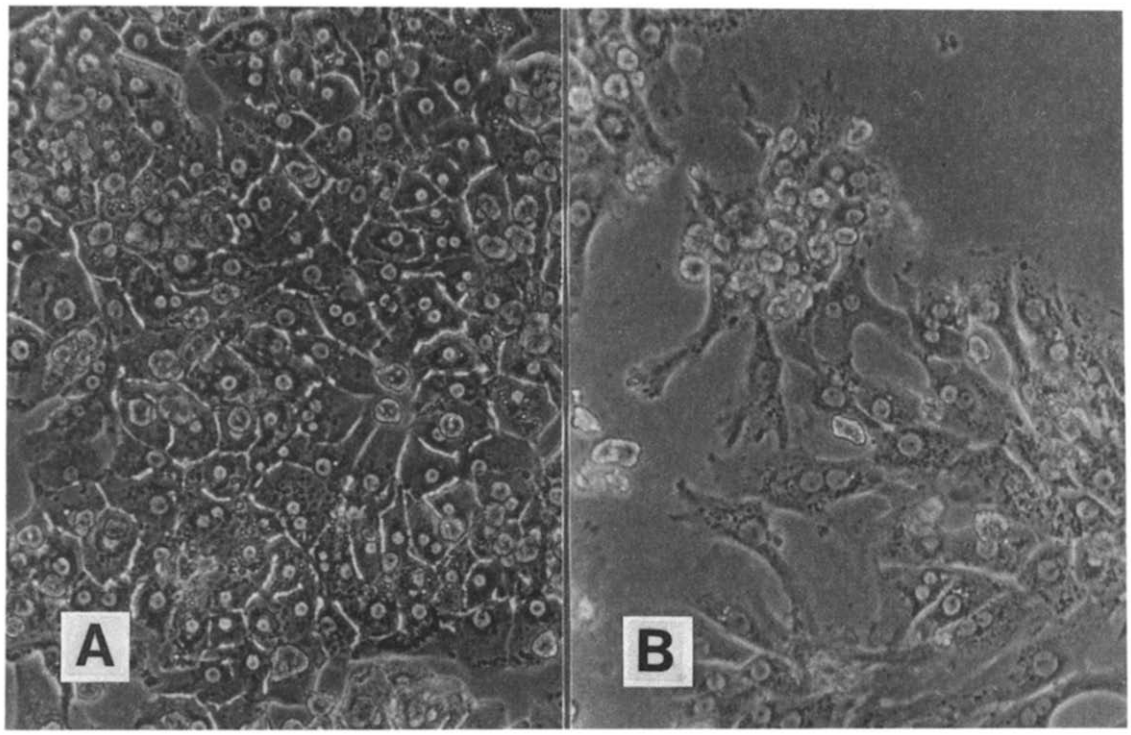

Fig. 2. Phase contrast microscopy of cultured rat liver parenchymal cells at 72 hr after plating (× 100). (A) cells maintained in Medium II. (B) cells maintained in the same medium as Medium II except that a concentration of sodium pyruvate was reduced to a conventional level (0.23 mM).

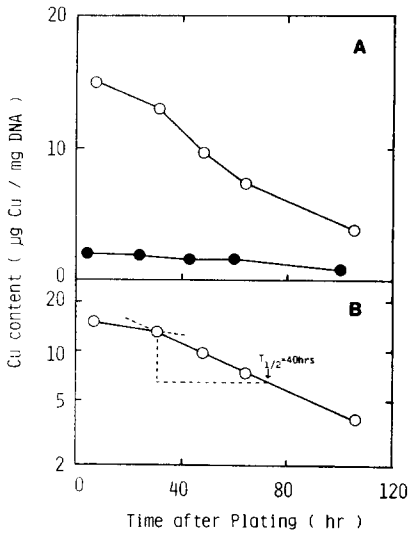

Fig. 3. Change of copper content in liver parenchymal cells. Copper content was expressed as μg copper per mg DNA. Cells harvested from 5 dishes were homogenized in a 1.5 ml solution of 0.1 M Tris/HCl buffer, pH 7.4 (25°) containing 0.25 M glucose. Amounts of copper and DNA in the homogenate were determined by fluorometry and atomic absorption spectrophotometry, respectively. Panel A, change of copper content in the pre-loaded liver parenchymal cells (—○—) and control liver parenchymal cells (—●—). Panel B, The change of copper content in the pre-loaded cells was expressed as semi-logarithmic plot. $T_{1/2}$ = half-life.

excretion rate of copper from the liver parenchymal cells.

Copper-efflux from liver parenchymal cells has been examined by observing ^{64}Cu-efflux from the cells within a short period (1 hr) after pre-labelling [16–18]. A small portion of ^{64}Cu was observed to be effluxed from the cells during the first 5–15 min of incubation while the remaining ^{64}Cu was shown to be retained in the cells afterward. The copper excreted at first is probably due to the efflux of intracellular free copper that was not chelated to proteins. On the other hand, the latter copper excreted at a slow rate seems to be the metal bound to proteins, mainly to metallothionein. In the present experiment, copper accumulated in the liver parenchymal cells was shown to be chelated to metallothionein. Therefore the excretion process observed in Figs. 3 is supposed to correspond to the slow excretion process in the previously reported data [16–18]. However, as the copper concentration in the pre-loaded cells was much greater than the normal level, it is possible that the rate of excretion of copper chelated to metallothionein, observed in the present study, may differ from that at the steady state.

We previously observed that MT II was a major iso-metallothionein in rat liver immediately after copper administration and that the relative ratio between MT I and MT II changed with time and MT I became a major isoform on the 3rd day after the administration [3]. The present observation indi-

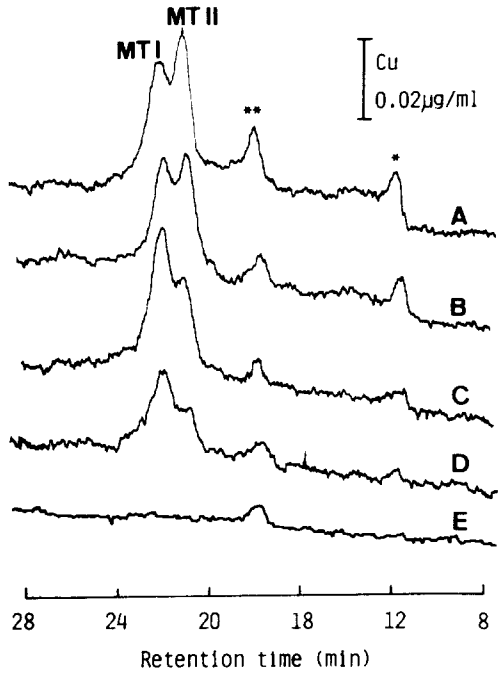

Fig. 4. Gel permeation–copper atomic absorption chromatograms of the 170,000 *g* supernatants from cultured rat liver parenchymal cells on SW 3000 column. A 100 μl portion of the 170,000 *g* supernatants was applied on an SW 3000 column and the column was eluted with 50 mM Tris/HCl buffer, pH 8.0 (25°) at a flow rate of 1.0 ml/min. The detector level of an atomic absorption spectrophotometer was set as indicated by the vertical bar. The 170,000 *g* supernatants were prepared from cultured copper pre-loaded cells harvested at 6 hr (A), 27 hr (B), 52 hr (C) and 95 hr (D) after plating. The control profile (E) corresponds to the 170,000 *g* supernatant prepared from cultured control cells harvested at 6 hr after plating. MT I (metallothionein I), MT II (metallothionein II). *and ** indicate the void volume of the column and superoxide dismutase, respectively.

cated that an identical change in the relative iso-metallothionein level also occurred *in vitro*. Our present finding suggests that the change in the ratio of the two iso-metallothioneins is an autonomously controlled process and is not affected by humoral factors in liver parenchymal cells. However, mechanism for this change is still unknown.

Amounts of intracellular proteins are known to be determined by the balance between synthesis and degradation of proteins. Cain and Holt reported that the degradation rate of MT I was faster than that of MT II [19]. Therefore, the present result suggests that the synthesis of MT I is predominant to that of MT II in liver parenchymal cells.

Disappearance rate of copper from liver parenchymal cells was shown to be parallel to decrease rate of copper metallothionein. The half-life of copper excretion (51 ± 9 hr) was also similar to the average half-life of protein degradation in liver parenchymal cells (about 40 hr) (S. Goto, unpublished results). The average half-lives of proteins in rat liver have likewise been reported to be between 2 and 3 days [20]. Therefore, the excretion of copper from the cells is suggested to be related closely to intracellular degradation of metallothionein and the degradation process of metallothionein is probably important as the excretion mechanisms for the copper accumulated in liver parenchymal cells.

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