Mitochondrial ultrastructure & release of proteins during liver regeneration

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Background & objectives: It has been reported that some proteins are released from mitochondria during liver regeneration after partial hepatectomy (PH), but the relationship between proteins release and mitochondrial permeability transition (MPT) remains unclear. We undertook this study to demonstrate the changes of mitochondrial ultrastructure and proteins release during liver regeneration and to determine the relationship between proteins release and MPT in liver regeneration in rats.

Methods: After PH and administration of cyclosporin-A (CsA, a specific inhibitor of MPT), ultrastructural morphology of mitochondria in the remnant liver were determined by electron microscopy. Catalytic activity of mitochondrial and cytosolic proteins including aspartate aminotransferase (AST) and glutamic acid dehydrogenase (GDH) was measured.

Results: The liver mitochondria at 24 and 72 h were quite variable in morphology and ultrastructure. The enzyme activities of AST and GDH in cytosol released from mitochondrial matrix changed significantly at 24 and 72 h. CsA can inhibit the permeability of mitochondria partly at the same time.

Interpretation & conclusions: The changes of mitochondria in ultrastructure reflected the feature of MPT, and the changes of enzymes activities released from mitochondrial matrix were consistent with those of mitochondrial ultrastructure. CsA can inhibit these changes to some extent. There was a close relationship of MPT with mitochondrial ultrastructure and proteins release during liver regeneration.

Key words Cyclosporin-A - liver regeneration - mitochondrial ultrastructure - protein release

The remnant liver has remarkable ability to regenerate after 70 per cent partial hepatectomy (PH), and it takes about 7-10 days to restore mass and function. Energy metabolism plays an important role during rat liver regeneration. Mitochondria not only provide the necessary energy for life activities, but also take part in many activities of cell life such as proliferation, apoptosis, injury, aging, etc. It has been found that mitochondrial permeability transition (MPT) has some remarkable changes during liver regeneration, suggesting that mitochondria play a dominant role, especially in priming and termination phases, associated
with initiation and termination of regeneration\textsuperscript{4,6}. The mitochondrial permeability transition pore (MPTP), a multi-protein complex including the voltage-dependent anion channel (outer membrane), the adenine nucleotide translocase (ANT; inner membrane), cyclophilin-D (mitochondrial matrix) and Bcl-2 family members (Bcl-2, Bad and Bax), is formed at the contact site between the mitochondrial inner and outer membranes\textsuperscript{7}.

MPT is a reversible phenomenon whereby the mitochondrial inner membrane becomes freely permeable to solutes of less than 1500 Da. The abnormal permeability can induce the disappearance of transmembrane potential and the release of apoptosis inducing factors. Many factors can regulate the opening of MPTP, such as cyclosporin-A (CsA, a specific inhibitor of the mitochondrial transition pore), ATP, \textsuperscript{Ca}\textsuperscript{2+}, reactive oxygen species (ROS), etc\textsuperscript{8}.

Mitochondrial swelling mainly results from insufficient cell energy in hypoxia condition, and is also caused by some factors, such as bacteriotoxin, viral infection, ionizing radiation, osmotic pressure injury, alimentary deficiency, etc\textsuperscript{9,10}. Slight swelling in physiological condition manifests an increase in mitochondrial functional compensation, while mitochondrial shrinkage shows the tightness of oxidative phosphorylation couple\textsuperscript{11-13}.

Miao et al\textsuperscript{14} found that the mitochondrion was first shrunk and then swollen during the early and late phases of liver regeneration, which suggested that the permeability of mitochondria first decreased and then increased. But the structure and function of mitochondria may be different in vivo and in vitro because of the limit of purification technology and instability. There has been no study on the structure and function of mitochondria except one by Ferri et al\textsuperscript{15} who found that mitochondria get swollen at 24 h after PH.

There is no systematic study on mitochondrial ultrastructure and proteins release during the whole process of liver regeneration. Therefore, we investigated the changes of mitochondrial ultrastructure and proteins release during liver regeneration in rats to study the relationship between MTP and liver regeneration.

### Material & Methods

**Materials**: Olive oil was purchased from Olivoila, Italy, CsA from Novartis, Basel, Switzerland, Araldite resin from Tab Laboratories Equipment LTD, Berkshire, England, and sodium cacodylate and OsO\textsubscript{4} were purchased from Sigma, USA. Bio-Rad kit was purchased from Bio-Rad Laboratories Inc, USA. Mannitol, Sucrose, EDTA, EGTA, HEPES, succinate, Tris, MOPS and other chemicals were purchased from AMRESCO Inc, USA.

**Animals**: A total of 180 male SD rats (aged, 8 wk; weighing, 200-250 g; purchased from Animal Experimental Center, The Second Military Medical University, China) were housed at a 12 h light:dark cycle, and allowed access to water and a standard rat pellet chow diet ad libitum.

**Groups**: The rats were randomly assigned to four groups (45 animals in each group) as follows: (i) SH group, animals were subjected to sham operation and received olive oil (vehicle); (ii) SH plus CsA group, animals were sham operated and received CsA; (iii) PH group, animals underwent 2/3 partial hepatectomy and were given the placebo (olive oil); (iv) PH plus CsA group, animals were administered CsA and underwent 2/3 partial hepatectomy. The animals in each group were further divided into nine sub-groups (0, 3, 6, 12, 24, 48, 72, 120 and 168 h, n=5).

**CsA treatment**: All rats were received either 20 mg/kg/day CsA dissolved in 5 mg/ml olive oil or equal volume of placebo (olive oil) 4 days pre- and 1 day post-operatively\textsuperscript{20}. The appropriate dose of CsA was administered by gavage, using an orogastric stainless steel animal feeding tube.

**Surgery**: All rats were subjected to either standard 2/3 partial hepatectomy (the median and left lateral lobes of the liver were excised, approximately 70 per cent resection of liver mass), or to sham operation\textsuperscript{21}. The animals in SH group were given a small midline abdominal incision without excision of the liver. Their livers were removed at each time point after the surgery, and then weighed and processed as follows: one-fourth was cut into sections for electron microscopy study and three-fourths were used for the isolation of cytosol and mitochondria. The study protocol was approved by the Institutional Animal Care and Ethics Committee of the Second Military of Medical University, Shanghai.
Liver regeneration index (LRI) and liver regeneration degree (LRD): In PH and PH plus CsA groups, during the liver excision, the removed wet liver and the animal body were weighed by Model 100A electronic balance (Denver Instrument Company, USA), and the values of LRI and LRD were calculated using following equation: \[ LRD = \frac{B - (A/0.684 - A)}{A}; \quad LRI = \frac{B}{G} \] (A: weight of the wet liver removed; B: weight of the liver regenerated after PH; G: weight of the animal body, and 0.684 is the ratio of the weight of liver removed to the one of the original liver).

Electron microscopy: Ultrastructural morphology of mitochondria was determined by electron microscopy. Liver specimens from control rats and from rats at 0, 3, 6, 12, 24, 48, 72, 120, and 168 h after surgery, were fixed with 4 per cent glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 4 h at 4 ºC. After fixation and an overnight wash in sodium cacodylate buffer, the specimens were postfixed with 1 per cent osmium tetroxide in sodium cacodylate buffer for 1 h at 4 ºC, dehydrated in alcohol and embedded in araldite resin. And semithin sections (1 µm) were removed for optical microscopy. According to Reynolds23, before examination, ultrathin sections were mounted on copper mesh grids and stained with uranyl acetate and lead citrate with electron microscope (HITACHI H-800, Japan). The ultrastructural morphology of mitochondria was evaluated in five rats of each group and 10 randomly selected electron micrographs of the hepatic lobule were observed in each rat (15000 x magnification).

Preparation of cytosol and mitochondria: Mitochondria and cytosol were prepared as described earlier. Briefly, the liver was quickly homogenized in ice-cold buffer A (250 mM mannitol, 75 mM sucrose, 0.1 mM K+-EDTA, 0.5 mM K+-EGTA, 10 mM K+-HEPES and bovine serum albumin 0.5%, pH 7.2). Homogenate was centrifuged at 1500 g x 15 min by high-speed centrifuge (HITACHI CR21, Japan), and supernatant was sequentially spun at 10000 g x 15 min to obtain mitochondrial fraction. Post-mitochondrial supernatant was centrifuged at 105000 g x 60 min by ultracentrifuge (HITACHI GX SERIES HIMAC CS 120 GXL, Japan) and resulting supernatant was designated as cytosolic fraction. Both cytosolic and mitochondrial fractions were used to estimate specific enzyme activities.

According to Bradford’s method22, protein concentration was determined using the Bio-Rad kit. Isolated mitochondria, suspended at about 10 mg protein/ml in a medium (5 mM succinate-tris, 10 mM tris-MOPS, 0.2 M sucrose, 1 mM Pi-tris, pH 7.2), were used for determination of aspartate aminotransferase (AST) and glutamic acid dehydrogenase (GDH).

The GDH activity in cytosol was measured29. There are two kinds of isoenzymes of aspartate aminotransferase: mitochondrial aspartate aminotransferase (mAST) and cytosolic AST. Differential assay between mitochondrial and cytosolic isozymes of AST in the cytosol was done using a method that depends on the thermal instability of mAST at 70 ºC under conditions where the cytosolic AST is stable27. One aliquot of the ultracentrifuged cytosol was incubated at 37 ºC for 15 min and another at 70 ºC for 15 min. After the incubation the AST activity in both samples was determined at 25 ºC using the coupled assay of Karmen28. The AST activity of the sample pre-incubated at 37 ºC was taken to be that of both isoenzymes, whereas that of the sample pre-incubated at 70 ºC was assumed to be solely due to the cytosolic isoenzyme. Activity of the mAST was taken as the difference between the two values.

Statistical analysis: Significance of differences between groups was analyzed by two-way ANOVA and by Student’s t test for independent samples. \( P<0.05 \) was considered significant.

Results

LRI and LRD during the liver regeneration: To study the influence of CsA on the liver regeneration, we observed the LRI and LRD at 24, 48, 72, 120 and 168 h after the surgery in the PH and PH plus CsA groups. The LRI at 72 h in PH group increased significantly compared with that in CsA group, the values of LRD were also consistent with the changes of LRI (Figs 1A, 1B).

Changes of mitochondrial ultrastructure after surgery: In SH group (Fig. 2A), mitochondria at 3, 24 and 168 h after surgery were morphologically characterized by the same basic architecture of the typical liver mitochondria with a folded internal membrane, cristae-rich and a dense matrix, and were normal in volume. In SH plus CsA group (Fig. 2B), mitochondria were a little big in volume at 0 h after surgery, and there was no significant difference at other time.

In PH group (Fig. 2C), mitochondria were morphologically normal at 0, 3 and 168 h after PH, the same as them in SH group; at 6 h and 120 h after PH, mitochondria were swollen to a lower degree, characterized as reduction in the number of cristae, destructurization of the matrix compartment, a dilated...
and pale matrix, and lack of dense granules, at 24 h after PH, mitochondria were altered significantly, with clear vacuolization of the matrix compartment, even rupture of mitochondrial outer membrane, much larger in volume, the cristae were even swollen; at 72 h, mitochondria were altered in a middle degree.

In PH plus CsA group (Fig. 2D), mitochondria were swollen to a lower degree at 0, 3 and 6 h after PH; and altered significantly at 24 h, the cristae were also swollen, but less swollen than those in PH group, not any clear vacuolization of the matrix compartment; and were normal at 120 h; at 72 and 168 h after PH, mitochondria were contracted, characterized as smaller in volume, a dense matrix.

Changes of proteins release during liver regeneration: During the liver regeneration, the changes of enzyme activities of AST in cytosol and mitochondrial matrix were nearly consistent with those of GDH in two places (Figs 3, 4). There was no significant difference of the enzymes between SH group and SH plus CsA group at any time point. In PH group, the enzyme activities of AST (Fig. 3A) and GDH (Fig. 4A) in cytosol increased significantly ($P<0.05$) at 3 h after PH compared with SH group, then reached the peak at 24 h, and then decreased and returned to normal at 120 h. On the contrary, the changes of enzyme activities of AST (Fig. 3B) and GDH (Fig. 4B) in mitochondria were opposite to the enzyme activities in cytosol.

In PH plus CsA group, the changes of enzyme activities of AST and GDH in cytosol and mitochondrial
Fig. 2C. Electron micrographs of mitochondria at different times (hour) during liver regeneration after partial hepatectomy (PH group).
I. At 0 h, 3 h and 168 h, internal membrane folded, cristae-rich and a dense matrix mitochondria; II. At 6 h and 120 h, cristae reduced, a dilated and paled matrix; III. At 24 h, mitochondria with clear vacuolization and cristae swollen; at 72 h, matrix and cristae also swollen. IV. Magnification: 15000 x. a: vacuolization; b: cristae swelling.

Fig. 2D. Electron micrographs of mitochondria at different times (hour) during liver regeneration interfered by CsA after partial hepatectomy (PH plus CsA group).
I. At 0, 3 and 6 h, a little swollen mitochondria; II. At 24 h, cristae swollen and reduced, a dilated and paled matrix (b); III. At 72 and 168 h, mitochondria contracted in volume, a dense matrix. IV. Magnification: 15000 x. b: cristae swelling; CsA: cyclosporine-A.

were almost consistent with those in PH group, but compared with PH group, the enzyme activities of AST and GDH in cytosol increased significantly at 0 and 3 h after surgery, and decreased significantly at 24, 48 and 72 h, and then returned to normal.

Discussion

In this study, we found that the enzyme activities of mAST and GDH in cytosolic fraction increased significantly at 3 h after PH, and reached the peak at 24 h, which showed that the permeability of mitochondria started increasing at 3 h and reached a maximum at 24 h, possibly causing the release of mitochondrial matrix enzymes. By electron microscopy, we found that mitochondria were swollen obviously at 24 h after PH, to a middle degree at 72 h, and then returned to normal at 120 and 168 h, which was in accordance with the changes of mitochondria proteins release. Guerrieri et al.\cite{18} found that, in the early phase of liver regeneration, mitochondria ultrastructure changed significantly; the increase of mitochondrial membrane permeability induced the release of AST and MDH; and the mitochondrial Ca\(^{2+}\) content increased. The ultrastructure of mitochondria, the membrane permeability properties and the Ca\(^{2+}\) content returned to normal during the replicative phase of liver regeneration. Morales-Gonzalez et al.\cite{19,29} also found that the selective increase
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mechanism need to be clarified. All these findings show that the release of proteins and changes of mitochondria ultrastructure are related to MPT.

In conclusion, the permeability of mitochondria varied during the whole course of liver regeneration, suggesting that the changes of mitochondria swelling is reversible, which is related to MPT. When the permeability of mitochondria increased, some enzymes of mitochondrial matrix were selective to release into cytosol, especially at 24 and 72 h, which is also the main period when DNA synthesis and cell division of liver cell is also the main period when DNA synthesis and cell division of liver cell[31]. The relationship between proteins release in matrix and protein synthesis in cell need to be studied further. It is also not clear whether other kinds of proteins and small molecules can also release from the mitochondrial matrix during liver regeneration. Further studies are required to clarify these issues.

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References


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