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Suppression of Lysosomal Proteolysis at Three Different Steps in Regenerating Rat Liver[1]

Kenichi Watanabe,*,† Kazumi Ishidoh,* Takashi Ueno,* Nobuhiro Sato,† and Eiki Kominami*,[2]

*Department of Biochemistry and †Department of Gastroenterology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421

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Decreased lysosomal proteolysis in regenerating liver after 70% hepatectomy was analyzed. The activities of cathepsins B and L increased transiently 4 h after hepatectomy, began to decrease gradually reaching about 30% of the control level at 24 h, then returned to near control level after 7 days. Immunoblot and RNA blot analyses confirmed that the changes in cathepsin activities coincided with changes in protein levels and mRNA levels. In parallel with the changes in cathepsins, we found that the amounts of LGP120, LGP110, and LGP85, three integral lysosomal membrane proteins, declined significantly after hepatectomy, suggesting that the lysosomal levels are also diminished in regenerating liver. We isolated dextran-loaded lysosomes and found that the protein content and marker enzyme activities of dextran-loaded lysosomes from partially hepatectomized livers are lower by 50 and 40%, respectively, compared with control livers. This indicates that there is a significant reduction in the cellular lysosomal level in regenerating liver. In addition, we used a sensitive biochemical assay to quantify leupeptin-induced autolysosomes and found that the autophagic activity is markedly suppressed in regenerating liver as compared with normal liver. Thus, the suppression of lysosomal proteolysis in regenerating liver is attained through three steps, *i.e.*, decreased biosynthesis of cathepsins, decreased lysosomal biogenesis, and decreased cellular autophagy.

Key words: autophagy, cathepsin, hepatectomy, lysosome, protein degradation.

Lysosomes play a principal role in protein turnover by degrading exogeneous and endogeneous proteins and supplying cells with amino acids necessary for protein synthesis or as an energy source (*1*). Lysosomal proteolysis is caused mainly by cysteine proteinases such as cathepsins B, H, and L (*2*), and is highly dependent on nutritional conditions and various stimuli including hormones (*1*). Previous studies using a liver perfusion system demonstrated that the rate of lysosomal protein degradation varies between 0.23-0.26% of total cell protein/h under feeding conditions and 4.5%/h under starvation conditions (*3*). This is a characteristic of the autophagic process by which long-lived cytoplasmic proteins are sequestered and degraded in lysosomes. Under starvation conditions, autophagic protein degradation is induced by deprivation of extracellular amino acids and/or carbon sources, whereas the degradation rate of endocytosed proteins remains unchanged under both feeding and starvation conditions (*3*).

It is well known that lysosomal proteolytic activities are inversely correlated with cell growth, *i.e.*, lysosomal proteolysis is lower in transformed cells than in parent cells (*4*). The amounts of mRNA for cathepsins B and L are higher in transformed cell lines than in the parent cells, but the activities and amounts of cathepsins B and L are lower since the newly synthesized proteinases are sorted to the extracellular medium instead of to the lysosomes. Furthermore, the suppression of autophagic protein degradation may also contribute to the decreased protein breakdown in transformed cells (*4*).

Further insight into the relationship between lysosomal proteolysis and cell growth has been obtained from the study of regenerating liver. In regenerating liver, decreased protein degradation rather than increased protein synthesis has been shown to be the main cause of the rapid increase in cell mass after hepatectomy (*5, 6*). It has also been found that autophagy is suppressed in regenerating liver (*7*), although the mechanism of this suppression is not

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[2] To whom correspondence should be addressed. Tel: +81-3-5802-1030, Fax: +81-3-5802-5889, E-mail: kominami@med.juntendo.ac.jp

Abbreviations: CI-MPR, cation-independent mannose 6-phosphate receptor; PBS, phosphate-buffered saline; SSC, saline sodium citrate; TBS, Tris-buffered saline; MCA, methylcoumaryl amide; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LGP, lysosomal membrane glycoprotein; LDH, lactate dehydrogenase; CA-074, *N*-(L-3-*trans*-propylcarbamoyloxirane-2-carbonyl)-L-isoleucyl-L-proline; E-64-c, (+)-(2*S*,3*S*)-3-[(*S*)-methyl-1-(3-methylbutylcarbamoyl)-butylcarbamoyl]-2-oxiranecarboxylic acid; Hsc, heat shock cognate protein.

well characterized. Since lysosomal proteolysis is regulated through many steps, *e.g.*, lysosomal proteinase levels, the presence of endogeneous proteinase inhibitors, autophagosome and lysosome formation, the fusion of autophagosomes with lysosomes, *etc.*, it is important to clarify which of these steps play key roles in the decline in lysosomal proteolysis in regenerating liver.

In this study, we analysed extensively the changes in lysosomal proteinase activities in 70% hepatectomized rat livers. We measured the amounts of mRNAs for and cellular levels of cathepsins B, H, and L by Northern blotting and immunoblotting. We also attempted to quantify directly the cellular levels of lysosomes and autophagolysosomes. Our data clearly indicate that lysosomal proteolysis in regenerating liver is suppressed principally through three steps, a decrease in cathepsin content, a change in lysosome quantity, and the formation of autophagolysosomes.

EXPERIMENTAL PROCEDURES

Animals and Materials—Male Wistar rats ranging from 180 to 230 g in weight were maintained in an environmentally controlled room (lights on from 6:00 to 20:00) with a standard pelleted laboratory diet and tap water *ad libitum* for at least 1 week. After starvation for 24 h, 70% hepatectomy was performed between 9 and 11 a.m. according to the method of Higgins and Anderson (*8*) to minimize the diurnal variations in liver DNA synthesis. After surgery, the animals were maintained under the same environmentally controlled conditions. Control rats (0 h) received neither anesthesia nor surgery. The animals were killed by decapitation at the times indicated. Liver samples harvested from both control and partially hepatectomized rats were rinsed in sterile PBS, blotted with filter paper to remove excess blood and fluid, then used to prepare RNA, frozen at −80°C for enzyme assays, or fixed in 4% paraformaldehyde/PBS for *in situ* hybridization experiments. Rat liver cathepsins B (*9*), L (*10*), and H (*11*), and cation-independent mannose 6-phosphate receptor (CI-MPR) (*12*) were purified as described previously and used to raise the rabbit antisera (*12, 13*). Polyclonal antisera directed against the carboxy terminus of Rab 7 (residues 175-191) (*14*), the carboxy-terminal sequence of rat LGP 120 (residues 375-386) (*15*), and the carboxy-terminal sequence of rat LGP 110 (residues 401-411) were used for immunoblot detection of Rab 7, LGP 120, and LGP 110, respectively. Rabbit anti-rat LGP 85 was kindly provided by Dr. Masaru Himeno (Kyushu University). Antibodies were purified by immunoaffinity chromatography using antigen-bound Sepharose 4B prior to use. The specificities of anti-cathepsins B, H, L, anti-CI-MPR, anti-LGPs 120, and 85 sera are the subjects of previous reports (*12, 15*). Reagents were obtained from the following sources: Z-Phe-Arg-methylcoumaryl amide (MCA), Z-Arg-Arg-MCA and Arg-MCA from the Peptide Institute (Osaka); 4-methylumbeliferyl (4-MU) *N*-acetyl-β-D-glucosaminide and 4-MU *N*-acetyl-β-D-glucuronide from Sigma Chemical; nylon membranes (GVHP) and the immunoblot assay kit (ECL) from Nihon Millipore (Tokyo) and Amersham International plc (Buckinghamshire, UK); horseradish-peroxidase-conjugated second antibody (goat anti-rabbit IgG) from Organon Teknika (PA, USA).

Measurement of Enzyme Activities—Cathepsins B, L, and H activities were determined according to the method of Barrett and Kirschke (*16*) with modifications by Katunuma and Kominami (*17*). All assays were performed in triplicate. For assays, the minced liver was sonicated in 9 volumes of PBS and the homogenate was centrifuged at 18,500×*g* for 10 min at 4°C. Aliquots of 10 μl of the supernatants were assayed for cathepsin activities. The substrates used were z-Arg-Arg-MCA for cathepsin B, Arg-MCA for cathepsin H, and z-Phe-Arg-MCA in the presence or absence of 20 μg/ml of CA-074 (*18*) for cathepsin L or cathepsins B+L, respectively. The activities of β-glucuronidase and β-hexosaminidase were assayed at pH 4.0 as described previously (*12*). LDH activity was measured by incubating 40 μl of sample at 25°C in a reaction mixture containing 45 mM potassium phosphate buffer (pH 7.0), 1 mM pyruvate, 0.1 mM NADH, and 0.1% Triton X-100. The decrease in the absorbance of NADH at 340 nm was measured. Catalase was assayed by the method of Daudhuin *et al.* (*19*).

RNA Blot Hybridization Analysis—Total RNA was extracted from fresh rat liver by the acid guanidium thiocyanate-CsCl ultracentrifugation method (*20*). One microgram of denatured RNA was electrophoresed in denatured 0.7% agarose gels containing 2.2 M formaldehyde. The fractionated RNA was transferred onto nylon membranes and fixed on the membranes by ultraviolet light. Prehybridization was performed in 50% formamide, 8.3% dextran sulfate, 0.75 M NaCl, 75 mM trisodium citrate, 50 mM sodium phosphate buffer (pH 7.0), 0.2% BSA, 0.2% polyvinylpyrrolidone 30, 0.2% Ficoll 400, 0.2% SDS, and 300 μg/ml of denatured salmon testis DNA at 42°C for 12 h. Hybridization was performed in the same mixture except for the addition of the ^{32}P-labeled probe for cathepsins B, H, or L at 42°C for 24 h (*21*). The filters were finally washed at 60°C in 15 mM NaCl, 1.5 mM trisodium citrate (0.1×SSC) containing 0.1% SDS, and analyzed with a Bio Image analyzer (BAS 2000).

In Situ Hybridization Analyses—For *in situ* hybridization analyses, a cDNA fragment for cathepsin L generated by *Bam*HI and *Eco*RI digestion (nucleotides −56 to 260 of the cDNA for rat cathepsin L; see Ref. *22*, Fig. 2) was cloned into pBluescript KS(+). After linearization with either *Eco*RI or *Bam*HI, the cRNA probes were transcribed by either T3 RNA polymerase for the anti-sense cRNA or T7 RNA polymerase for the sense cRNA labeled with DIG-11-dUTP using a DIG RNA labeling kit (Boehringer Mannheim). Liver pieces (5×5 mm) were fixed in fresh 4% paraformaldehyde in PBS (w/v) for 16 h at 4°C, incubated in 5, 10, 15, and 30% sucrose in PBS (w/v) for 1 h each at 4°C, and frozen in Optimum Cutting Temperature Compound (Tissue Tek, Miles) before freezing at −70°C. *In situ* hybridization was carried out essentially as described in Ref. *23*. Tissue sections (5 μm thick) were treated with proteinase K in PBS (5 μg/ml) for 30 min at 37°C, fixed with 4% paraformaldehyde in PBS (w/v) for 5 min at room temperature, and incubated with 0.1 M triethanolamine, 0.25% acetic acid for 20 min. The sections were prehybridized for 30 min at 50°C in prehybridization buffer consisting of 50% formamide, 10 mM Tris-HCl (pH 7.6), 200 μg/ml tRNA, 0.1% BSA, 0.1% polyvinylpyrrolidone K30, 0.1% Ficoll 400, 10% dextran sulfate, 0.6 M NaCl, and 0.25% SDS, and hybridized for 16 h at 50°C in prehybridization

buffer supplemented with 1 μg/ml digoxigenin-labeled RNA probe. After consecutive washings with 2×SSC, 50% formamide for 1 h at 55°C (2 times), 2×SSC for 30 min at 55°C, 0.2×SSC for 20 min at room temperature, and 0.1× SSC for 20 min at room temperature, the sections were incubated with RNase A (50 μg/ml in TEN) for 30 min at 37°C, then washed twice with TBS (pH 7.2) for 5 min at room temperature. The sections were then blocked with blocking buffer for 30 min at room temperature to reduce the background and incubated with an alkaline phosphatase conjugate of the anti-digoxigenin antibody for 16 h at 4°C. After washing three times in TBS (pH 7.2) for 5 min each time and in 20 mM Tris-HCl (pH 9.5), 0.9% NaCl for 5 min, the samples were incubated with substrate solution [0.19 μg/μl of 5-bromo-4-chloro-3-indolyl phosphate, 0.5 μg/μl of 4-nitro blue tetrazolium chloride with 20 mM Tris-HCl (pH 9.5), 0.9% NaCl] for 1 h at 37°C in the dark. The samples were then fixed in 10% formalin for 10 min at room temperature, washed with water, and counterstained with Kernechtrot (nuclear fast red).

Isolation of Highly Purified Lysosomes and Autophagolysosomes—Lysosomes were obtained from dextran-injected rat livers as described in Ref. *15*. Dextran (M_r = 70,000) dissolved in PBS was injected intraperitoneally into fasted rats at a dose of 0.2 g/100 g body weight. Twelve hours after injection, the livers were excised, homogenized, and the post-nuclear supernatant was carefully removed. The post-nuclear supernatants were centrifuged at 10,000×g for 20 min. The pelleted mitochondrial-lysosomal fraction was suspended in 2 ml of 0.3 M sucrose. The suspension was loaded onto 23 ml of 57% Percoll containing 0.3 M sucrose and centrifuged at 50,000×g for 40 min. The heavier lysosomal fraction was collected and centrifuged at 100,000×g for 1 h at 4°C to sediment the excess Percoll. The supernatant was diluted with 0.25 M sucrose and centrifuged at 12,000×g for 20 min at 4°C. The pellets were used as the dextran-lysosome fraction for assays of cathepsins B+L and β-glucuronidase and for protein determination. Autophagolysosomes were prepared from rats injected with leupeptin/E64c as previously described (*15*). A leupeptin/E64c mixture (3.5 mg each in 1.0 ml of PBS) was injected intraperitoneally into rats that had been starved for 12 h. One hour after injection, the mitochondrial-lysosomal fraction was prepared as described for the preparation of dextran-lysosomes. The mitochondrial-lysosomal fraction suspended in 0.3 M sucrose was loaded onto 23 ml of 57% Percoll containing 0.3 M sucrose, and centrifuged at 50,000×g for 40 min. Fractions of 1 ml were collected from the bottom to the top. Dense autophagolysosome fractions (1-10 in Fig. 7, panel A) were pooled and centrifuged at 100,000×g for 1 h. To remove Percoll, concentrated autophagolysosomes were suspended in 0.3 M sucrose and centrifuged at 12,000×g for 20 min. The resulting pellets were used for assay of lactic dehydrogenase and catalase. As a reference, the post-nuclear supernatant and its supernatant centrifuged at 100,000×g for 1 h were used for enzyme activities.

Analytical Methods and Reagents—Protein was determined by the BCA protein assay following the manufacturer's protocol (Pierce). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (*24*). Immunoblot analyses were carried out by the method of Towbin *et al.* (*25*). DNA content was measured by the method of Erwin *et al.* (*26*).

Statistics—Values are expressed as means±SEM, and significant differences between controls and hepatectomized rats were analysed by ANOVA.

RESULTS

Changes in the Activities of Lysosomal Cathepsins—The changes in cathepsin activities after 70% hepatectomy showed slightly complicated patterns. Cathepsin B and cathepsin L activities both increased initially by 16% within 4 h after hepatectomy, then began to decrease gradually, reaching about 30% of the control level within 24 h after surgery (Fig. 1, A and C). Over the following days, the activities recovered further, but still remained at 60% (cathepsin B) or 80% (cathepsin L) of control levels 168 h (7 days) after surgery. A similar pattern was observed for the changes in cathepsin H activity, although there was no initial increase (Fig. 1B). The activity of β-glucuronidase, a lysosomal non-proteolytic enzyme, gradually decreased by 50% between 24 and 48 h after hepatectomy, then returned to 70% of the control level at 7 days.

Immunoblot Analyses of Cathepsins B, H, and L—To evaluate whether the apparent changes in cathepsins B, H, and L activities are due to decreases in the amounts of proteinases or to an increase in the amount of an endogenous inhibitor such as cystatin, we next carried out immunoblot analyses to measure changes in the amounts of cathepsins B, H, and L in the regenerating liver.

The single-chain form of cathepsin B with a molecular mass of 29 kDa and the heavy chain with a molecular mass of 25 kDa for each of the two chains could be detected in control liver (Fig. 2A, lane 1). The 5-kDa light chain was not recognized by our antibody. The amount of cathepsin B decreased in the regenerating liver and leveled off 24 h after hepatectomy; the level then began to increase 48 h after surgery, but did not reach the control level. During the course of regeneration, both the single-chain and two-chain forms of cathepsin B were detected. These results are consistent with the changes in cathepsin B activity. Cathepsin H in the control liver migrated as a 31 kDa band that corresponds to the single-chain form (Fig. 2B). Immunoblot analyses with anti-cathepsin H antibody detected only the single chain form of cathepsin H in both control and regenerating liver. The amount of the single-chain form appeared to decrease gradually after hepatectomy, remain at a minimal level for a further 24-48 h, and then increase slowly to reach a level comparable to that of the control. However, these changes were not observed as clearly as those for cathepsins B and L, probably due to a smaller amount of cathepsin H and the weaker immunoreactivity of our antibody. Immunoblot analysis with anti-cathepsin L antibody detected both a 39 kDa band corresponding to the proform of cathepsin L and a 25 kDa band corresponding to the heavy chain of the two-chain form of mature cathepsin L in samples prepared from both control and regenerating livers (Fig. 2C). The two-chain form of cathepsin L, the predominant form in control liver, decreased gradually after hepatectomy. At 72 h after surgery, the cathepsin L content had recovered to a level slightly lower than that in control liver. These results correspond to those for cathepsin L activity. Thus, the changes in cathepsin B, H, and L

activities in regenerating liver can be explained primarily by changes in their amounts.

RNA Blot Hybridization Analyses with ^{32}P-Labeled cDNA Probes for Cathepsins B, H, and L—For hybridization assays, total RNA samples prepared from control and regenerating liver were probed with ^{32}P-labeled cathepsin B cDNA. The mRNA for cathepsin B migrated as a 2.2-kb band (Fig. 3, top). In addition, samples prepared from control and regenerating livers 4 and 8 h after 70% hepatectomy contained small amounts of 4.1-kb RNA. The amount of mRNA for cathepsin B was increased 4 and 8 h after hepatectomy, and then decreased and remained low for 1 week. Twenty-four hours after surgery, the regenerating liver contained the lowest amount of mRNA for cathepsin B. The cathepsin H mRNA migrated as a 1.7-kb band in samples prepared from control and regenerating liver (Fig. 3, middle). The amount of mRNA for cathepsin H in regenerating liver compared with control liver was slightly higher at 4 h, decreased markedly to its lowest levels at 12 and 24 h, and returned to near control levels more than 48 h after operation. The mRNA for cathepsin L migrated as a 1.7-kb band in all samples (Fig. 3, bottom). After partial hepatectomy, the amount of cathepsin L mRNA reached a maximum at 4 h, then decreased rapidly reaching a barely detectable level after 24 h. The mRNA for cathepsin L was scarcely detectable after 72 h. The amounts of all samples were normalized to the amount of GAPDH mRNA in all lanes (data not shown).

Changes in the Distribution of the mRNA for Cathepsin L as Revealed by an In Situ Hybridization Technique—

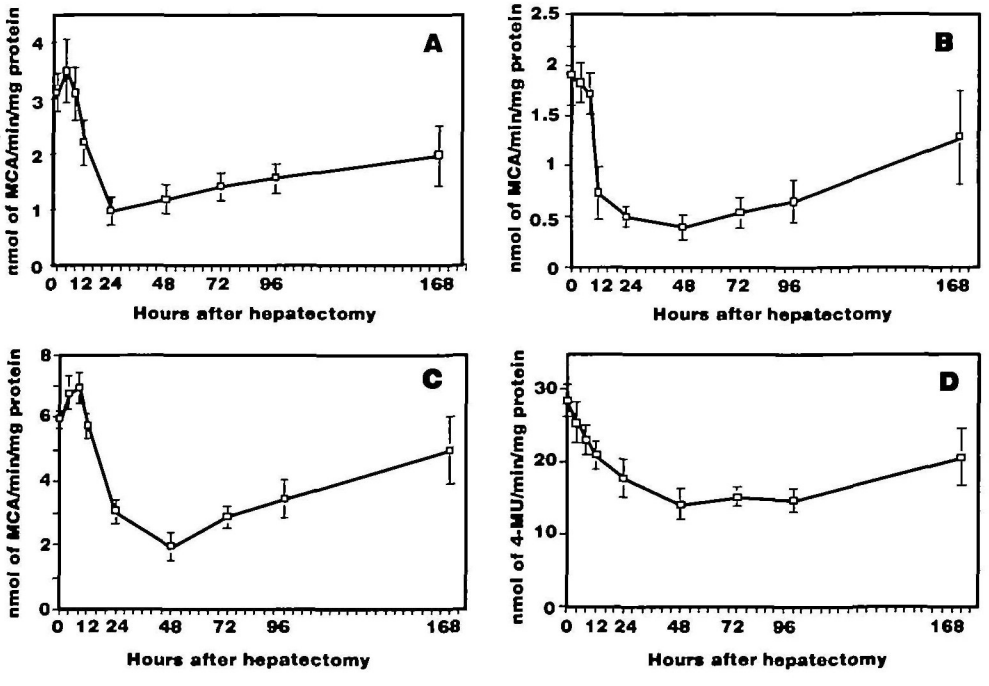

Fig. 1. **Sequential changes in (A) cathepsin B, (B) cathepsin H, (C) cathepsin L, and (D) β-glucuronidase activity after 70% hepatectomy.** For assays, the minced liver was sonicated in 9 volumes of PBS and the homogenate was centrifuged at 18,500×g for 10 min at 4°C. Aliquots of 10 μl of the supernatants were assayed for activities of cathepsins and β-glucuronidase as described in "EXPERIMENTAL PROCEDURES." Control rats, $n=4$; hepatectomized rats, $n=3$. Values are expressed as means± SEM.

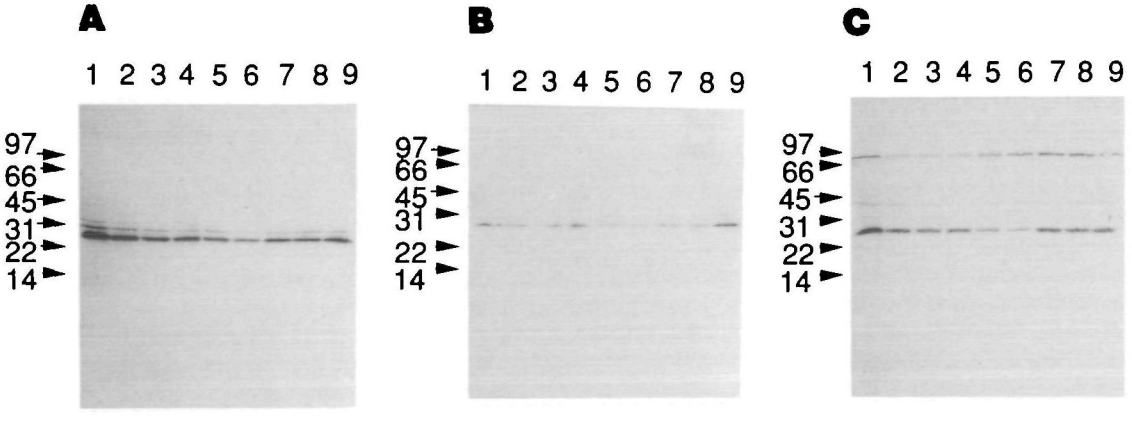

Fig. 2. **Immunoblot analysis of cathepsins B, H, and L in 70% hepatectomized and control rat livers.** SDS-15% polyacrylamide gel electrophoresis was performed as described in "EXPERIMANTAL PROCEDURES." Data from immunoblot analyses with anti-cathepsin B antibody (A), anti-cathepsin H antibody (B), and anti-cathepsin L antibody (C) are shown. The molecular species recognized by these antibodies are: the single-chain form (29 kDa) and the heavy-chain (25 kDa) of mature cathepsin B, the single-chain form (31 kDa) of mature cathepsin H, 39-kDa procathepsin L, and the 25-kDa heavy chain of mature cathepsin L. Lane 1, 0 h (control); lane 2, 4 h; lane 3, 8 h; lane 4, 12 h; lane 5, 24 h; lane 6, 48 h; lane 7, 72 h; lane 8, 96 h; lane 9, 168 h.

Since the hepatic lobe has a complicated structure including parenchymal cells, Kupffer cells, hepatic arteries, central veins, portal veins, and bile ducts, and, in addition, the hepatic parenchymal cells are exposed to metabolic zonation, we analyzed changes in the intralobular distribution of cathepsin mRNAs after 70% hepatectomy. Because RNA blot hybridization analyses showed that the changes in the amount of the mRNA for cathepsin L were much more evident than the changes in the mRNAs for cathepsin B or H, we analyzed changes in the distribution of cathepsin L mRNA by *in situ* hybridization. As shown in Fig. 4A, the mRNA for cathepsin L is distributed homogeneously in the parenchimal cells of control liver. The picture with high magnification is shown in Fig. 4E. After partial hepatectomy, the distribution of cathepsin L mRNA changed so as to exhibit a predominantly peripheral profile in the liver lobules (Fig. 4, B and G, with high magnification). This change in the distribution of cathepsin L mRNA occurred 4 h after hepatectomy when the mRNA levels for cathepsin L increased transiently (Fig. 3). At 12 h, the pattern returned to a homogeneous distribution characteristic of control liver (Fig. 4D). As shown in Fig. 4F, the digoxygenin-labeled sense strand probe for cathepsin L cRNA was not hybridized in the liver under the experimental conditions used. Thus, the hybridization signal is specific for the mRNA for cathepsin L.

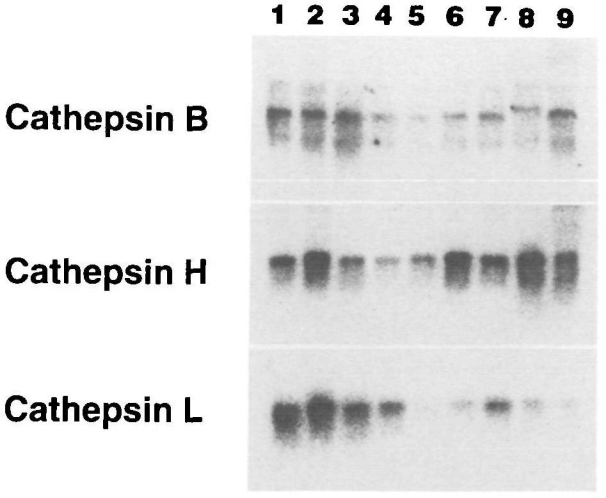

Fig. 3. **RNA blot analysis of the mRNA for cathepsins B, H, and L in rat liver following 70% hepatectomy.** Total RNA from control and partially hepatectomized rat livers was analyzed by RNA blot hybridization using ^{32}P-labeled cDNA probes specific for cathepsins B, H, and L. The amounts of all samples were normalized to the amount of mRNA for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in all lines. Lane 1, 0 h (control); lane 2, 4 h; lane 3, 8 h; lane 4, 12 h; lane 5, 24 h; lane 6, 48 h; lane 7, 72 h; lane 8, 96 h; lane 9, 168 h.

Changes in the Amounts of CI-MPR, rab 7, and Lysosomal Membrane Proteins—Newly synthesized lysosomal hydrolases are sorted in the *trans*-Golgi network by binding to the mannose 6-phosphate receptors (MPR) and are selectively transported to lysosomes through endosomes (*27*). Rab 7 is known to be involved in trafficking CI-MPR and associated lysosomal hydrolases from early endosomes to later endocytic compartments, including late endosomes and lysosomes (*28*). In contrast to soluble hydrolases, lysosomal membrane proteins are delivered to lysosomes independently of MPRs from the *trans*-Golgi network (*29*). Therefore, we analyzed changes in the amounts of CI-MPR, Rab 7, and three major integral lysosomal membrane proteins, LGP-120, LGP-110, and LGP-85, in regenerating liver. Immunoblot analysis of CI-MPR showed a marked increase between 1 to 6 days after 70% hepatectomy following a transient decrease at 12 h. Rab 7 was also increased between 1 and 6 days after hepatectomy. Of

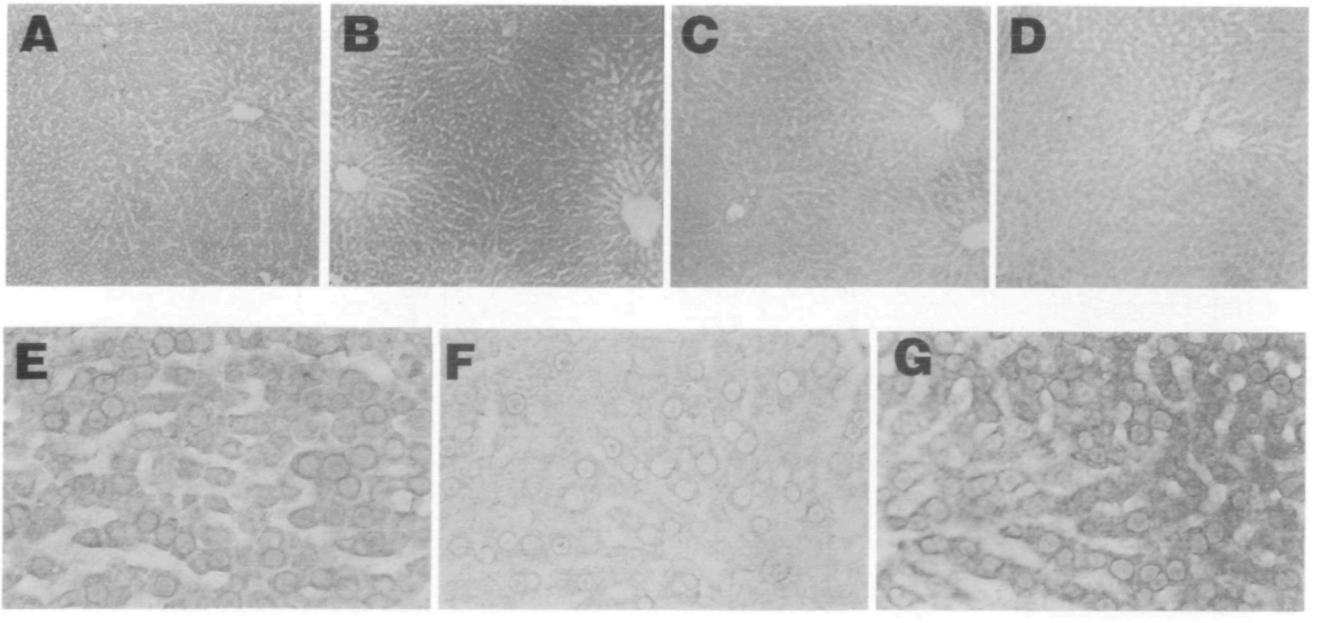

Fig. 4. **Changes in the distribution of the mRNA for cathepsin L in rat livers after 70% hepatectomy and control rat livers *in situ*.** Histological sections (5 μm) of rat liver [control (A, E, and F) or 4 h (B, G), 8 h (C) and 12 h (D) post-partial hepatectomy] were hybridized to either a cathepsin L antisense (A, B, C, D, E, and G) or cathepsin L sense (F) probe. Panels A, B, C, and D, ×30; panels E, F, and G, ×120.

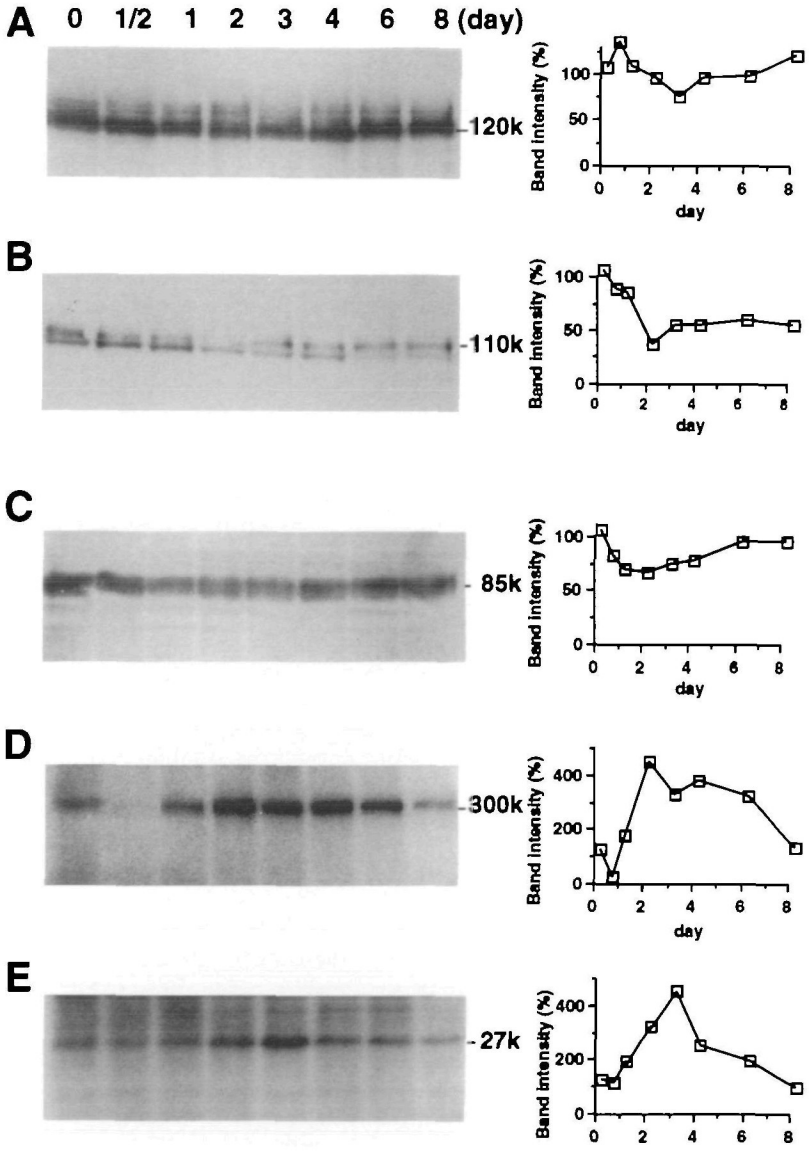

Fig. 5. **Immunoblot analyses of LGP120, LGP110, LGP85, CI-MPR, and Rab 7 in control and 70% hepatectomized rat livers.** SDS-PAGE using 10% acrylamide for LGPs 120, 110, and 85, 7% acrylamide for CI-MPR, and 15% acrylamide for Rab 7 was performed as described in "EXPERIMENTAL PROCEDURES." Data from immunoblot analyses with anti-LGP120 antibody (A), anti-LGP110 antibody (B), anti-LGP85 antibody (C), anti-CI-MPR antibody (D), and anti-rab 7 antibody (E) are shown. Quantitation was by densitometric scanning of fluorograms produced from the immunoblots.

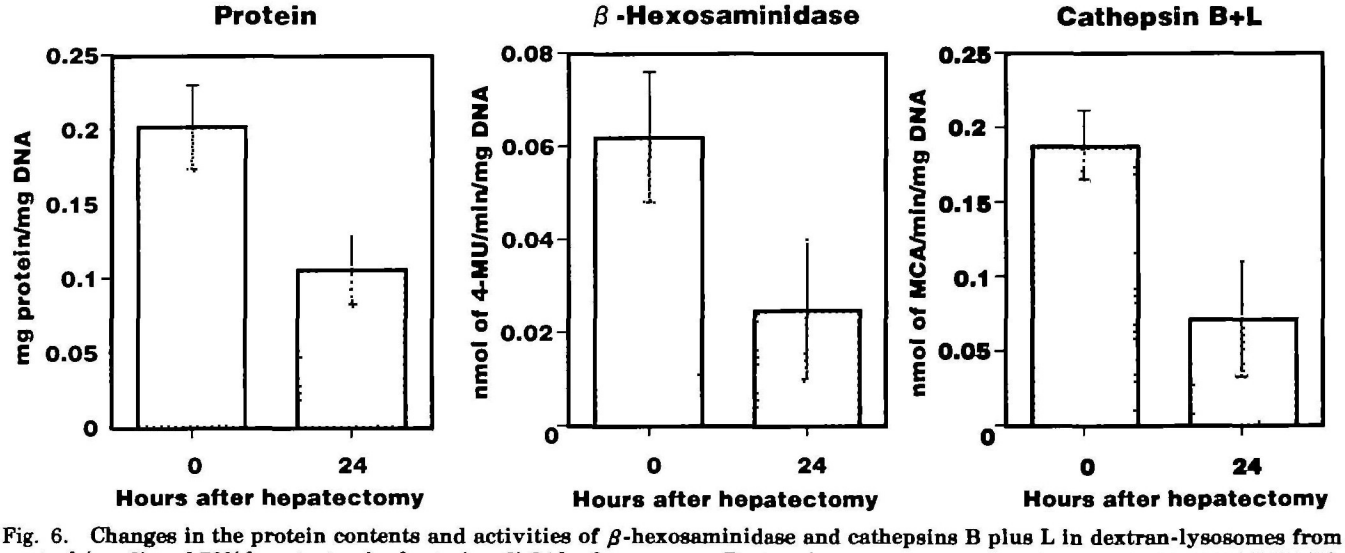

Fig. 6. **Changes in the protein contents and activities of β-hexosaminidase and cathepsins B plus L in dextran-lysosomes from control ($n=3$) and 70% hepatectomized rats ($n=3$) 24 h after surgery.** Dextran-lysosomes were prepared as described in "EXPERIMENTAL PROCEDURES" and used for assay of β-hexosaminidase, cathepsins B plus L, and DNA content. There are significant differences between control and hepatectomized rats ($p<0.5$).

importance was the finding that the levels of both CI-MPR and Rab 7 are elevated during liver regeneration.

The amounts of LGP120 (Fig. 5A) and LGP85 (Fig. 5C), which migrate as bands of 120 and 85 kDa, respectively, appeared to decrease gradually after hepatectomy, reaching 60-65% of the control level within 2-3 days, then increase slowly to levels comparable to those of controls. The amount of LGP110 decreased gradually by 35% 48 h after hepatectomy, and returned to 50% of the control level at 8 day (Fig. 5B). Thus, the amounts of all soluble lysosomal enzymes and membrane proteins tested so far decreased in the liver after partial hepatectomy.

Numbers of Lysosomes in the Liver after Partial Hepatectomy—The decreases in the amounts of all soluble lysosomal enzymes and membrane proteins tested so far in the liver after partial hepatectomy could reflect a reduction in the cellular level of lysosomes. To examine this possibility, we quantified lysosomes and autophagolysosomes. First, we isolated lysosomes from the livers of rats injected with dextran (0.2 g/100 g body weight). Dextran-loaded lysosomes have a much higher density than any other cell organelle and membrane and can be separated by Percoll gradient centrifugation. As shown in Fig. 6, both the protein content and the β-hexosaminidase and cathepsin B plus L activities in lysosomes isolated from rat livers 24 h after 70% hepatectomy were markedly decreased as compared with the levels in control livers; the ratios of protein content, β-hexosaminidase, and cathepsins B plus L activities between the experimental and control livers were 0.53, 0.40, and 0.38, respectively. These results suggest a reduced level of hepatic lysosomes in the partially hepatectomized livers in comparison with control livers, but the extent of the suppression of enzymatic activities in the isolated lysosomes was smaller than that of lysosomal enzyme activities determined in liver extracts.

We next analyzed the ability to form autophagolysosomes. The inhibition of lysosomal proteolysis by E64c and leupeptin results in an accumulation of autophagolysosomes filled with various undigested cytoplasmic components (*15, 30, 31*). Owing to their sequestered cytosolic

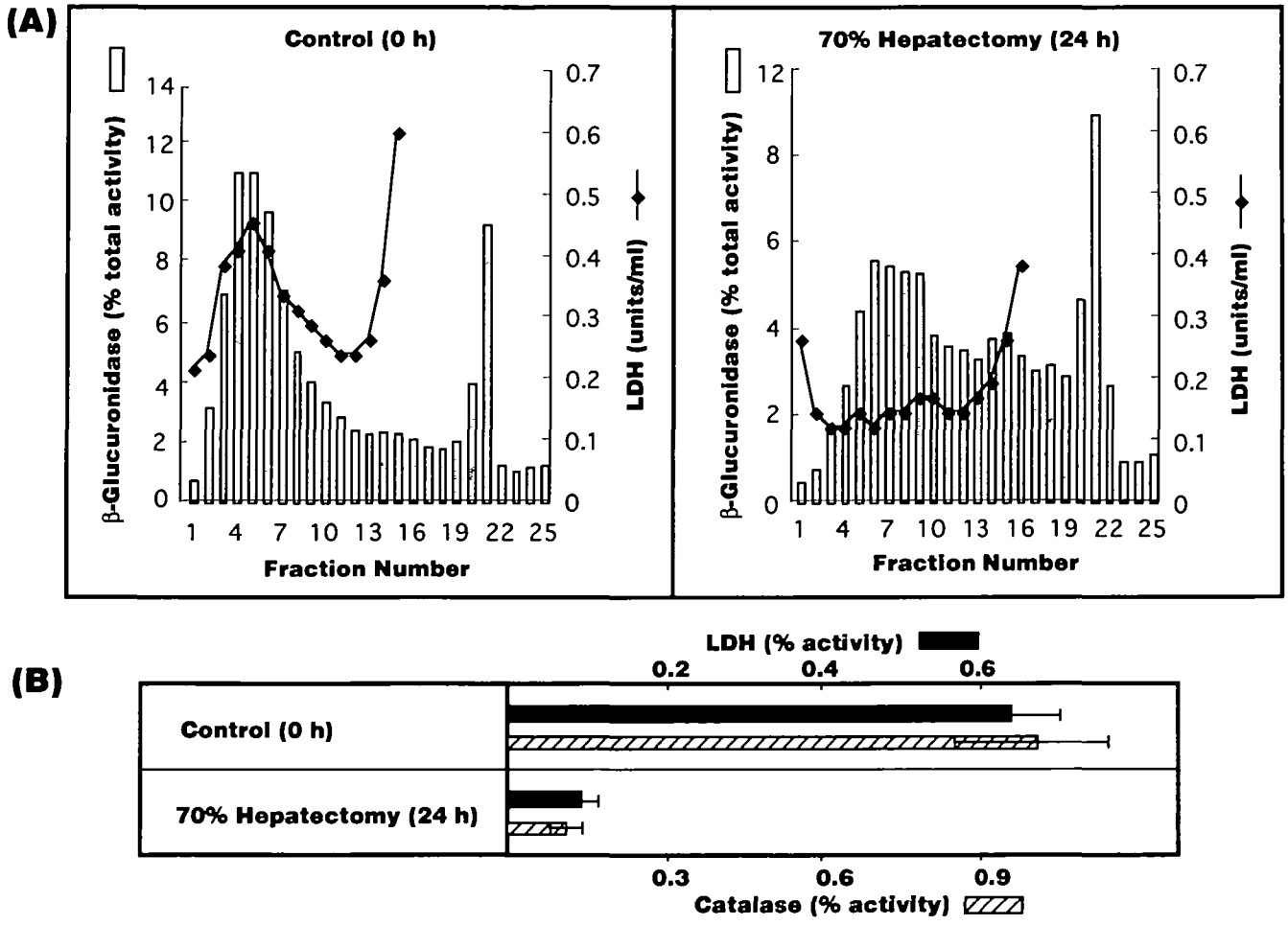

Fig. 7. **Analyses of autophagolysosome formation.** Autophagolysosomes were isolated from control and 70% hepatectomized rats 24 h after surgery as described in "EXPERIMENTAL PROCEDURES." Dense autophagolysosome fractions (1-10), separated from the bulky cellular membranes including unfused lysosomes by Percoll gradient centrifugation, were assayed for β-glucuronidase (lysosomal marker) and sequestered cytoplasmic enzyme (LDH) activities (panel A). Dense autophagolysosome fractions (1-10) were pooled and centrifuged at 100,000×*g* for 1 h to remove Percoll. Concentrated autophagolysosomal fractions were used for assay of LDH and catalase in the presence of 0.1% of Triton X-100. As a reference, the postnuclear supernatant and its supernatant centrifuged at 100,000×*g* for 1 h were assayed for catalase and LDH, respectively. Activities in autophagolysosomes are given as percentage of activities in the supernatant (panel B).

components, autophagolysosomes have much higher density than any other cell organelle, and hence autophagolysosomes can be separated by Percoll density gradient centrifugation. The activities of lactate dehydrogenase (LDH), a cytosolic enzyme, and of catalase, a peroxisomal enzyme sequestered in the heavy autolysosome fraction, are a useful and sensitive indicator of cellular autophagic activity (*31, 32*). In control liver (Fig. 7A), LDH activity was detected in the heavy-density autolysosomal fraction that also possesses lysosomal marker β-hexosaminidase activity. In contrast, the heavy density fraction from partially hepatectomized liver lacked this LDH activity (Fig. 7A). Moreover, the shift in β-hexosaminidase activity to the heavy density fraction was markedly impaired in hepatectomized livers. The isolated autophagolysosome fractions from partially hepatectomized liver showed 7-9-fold decreases in the activities of sequestered LDH and catalase (Fig. 7B). Taken together, the results suggest that autophagolysosome formation, as well as the number of lysosomes, is severely suppressed in partially hepatectomized rat liver.

DISCUSSION

During regeneration, the liver falls into a growth phase immediately after 70% hepatectomy with hepatocyte proliferation accompanying the degradation of extracellular matrix proteins to reconstitute the perihepatic area. Thus, during the early stages of liver regeneration, the digestion of extracellular matrix proteins taken up into the lysosomes by proteolytic enzymes plays a role in extrahepatic remodeling. It has been reported that the lysosomal activities of hyauronidase, β-*N*-acetylglucosaminidase, acid phosphatase, and cathepsin D change following partial hepatectomy (*33*): both cathepsin D and hyauronidase activities are found to be elevated 9 h after surgery, at which time neither the β-glucosaminidase nor acid phosphatase activity shows any significant change, suggesting that hyauronidase and cathepsin D are likely to be involved in extrahepatic remodeling. The activities of these four enzymes are all decreased 18 h after partial hepatectomy (*33*). To evaluate changes in the activities of other lysosomal enzymes, such as cathepsins B, H, and L, that might also be involved in the degradation of proteins taken up by the lysosomes from the extracellular space, we measured their activities at various times after partial hepatectomy.

The results presented in this paper clearly show that the time-dependent changes in the activities of cathepsins B and L are similar to those of cathepsin D and hyauronidase, whereas changes in the activities of cathepsin H and β-glucuronidase resemble those of β-*N*-acetylglucosaminidase and acid phosphatase (*33*). Our results showing the recovery of cathepsins B and L activities 3 days after hepatectomy are consistent with those of a recently published report (*34*). Cathepsins B and L, as well as cathepsin D and hyauronidase, are thought to participate in the degradation of extracellular matrix proteins including collagens, fibronectin, and laminin (*35, 36*). We showed recently that procathepsin L and its processed form secreted from ras-transformed NIH 3T3 cells can degrade types I and IV collagens, fibronectin, and laminin (*37*). The transient induction of cathepsins B and L during the early stages of liver regeneration (within 12 h after hepatectomy) can, therefore, be related to the digestion of extracellular matrix proteins taken up from the perihepatic space.

After more than 12 h, the activities of cathepsins B, H, and L decrease synchronously as reported by Suleiman *et al.* (*38*) and Fiszer-Szafarz and Nadal (*33*). In view of the finding by Barett and Kirschke (*16*) that collagen can be hydrolyzed by cathepsin L but not by cathepsin B or H, our data indicate that cathepsin L is the key lysosomal enzyme involved in the degradation and resynthesis of the extrahepatic matrix, supporting the previous observation by Yamamoto *et al.* (*34*) that the amount of collagen synthesis is inversely correlated with the activities of cathepsins B and L in regenerating liver.

The changes in the activities of cathepsins B, H, and L can be explained by the changes in the amounts of the enzymes following the induction of their mRNAs. *In situ* hybridization analysis using a cathepsin L probe showed that the signals for cathepsin L mRNA occur mainly in the periportal region (zone 1) of the liver after partial hepatectomy. Although the amounts of lysosomal enzyme transport receptor, CI-M6PR, increased markedly at 24-48 h after hepatectomy (Fig. 5), it is possible that newly synthesized procathepsin L is excreted from parenchymal cells because of its lower affinity for CI-M6PR than other cathepsins. Since it has been reported that intraperitoneally-injected cathepsin L augments hepatic DNA synthesis *in vivo* (*39*), it is possible that the secreted cathepsin L participates in DNA synthesis in the periportal region, where DNA synthesis starts early after hepatectomy (*40*).

In parallel with the changes in cathepsins, we found that the amounts of LGP120, LGP110, and LGP85, three integral lysosomal membrane proteins, decline significantly after hepatectomy. Most lysosomal hydrolases and membrane proteins return to control levels by 7-8 days after hepatectomy along with the cessation of proliferation and restoration of the hepatic architecture. The marked induction of CI-MPR and Rab 7 during regeneration appears to be related to the restoration of differentiated hepatic function and lysosomal biogenesis. Jirtle *et al.* (*41*) reported that CI-MPR is upregulated during liver regeneration and that the increased expression of this receptor leads to the accumulation of a high concentration of TGF-β1 in hepatocytes. Although CI-MPR may function mainly in the binding and endocytosis of latent TGF-β1 as the authors suggest during early phase of liver regeneration, it may also help to restore lysosomal hydrolytic functions during the late phase.

Previous morphological analysis (*7*) has shown that a loss of autophagic vacuoles occurs one day (19-33 h) after partial hepatectomy, suggesting that autophagy is suppressed in the regenerating liver. In a sensitive, quantitative assay for autophagolysosome formation, we demonstrated that LDH and catalase activities that are sequestered into the heavy-density autophagolysosomes are remarkably decreased in regenerating liver. Thus, our biochemical data are consistent with the above histochemical data (*7*). In addition, we also demonstrated that hepatic lysosomal levels are reduced in the regenerating liver. This is a new finding suggesting that partial hepatectomy may cause the inhibition of lysosome biogenesis. The data indicate that the observed suppression of autophagolysosome formation is probably due at least in part to the decrease in lysosomes, because autophagolysosomes

are formed by the fusion of autophagosomes and lysosomes. Of course, our results do not rule out the possibility that autophagosome formation itself is also suppressed after partial hepatectomy. In order to obtain a more thorough understanding of the mechanisms of the reduced autophagy in regenerating liver, separate experiments examining autophagosome formation and lysosome biogenesis are necessary.

Recently, it was reported that there are two species of lysosomes in rat liver (*42*), Hsc 73-positive lysosomes and Hsc 73-negative lysosomes. The former participate in the degradation of cytosolic proteins through an autophagic process, while the latter are not involved in the degradation of cytosolic proteins. The degradation of Hsc 73 occurs faster in Hsc-negative lysosomes than in Hsc-positive lysosomes. This difference derives from the difference in intralysosomal pH, *i.e.*, the intralysosomal pH of Hsc 73-positive lysosomes is 5.2, while that of Hsc 73-negative lysosomes is 6.0 (*42*). In the present study, autophagolysosomes were not detected in regenerating liver, while the number of lysosomes was about 50% of that in control liver. Thus, intralysosomal Hsc 73 is presumably degraded in regenerating liver. Since the intralysosomal pH in Hsc 73-negative lysosomes is higher than in Hsc 73-positive lysosomes, intralysosomal cathepsins would also be degraded faster in Hsc 73-negative lysosomes than in Hsc 73-positive lysosomes. In this context, the lower amounts of lysosomal cathepsins in regenerating liver than in control liver might arise not only from the suppression of *de novo* cathepsin synthesis but also from acceleration of the degradation of cathepsins. These hypotheses can be confirmed by measuring intralysosomal pH and the degradation rates of cathepsins in regenerating liver.

In summary, we found that cathepsin activities change in a complicated manner depending on the time after hepatectomy. A small increase in activities occurs within 12 h of hepatectomy followed by a steep decline from 12-24 h. After 24 h, the activities are gradually restored, finally reaching about 70% of control levels 1 week after hepatectomy. The changes in cathepsin activities correlate well with changes in the cellular enzyme levels; in the case of cathepsin L, the cellular content follows the quantitative changes in its mRNA. The signals for cathepsin L mRNA occur in the periportal region as early as 4 h after surgery. In accordance with the changes in the lysosomal enzyme activities, the number of lysosomes was found to be diminished in regenerating liver. This decrease in the number of lysosomes might be an important mechanism by which autophagy is markedly suppressed in hepatectomized liver.

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