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GENE TARGETING IN HEMOSTASIS. HEPSIN

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1. ABSTRACT

Hepsin is a type II transmembrane serine protease abundantly expressed on the surface of hepatocytes. Biochemical studies have shown that hepsin is an enzyme of 51 kDa with the trypsin-like substrate specificity. Several *in vitro* studies have suggested that hepsin may play a role in blood coagulation, hepatocyte growth, and fertilization. To determine the functional importance of hepsin, hepsin-deficient mice were generated by homologous recombination. Homozygous *hepsin-/-* mice were viable and fertile, and grew normally. When analyzed in hemostasis assays, such as tail bleeding time and plasma clotting times, and *in vivo* modes, such as disseminated intravascular coagulation, septic shock, and acute liver regeneration, *hepsin-/-* mice had similar phenotypes as wild-type controls. Liver weight and serum concentrations of liver-derived proteins or enzymes were also similar in *hepsin-/-* and wild-type mice. No abnormalities were identified in major organs in *hepsin-/-* mice in histological examinations. These results indicate that hepsin is not an essential enzyme for normal hemostasis, embryogenesis, and maintenance of normal liver function. Unexpectedly, serum concentrations of bone-derived alkaline phosphatase were approximately two-fold higher in both male and female *hepsin-/-* mice than those in wild-type controls. The underlying mechanism for this phenotype and long-term effects of hepsin deficiency remain to be determined.

2. INTRODUCTION

Serine proteases are important for a variety of biological processes including food digestion, complement activation, and blood coagulation (1-4). In *Drosophila*, serine proteases-mediated pathways are also essential for embryonic development (5). For example, serine proteases encoded by the *nudel*, *gastrulation defective*, *easter* and *snake* genes are key components of a proteolytic cascade critical for the establishment of the dorsal-ventral pattern in developing embryos. Genetic defects in these genes often

lead to the disruption of the dorsal-ventral axis, resulting in embryonic lethality (6-9).

Most serine proteases of the trypsin family are secreted proteins. More recently, new members have been identified that contain an integral transmembrane domain, making them a structurally distinct subclass in this extended trypsin-like serine protease superfamily. The membrane bound serine proteases include enterokinase (10-13), hepsin (14), corin (15-17), membrane-type serine protease 1 (MT-SP1) (18-20), human airway trypsin-like protease (21), TMPRSS2 (22,23), TMPRSS3 (24), and Stubble-stubbloid (25). Structurally, these serine proteases are type II transmembrane proteins with the transmembrane domain near the amino terminus and the protease domain at the carboxyl terminus in the extracellular region. The importance of this emerging class of transmembrane serine proteases already has been recognized (26).

Hepsin is an early member of this new class of transmembrane serine protease. Human hepsin cDNA was first isolated by homologous cloning from a liver library (14). Hepsin mRNA is most abundantly expressed in hepatocytes. Several studies have implicated that hepsin may have functions in blood coagulation (27), cell growth and differentiation (28), and early embryonic development (29). This article will review the molecular biology, biochemistry and functions of hepsin, and summarize phenotypic analysis of hepsin-deficient mice.

3. HEPSIN cDNA AND GENE STRUCTURES

3.1. Cloning of hepsin cDNA

To identify novel serine protease genes, a human liver cDNA library was screened with degenerate oligodeoxynucleotides synthesized based on a conserved amino acid sequence near one of the serine protease active sites (14). A new cDNA was identified that was approximately 1.8 kb in length and encoded a novel serine protease. The serine protease was designated hepsin for its abundant expression in hepatocytes. Northern analysis showed that, in addition to the liver, low levels of human hepsin mRNA were present in other tissues such as kidney, pancreas, prostate and thyroid (30, 31). Cellular distribution of hepsin mRNA in these tissues has not been determined. Most recently, hepsin expression was reported in several types of cancer including ovarian cancer (31) and renal cell carcinoma (32). The hepsin expression in cancer cells may contribute to protease-mediated tumor invasion and metastasis.

After the cloning of human hepsin cDNA, rat and mouse hepsin cDNA clones were also isolated (29, 33, 34). Both rat and mouse hepsin cDNAs share significant (~88 %) sequence homology with human hepsin cDNA. In mice, two alternatively spliced forms of hepsin mRNA were identified (29). On Northern blots, these two alternatively spliced forms appeared as 1.8- and 1.9-kb bands, respectively. The abundance of these two bands was similar. Further PCR analysis and cDNA cloning showed that the 1.9-kb species was generated by an in-frame insertion of a 60-bp sequence near the 5'-end of the open reading frame. The insertion results in an addition of 20 amino acids within the cytoplasmic domain of mouse hepsin protein. To date, such alternatively spliced forms have not been reported in other speices and the functional significance of these isoforms remains to be determined.

3.2. Hepsin gene structure

The human hepsin gene was mapped to chromosome 19 at q11-13.2 by Southern analysis of a panel of rodent-human somatic hybrid cell lines (30). To date, no human genetic diseases have been reported to be closely associated with the hepsin gene locus. The mouse hepsin gene has been isolated from genomic libraries prepared from 129Sv strain mice (34, 35). The structure of the mouse hepsin gene was determined by restriction digestion and DNA sequencing. The gene is approximately 17 kb in length and contains 14 exons (34). Analysis of cDNA clones from RT-PCR reactions indicated that at least four mRNA forms of mouse hepsin were generated by alternative splicing of the first three exons. The data are consistent with the first report of the alternatively spliced forms of mouse hepsin mRNA (29). It is interesting to note that the exon/intron organization of the hepsin gene is closer to those of the plasminogen and prothrombin genes than that of trypsin (36-38). This suggests that the serine proteases with multiple domain structures may have a similar evolutionary history.

3.3. The promoter sequence of the mouse hepsin gene

A sequence of 274 bp upstream of the transcription initiation site was isolated in the mouse hepsin gene (34). Within this sequence, potential binding sites for several transcription factors such as SP1, AP2, C/EBP, LF-A1 and E box have been identified. To determine the promoter activity of the 5'-flanking sequence, expression plasmids were constructed and assayed in HepG2 cells (34). A strong promoter activity was detected in HepG2 cells, indicating that the sequence contained the essential structural elements required for the gene expression of hepsin. Interestingly, inclusion of an intron sequence from the 5'-UTR in the expression vector greatly enhanced the hepsin promoter activity. Further characterization of the hepsin gene promoter may help to understand the mechanism for the predominant liver expression pattern of hepsin.

4. HEPSIN PROTEIN

4.1. The domain structure of hepsin

The open reading frame of the human hepsin cDNA encodes a polypeptide of 417 amino acids with a calculated mass of 45 kDa (Figure 1). At the amino terminus of the predicted hepsin protein, there is no discernible signal peptide sequence. Hydropathy plots identified a highly hydrophobic region between amino acid residues 18 and 44 that could serve as a transmembrane domain (14). There are more positively charged amino acid residues immediately preceding the putative transmembrane segment, suggesting that hepsin is a type II transmembrane protein with the amino terminus present the cytosol (39).

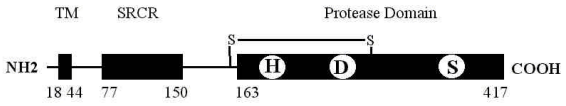

Figure 1. A schematic presentation of the domain structure of hepsin protein. The transmembrane domain (TM), scavenger receptor cysteine-rich domain (SRCR) and serine protease catalytic domain (Protease Domain) with active-site residues histidine (H), aspartate (D) and serine (S) are indicated. Numbers correspond to the amino acid residues of human hepsin. A disulfide bond (s-s) connecting hepsin two subunits after the activation cleavage is also shown.

Immediately after the transmembrane domain, there is a cysteine-rich region between amino acid residues 45 and 162 in human hepsin, which is homologous to the cysteine-rich motif originally found in the macrophage scavenger receptor (40, 41). Such a motif is also present in several other transmembrane serine proteases including enterokinase (10), TMPRSS2 (22) and corin (15), suggesting that the cysteine-rich domains may be functionally important. Within this region, a potential *N*-linked glycosylation site at amino acid residue 112 in human hepsin is noted.

The trypsin-like protease domain of hepsin is located at the carboxyl terminus of the protein between amino acid residues 163-417 with essential features of serine protease sequences being well conserved. The active site residues of the catalytic triad are located at His203, Asp257, and Ser353. The amino acid residues forming the substrate specificity pocket are located at Asp347, Gly378, and Gly388, predicting that hepsin cleaves its substrate(s) after basic residues, such as lysine and arginine. In addition, a putative activation cleavage site is found at Arg162, suggesting that hepsin is synthesized as an inactive zymogen and that a proteolytic cleavage is required for its activation. After the activation cleavage, the catalytic domain of hepsin is predicted to be attached to the rest of molecule by a disulfide bond between residues Cys153 and Cys277. The corresponding cysteine residues are commonly found in other two-chain serine proteases, such as chymotrypsin (42, 43), prekallikrein (44) and prothrombin (45).

4.2. Biochemical properties of hepsin

4.2.1. Molecular weight

Tsuji *et al*. purified human hepsin from HepG2 cells (30, 46). Western analysis following SDS-PAGE identified hepsin as a major band of 51 kDa. In cell-free translation assays, recombinant human hepsin appeared as a band of 44 kDa in SDS-PAGE. The results were consistent with the calculated mass of 45 kDa based on the human hepsin cDNA sequence. When subcellular components of HepG2 cells were fractionated by Percoll density gradient centrifugation, hepsin was detected in cell membrane fractions but not in the cytosol or conditioned medium, confirming the prediction from sequence analysis that hepsin is a transmembrane protein (30). In HepG2 cells, hepsin appeared to be synthesyzed as a zymogen because the major 51 kDa band was detected in Western analysis under both reducing and non-reducing conditions (30). A smaller minor band of 28 kDa was also detected in the Western analysis, which may represent the catalytic subunit of hepsin derived from proteolytic cleavage. It is not clear, however, if the proteolytic cleavage occurred on the HepG2 cell surface or during the purification.

In another study, rat hepsin was purified from liver microsomal membranes to homogeneity by hydroxyapatite, DEAE-Sepharose and benzamidine-Sepharose chromatography (47). Analysis of the purified protein showed that rat hepsin had a molecular mass of 50 kDa in SDS-PAGE and that rat hepsin was activated during the purification by proteolytic cleavage at the predicted activation cleavage site between residues Arg161 and Ile162. On reducing gels, the activated rat hepsin appeared as a 31 kDa band and a 19 kDa band, representing two subunits of the mature protein.

4.2.2. Membrane topology

Analysis of hepsin protein sequence suggests that hepsin is a type II transmembrane protein with its protease catalytic domain present on the cell surface (14). The predicted membrane topology was confirmed in several independent experiments. Tsuje *et al* showed that a specific antibody raised against synthetic peptides derived from the catalytic domain of hepsin immunostained HepG2 cells in the presence or absence of Triton X-100 (30, 48). In addition, limited proteolysis of intact HepG2 cells by low concentrations of either trypsin or proteinase K resulted in significant degradation of the carboxyl terminal portion of hepsin. In a separate study, rat hepsin from the liver microsomal vesicles was protected from proteinase K digestion in the absence of detergent, confirming the topology of a type II transmembrane protein (47).

4.2.3. Substrate specificity

The substrate specificity of hepsin toward synthetic small peptides was examined using purified human and rat hepsin (46, 47). In a series of assays using chromogentic and fluorogenic peptide substrates, hepsin was found to cleave basic but not aromatic or aliphatic amino acid residues. For the basic residues at the S1 position, an Arg residue was much more favored than a Lys residue. Interestingly, an Arg residue also appeared to be favored at the S2 position (47). The results were consistent with the amino acid residues of hepsin that form the substrate specificity pocket: Asp347 at the bottom and Gly378 and Gly388 on two sides (14).

4.2.4. Effect of inhibitors

In small peptide substrate-based assays, the activity of hepsin was inhibited by inhibitors specific for trypsin-like serine protease, such as leupeptin, antipain, N^{α}-tosyl-L-lysine chloromethyl ketone and soybean trypsin inhibitor (47). EDTA had little effect on the activity of hepsin, suggesting that divalent ions are not required for its proteolytic activity toward small peptides. In a separate study, effects of serine protease inhibitors were tested on

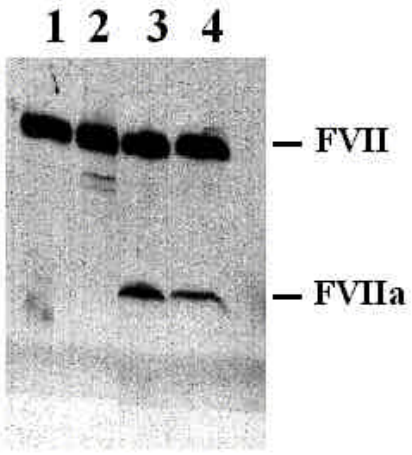

Figure 2. Conversion of FVII to FVIIa by hepsin expressing BHK cells. Human recombinant hepsin was stably expressed in BHK cells. The hepsin expressing cells (~ 8 x 10^5) (lanes 3 and 4), or control BHK cells (lanes 1 and 2), were incubated in a phosphate-buffered saline (pH 7.4) containing purified human plasma FVII (0.2 μg/ml) at 37°C for 30 min. Conversion of FVII to FVIIa was analyzed by Western blotting using a sheep anti-human FVII antibody. The activity of FVIIa was confirmed in a chromogenic substrate-based assay.

human hepsin expressed on the cell surface (27). Human hepsin activity was inhibited by 4-amidiophenylmethylsulfonyl fluoride, aprotinin and antithrombin III. Soybean trypsin inhibitor, however, did not inhibit hepsin in the cell-based assay (27). In addition, EDTA inhibited hepsin-mediated activation of factor VII in this assay. The apparently different effects of soybean trypsin inhibitor and EDTA on hepsin may be due to different properties of soluble and membrane-associated hepsin forms toward the small and macromolecular substrates.

5. FUNCTIONS OF HEPSIN

5.1. Activation of blood coagulation factor VII

Despite its interesting structural features and tissue-specific expression patterns, the biological function of hepsin is poorly understood. Kazama *et al*. first suggested that hepsin may play a role in the initiation of blood coagulation (27). In an *in vitro* study, recombinant human hepsin expressed on the surface of baby hamster kidney (BHK) cells activated blood coagulation factor VII (FVII), an essential enzyme that acts at the top of the tissue factor-dependent coagulation pathway (4). The reaction appeared to be highly specific because hepsin did not activate other structurally related proteases such as factors IX and X, prothrombin, and protein C under similar experimental conditions. The hepsin-mediated FVII activation was shown to be sufficient to initiate the coagulation pathway leading to thrombin generation (27). We have confirmed the activity of hepsin in factor VII activation in a similar cell-based experiment. In our study, recombinant human hepsin was expressed in stably transfected BHK cells. Incubation of FVII with hepsin expressing BHK cells, but not control BHK cells, resulted in the conversion of FVII to FVIIa, as demonstrated by Western analysis (Figure 2) and chromogenic substrate-based assays (data not shown). Similar results were also obtained in transient transfection experiments using COS-7 cells. Kazama *et al*. also showed that the hepsin-dependent factor VII activation was inhibited by antithrombin III but not tissue factor pathway inhibitor (27).

5.2. Promotion of hepatocyte growth

In addition to FVII activation, hepsin was reported to play a role in hepatocyte growth in a cell culture system. Torres-Rosado *et al*. showed that the presence of anti-hepsin antibodies or hepsin-specific antisense oligonucleotides significantly altered the morphology of PLC/PRF/5 hepatoma cells and inhibited their growth (28). A similar inhibitory effect of the antibodies was also observed in HepG2 cells. This finding is intriguing because serine proteases are known to have growth factor-like activities. For example, thrombin is a potent mitogen for vascular fibroblast and smooth muscle cells (49, 50). Furthermore, several growth factors, such as hepatocyte growth factor (HGF) and the product of growth arrest-specific gene 6 (Gas6), share striking sequence and structural similarities with trypsin-like serine proteases (51, 52), and HGF is critical for the development of several epithelial organs including liver and placenta (53, 54). It is possible that hepsin acts either directly as a growth factor or indirectly as an enzyme that processes a growth factor important for cell growth and differentiation.

5.3. Embryonic development

Membrane-bound serine proteases are known to be important in development. For example, the serine protease Stubble-stubbloid, structurally related to hepsin, is essential for epithelial morphogenesis of imaginal discs in *Drosophila melanogaster* (25). Genetic studies demonstrated that defects in the *Stubble-stubbloid* gene lead to malformation of legs, wings and bristles in the fruitfly (55). In mammalian development, serine proteases are implicated in digestion of the zona pellucida, the extracellular coat of blastocysts (56). The proteolysis-mediated hatching is critical for a fertilized embryo to be implanted in the uterus. In a study to identify membrane-associated serine proteases expressed in mammalian preimplantation embryos, Vu *et al*. isolated and cloned mouse hepsin in early blastocysts (29). PCR analysis showed that hepsin mRNA was present in mouse embryos as early as the two-cell stage and that the mRNA was most abundant in early blastocysts prior to hatching. It was hypothesized that hepsin may be the elusive enzyme that is involved in blastocyst hatching (29).

6. HEPSIN-DEFICIENT MICE

6.1. Disruption of the hepsin gene

To assess the biological importance of hepsin, two laboratories independently create hepsin-deficient mice by homologous recombination techniques (35, 57). The design of targeting vectors was very similar in these two

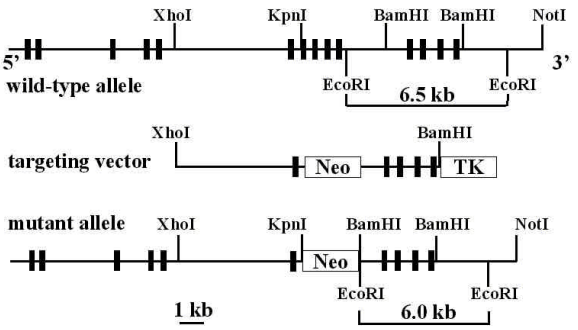

Figure 3. Disruption of the mouse hepsin gene by homologous recombination. The genomic structure of the mouse hepsin gene is shown on top. Exons (black boxes) and selected restriction sites in the hepsin gene (wild-type allele) are indicated. The targeting vector was constructed by replacing of the KpnI/BamHI fragment with a neo gene cassette. A tk gene cassette was included at the 3'-end of the vector. The predicted genomic structure after homologous recombination (mutant allele) is also shown.

studies. The targeting vectors were constructed to replace either exons 7-10 (Figure 3) (35) or exons 9 and 10 (57) with a *neo* cassette. In mouse hepsin gene, exons 7-8 encode for amino acid residues 97-150 and exons 9-10 for amino acid residues 151-269 (34). These amino acid sequences contain the activation cleavage site, Arg161-Ile162, and two serine protease active sites, His202 and Asp256. Deletion of these functional residues is expected to abolish the catalytic activity of hepsin completely. The targeting vectors were transfected into embryonic stem (ES) cells to disrupt the hepsin gene. Mutant ES cells were selected and used to generate chimeric mice that were further bred to obtain heterozygous and homozygous hepsin-deficient mice. In homozygous hepsin-deficient mice, the absence of hepsin mRNA expression in liver samples was confirmed by Northern and RT-PCR analyses (35, 57). In both studies, no truncated forms of hepsin mRNA were detected in liver samples from hepsin-/- mice. As a control, prothrombin mRNA expression was not affected in liver samples from hepsin-/- mice (35).

6.2. Viability, growth, and fertility of hepsin-/- mice

Genotyping of over 600 offspring from the breeding of hepsin+/- mice showed an approximately 1:2:1 ratio for wild-type, hepsin+/- and hepsin-/- mice. The observed Mendelian pattern of inheritance indicates that homozygous hepsin deficiency is compatible with normal embryonic development. When examined at 3 weeks, male to female ratio was approximately 1:1 in wild-type, hepsin+/-, and hepsin-/- mice. When followed up to two years, hepsin-/- mice appeared to develop normally and exhibited similar body weight gain as wild-type and hepsin+/- littermates. No obvious physical abnormalities were observed in hepsin-/- mice. Both male and female hepsin-/- mice were fertile and produced viable offspring, suggesting that hepsin is not essential for reproduction and fertilization.

6.3. Hemostasis in hepsin-/- mice

In hematologic examinations performed between 6 and 24 weeks, values for red blood cells, white blood cells, and platelet counts, hematocrit and hemoglobin were similar for hepsin-/- and wild-type mice (35). To examine the importance of hepsin in hemostasis, tail bleeding time and plasma clotting times (aPTT and PT) were measured in hepsin-/- mice. The tail bleeding time measures both platelet and blood coagulation activities, whereas aPTT and PT assays measure the function of the intrinsic and extrinsic blood coagulation pathways, respectively. In hepsin-/- mice, no prolongation in time was found in either the tail bleeding or the clotting assays, indicating that hepsin deficiency had a minimum effect on normal hemostasis in mice (35, 57). These data are consistent with the observation that no spontaneous bleedings occurred in hepsin-/- mice.

6.4. Disseminated intravascular coagulation (DIC) and septic shock models

To examine the potential function of hepsin under pathological conditions, hepsin-/- mice were tested in both thromboplastin-induced DIC and lipopolysaccharide (LPS)-induced septic shock models (35). In the DIC model, thromboplastin was injected intravenously to initiate the tissue factor-dependent coagulation pathway. The times at which breathing or heart beating stopped were measured. The results were very similar for both hepsin-/- mice and wild-type controls. In the septic shock model, LPS (40 mg/kg) from *Escherichia coli* serotype O111:B4 was injected intraperitoneally. The mice were monitored for signs of endotoxemia and lethality for up to 9 days. Again, the survival rates were similar for hepsin-/- and wild-type mice after the challenge of the high dose of endotoxin. Together, these data strongly indicate that the tissue factor-mediated coagulation pathway was not significantly altered in hepsin-/- mice under these experimental conditions.

6.5. Blood chemistry analysis for liver function

To examine the effects of hepsin deficiency on liver function, blood samples from hepsin-/- mice were analyzed. In hepsin-/- mice, serum concentrations of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were similar to or slightly higher than those of wild-type mice (35, 57). It is not clear if the mild elevation of serum ALT and AST in hepsin-/- mice was statistically significant (57). Serum concentrations of total protein, albumin, globulin, total bilirubin, γ-glutamyltranspeptidase (GGT), amylase, creatine kinase, and lactate dehydrogenase were also similar in hepsin-/- and wild-type control mice, indicating that the liver function was not significantly affected in hepsin-/- mice (35, 57).

Unexpectedly, serum alkaline phosphatase levels were found to be approximately twofold higher in hepsin-/- mice than those of wild-type (Figure 4). The elevation of serum alkaline phosphatase was detected in both male and

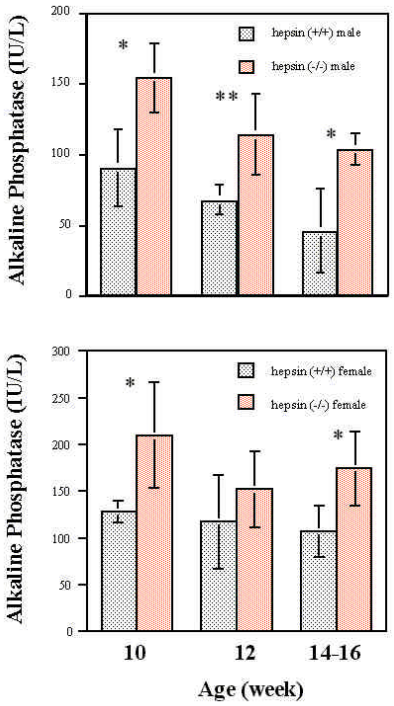

Figure 4. Serum alkaline phosphatase levels in hepsin-deficient and control mice. Serum samples were prepared from male (top) and female (bottom) hepsin-/- and wild-type mice at 10, 12, and 14-16 weeks of age. The number of mice in each group is four to five. Total serum alkaline phosphatase activity was measured by hydrolysis of *p*-nitrophenylphosphate at pH 9.2. Data are presented as mean ± SD. Statistical difference between hepsin-/- (grey bars) and wild-type mice (pink bars) are indicated by asterisks: *P<0.05; **P<0.01 by Student's *t* test. Reproduced from ref. (35) with permission.

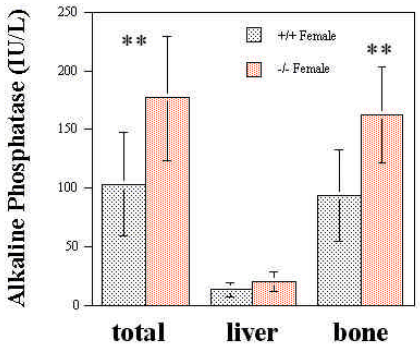

Figure 5. Analysis of alkaline phosphatase isoenzymes. Serum samples were collected from 14-16-week-old female mice. Total serum alkaline phosphatase activity was assayed by hydrolysis of *p*-nitrophemylphosphate at pH 9.2. Isoenzyme activities was measured by a quantitative assay described by Hoffmann *et al*. (59). Data are presented as mean ± SD from 8 wild-type (grey bars) and 14 hepsin-/- (pink bars) mice. Statistical difference are indicated by asterisks, **P<0.01 by Student's *t* test. Reproduced from ref. (35) with permission.

female hepsin-/- mice when measured at 10, 12, and 14-16 weeks of age. Further analysis of alkaline phosphatase isoenzymes showed that the elevated alkaline phosphatase in the serum from hepsin-/- mice was mostly of bone origin (Figure 5). This phenotype appeared to be strain-independent because similar results were found in hepsin-/- mice bred in either National Institute of Health (NIH) Swiss or C57BL/6J background (35, 57). In addition, elevated serum alkaline phosphatase levels were also observed in hepsin transgenic mice. Yu *et al*. generated transgenic mice to overexpress either wild-type hepsin or an active site mutant hepsin in the liver under an albumin promoter (58). Transgenic mice overexpressing wild-type or mutant hepsins were viable and appeared to develop normally. Interestingly, elevated alkaline phosphatase levels were detected in transgenic mice overexpressing the active site mutant hepsin but not in mice overpressing wild-type hepsin. The molecular mechanism responsible for the mild elevation of serum alkaline phosphatase and its long-term consequence in hepsin-/- mice remain unknown.

6.6. Morphologic and histologic analyses

Necropsies and histologic examinations performed on hepsin-/- mice at various ages did not identify any gross abnormalities in major organs including liver, kidney, brain, lung, heart, pancreas, spleen and bone. Liver samples from 8-12 week old hepsin-/- and wild-type mice appeared similar and had comparable weights. In H & E-strained sections, tissues including liver and bone from hepsin-/- mice were histologically indistinguishable from those of wild-type controls. No inflammatory cell infiltration and hepatocyte degeneration, necrosis or apoptosis were detected in liver samples from hepsin-/- mice (35, 57). X-ray examinations were also performed to detect potential defects in the skeleton. No structural abnormalities were identified in long bones, pelvis and vertebrae in hepsin-/- mice at the age of 5 months (35).

6.7. Liver regeneration model

To examine the potential function of hepsin in hepatocyte growth in mice, an acute liver regeneration model was tested in 8-10-week-old hepsin-/- mice (57). In this model, a partial hepatectomy was performed to remove two-thirds of the liver. The liver mass restoration was examined at 24, 48, and 96 hours after the surgery. At each of these time points, the rate of liver mass restoration was similar in hepsin-/- and wild-type control mice. By 96 hours after the hepatectomy, the resected livers in these animals recovered up to 70% of their original mass (57). The results indicate that hepsin is not essential for hepatocyte proliferation during liver regeneration in adult mice.

7. PERSPECTIVE

Hepsin is a hepatic serine protease identified by homologous cloning. Structurally, hepsin belongs to a new class of transmembrane serine proteases. The unique structural features and tissue distribution patterns of hepsin have attracted a number of investigators to study its

function. Biochemical analyses have confirmed that hepsin is an enzyme with the trypsin-like substrate specificity. Several *in vitro* studies further indicated that hepsin may play a role in blood coagulation, hepatocyte growth, and fertilization. However, characterization of hepsin-deficient mice showed that hepsin is not an essential enzyme for normal hemostasis, embryonic development, and maintenance of normal liver function. The phenotype of mild increase in serum alkaline phosphatase in hepsin-/- mice is interesting, although the underlying mechanism remains unknown at this time. Further biochemical and genetic studies are needed to elucidate the physiological function of this hepatic transmembrane serine protease.

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9. REFERENCES

1. Huber, R., & W. Bode: Structural basis of the activation and action of trypsin. *Acc Chem Res* 11, 114-122 (1978)
2. Neurath, H: Evolution of proteolytic enzymes. *Science* 224, 350-357 (1984)
3. Neurath, H: The versatility of proteolytic enzymes. *J Cell Biochem* 32, 35-49 (1986)
4. Davie, E.W., K. Fujikawa, & W. Kisiel: The coagulation cascade: initiation, maintenance, and regulation. *Biochemistry* 30, 10363-10370 (1991)
5. Anderson, K.V., D.S. Schneider, D. Morisato, Y. Jin, & E.L. Ferguson: Extracellular morphogens in Drosophila embryonic dorsal-ventral patterning. *Cold Spring Harb Symp Quant Biol* 57, 409-417 (1992)
6. Morisato, D., & K.V. Anderson: Signaling pathways that establish the dorsal-ventral pattern of the Drosophila embryo. *Annu Rev Genet* 29, 371-399 (1995)
7. LeMosy, E.K., D. Kemler, & C. Hashimoto: Role of Nudel protease activation in triggering dorsoventral polarization of the Drosophila embryo. *Development* 125, 4045-4053 (1998)
8. Konrad, K.D., T.J. Goralski, A.P. Mahowald, & J.L. Marsh: The gastrulation defective gene of Drosophila melanogaster is a member of the serine protease superfamily. *Proc Natl Acad Sci U S A* 95, 6819-6824 (1998)
9. Han, J.H., S.H. Lee, Y.Q. Tan, E.K. LeMosy, & C. Hashimoto: Gastrulation defective is a serine protease involved in activating the receptor toll to polarize the Drosophila embryo. *Proc Natl Acad Sci U S A* 97, 9093-9097 (2000)
10. Kitamoto, Y., X. Yuan, Q. Wu, D.W. McCourt, & J.E. Sadler: Enterokinase, the initiator of intestinal digestion, is a mosaic protease composed of a distinctive assortment of domains. *Proc Natl Acad Sci U S A* 91, 7588-7592 (1994)
11. Kitamoto, Y., R.A. Veile, H. Donis-Keller, & J.E. Sadler: cDNA sequence and chromosomal localization of human enterokinase, the proteolytic activator of trypsinogen. *Biochemistry* 34, 4562-4568 (1995)
12. Yuan, X., X. Zheng, D. Lu, C. Rubin, C.Y. Pung, & J.E. Sadler: Structure of murine enterokinase (enteropeptidase) and expression in small intestine during development. *Am J Physiol* 274, G342-349 (1998)
13. Matsushima, M., M. Ichinose, N. Yahagi, N. Kakei, S. Tsukada, K. Miki, K. Kurokawa, K. Tashiro, K. Shiokawa, K. Shinomiya: Structural characterization of porcine enteropeptidase. *J Biol Chem* 269, 19976-19982 (1994)
14. Leytus, S.P., K.R. Loeb, F.S. Hagen, K. Kurachi, & E.W. Davie: A novel trypsin-like serine protease (hepsin) with a putative transmembrane domain expressed by human liver and hepatoma cells. *Biochemistry* 27, 1067-1074 (1988)
15. Yan, W., N. Sheng, M. Seto, J. Morser, & Q. Wu: Corin, a mosaic transmembrane serine protease encoded by a novel cDNA from human heart. *J Biol Chem* 274, 14926-14935 (1999)
16. Yan, W., F. Wu, J. Morser, & Q. Wu: Corin, a transmembrane cardiac serine protease, acts as a pro-atrial natriuretic peptide-converting enzyme. *Proc Natl Acad Sci U.S.A* 97, 8525-8529 (2000)
17. Tomita, Y., D.H. Kim, K. Magoori, T. Fujino, & T.T. Yamamoto: A novel low-density lipoprotein receptor-related protein with type II membrane protein-like structure is abundant in heart. *J Biochem (Tokyo)* 124, 784-789 (1998)
18. Takeuchi, T., M.A. Shuman, & C.S. Craik: Reverse biochemistry: use of macromolecular protease inhibitors to dissect complex biological processes and identify a membrane-type serine protease in epithelial cancer and normal tissue. *Proc Natl Acad Sci U S A* 96, 11054-11061 (1999)
19. Takeuchi, T., J.L. Harris, W. Huang, K.W. Yan, S.R. Couphlin, & C.S. Craik: Cellular localization of membrane-type serine protease 1 and identification of protease-activated receptor-2 and single-chain urokinase-type plasminogen activator as substrates. *J Biol Chem* 275, 26333-26342 (2000)
20. Lin, C.Y., J. Anders, M. Johnson, Q.A. Sang, & R.B. Dickson: Molecular cloning of cDNA for matriptase, a matrix-degrading serine protease with trypsin-like activity. *J Biol Chem* 274, 18231-18236 (1999)
21. Yamaoka, K., K. Masuda, H. Ogawa, K. Takagi, N. Umemoto, & S. Yasuoka: Cloning and characterization of the cDNA for human airway trypsin-like protease. *J Biol Chem* 273, 11895-11901 (1998)
22. Paoloni-Giacobino, A., H. Chen, M.C. Peitsch, C. Rossier, & S.E. Antonarakis: Cloning of the TMPRSS2 gene, which encodes a novel serine protease with transmembrane, LDLRA, and SRCR domains and maps to 21q22.3. *Genomics* 44, 309-320 (1997)
23. Jacquinet, E., N.V. Rao, G.V. Rao, & J.R. Hoidal: Cloning, genomic organization, chromosomal assignment and expression of a novel mosaic serine proteinase: epitheliasin. *Febs Lett* 468, 93-100 (2000)
24. Wallrapp, C., S. Hahnel, F. Muller-Pillasch, B. Burghardt, T. Iwamura, M. Ruthenburger, M.M. Lerch, G. Adler, & T.M. Gress: A novel transmembrane serine protease (TMPRSS3) overexpressed in pancreatic cancer. *Cancer Res* 60, 2602-2606 (2000)

25. Appel, L.F., M. Prout, R. Abu-Shumays, A. Hammonds, J.C. Garbe, D. Fristrom, & J. Fristrom: The Drosophila Stubble-stubbloid gene encodes an apparent transmembrane serine protease required for epithelial morphogenesis. *Proc Natl Acad Sci U S A* 90, 4937-4941 (1993)
26. Hooper, J.D., J.A. Clements, J.P. Quigley, & T.M. Antalis: Type II transmembrane serine proteases - Insights into a emerging class of cell surface proteolytic enzymes. *J Biol Chem* 276, 857-860 (2001)
27. Kazama, Y., T. Hamamoto, D.C. Foster, & W. Kisiel: Hepsin, a putative membrane-associated serine protease, activates human factor VII and initiates a pathway of blood coagulation on the cell surface leading to thrombin formation. *J Biol Chem* 270, 66-72 (1995)
28. Torres-Rosado, A., K.S. O'Shea, A. Tsuji, S.H. Chou, & K. Kurachi: Hepsin, a putative cell-surface serine protease, is required for mammalian cell growth. *Proc Natl Acad Sci U S A* 90, 7181-7185 (1993)
29. Vu, T.K.H., R.W. Liu, C.J. Haaksma, J.J. Tomasek, & E.W. Howard: Identification and cloning of the membrane-associated serine protease, hepsin, from mouse preimplantation embryos. *J Biol Chem* 272, 31315-31320 (1997)
30. Tsuji, A. A. Torres-Rosado, T. Arai, M.M. Le Beau, R.S. Lemons, S. H. Chou, & K. Kurachi: Hepsin, a cell membrane-associated protease. Characterization, tissue distribution, and gene localization. *J Biol Chem* 266, 16948-16953 (1991)
31. Tanimoto, H., Y. Yan, J. Clarke, S. Korourian, K. Shigemasa, T.H. Parmley, G.P. Parham, & T.J. O'Brien: Hepsin, a cell surface serine protease identified in hepatoma cells, is overexpressed in ovarian cancer. *Cancer Res* 57, 2884-2887 (1997)
32. Zacharski, L.R., D.L. Ornstein, V.A. Memoli, S.M. Rousseau, & W. Kisiel: Expression of the factor VII activating protease, hepsin, in situ in renal cell carcinoma. *Thromb Haemost* 79, 876-877 (1998)
33. Farley, D., F. Reymond, & H. Nick: Cloning and sequence analysis of rat hepsin, a cell surface serine proteinase. *Biochim Biophys Acta* 1173, 350-352 (1993)
34. Kawamura, S., S. Kurachi, Y. Deyashiki, & K. Kurachi: Complete nucleotide sequence, origin of isoform and functional characterization of the mouse hepsin gene. *Eur J Biochem* 262, 755-764 (1999)
35. Wu, Q., D. Yu, J. Post, M. Halk-Miller, J.E. Sadler & J. Morser: Generation and characterization of mice deficient in hepsin, a hepatic transmembrane serine protease. *J Clin Invest* 101, 321-326 (1998)
36. Petersen, T.E., M.R. Martzen, A. Ichinose, & E.W. Davie: Characterization of the gene for human plasminogen, a key proenzyme in the fibrinolytic system. *J Biol Chem* 265, 6104-6111 (1990)
37. Degen, S.J., & E.W. Davie: Nucleotide sequence of the gene for human prothrombin. *Biochemistry* 26, 6165-6177 (1987)
38. Craik, C.S., Q.L. Choo, G.H. Swift, C. Quinto, R.J. MacDonald, & W.J. Rutter: Structure of two related rat pancreatic trypsin genes. *J Biol Chem* 259, 14255-14264 (1984)
39. Hartmann, E., T.A. Rapoport, & H.F. Lodish: Predicting the orientation of eukaryotic membrane-spanning proteins. *Proc Natl Acad Sci U S A* 86, 5786-5790 (1989)
40. Matsumoto, A., M. Naito, H. Itakura, S. Ikemoto, H. Asaoka, I. Hayakawa, H. Kanamori, H. Aburatani, F. Takaku, & H. Suzuki: Human macrophage scavenger receptors: primary structure, expression, and localization in atherosclerotic lesions. *Proc Natl Acad Sci U S A* 87, 9133-9137 (1990)
41. Resnick, D., A. Pearson, & M. Krieger: The SRCR superfamily: a family reminiscent of the Ig superfamily. *Trends Biochem Sci* 19, 5-8 (1994)
42. Brown, J.R. & B.S. Hartley: Location of disulphide bridges by diagonal paper electrophoresis. The disulphide bridges of bovine chymotrypsinogen A. *Biochem J* 101, 214-228 (1966)
43. Hartley, B.S. & D.L. Kauffman: Corrections to the amino acid sequence of bovine chymotrypsinogen A. *Biochem J* 101, 229-231 (1966)
44. Chung, D.W., K. Fujikawa, B.A. McMullen, & E.W. Davie: Human plasma prekallikrein, a zymogen to a serine protease that contains four tandem repeats. *Biochemistry* 25, 2410-2417 (1986)
45. Degen, S.J., R.T. MacGillivray, & E.W. Davie: Characterization of the complementary deoxyribonucleic acid and gene coding for human prothrombin. *Biochemistry* 22, 2087-2097 (1983)
46. Kurachi, K., A. Torres-Rosado, & A. Tsuji: Hepsin. *Methods Enzymol* 244, 100-114 (1994)
47. Zhukov, A., U. Hellman, & M. Ingelman-Sundberg: Purification and characterization of hepsin from rat liver microsomes. *Biochim Biophys Acta* 1337, 85-95 (1997)
48. Tsuji, A., A. Torres-Rosado, T. Arai, S.H. Chou, & K. Kurachi: Characterization of hepsin, a membrane bound protease. *Biomed Biochim Acta* 50, 791-793 (1991)
49. Fenton, J.W: Thrombin. *Ann N Y Acad Sci* 485, 5-15 (1986)
50. Vu, T.K., D.T. Hung, V.I. Wheaton, & S.R. Coughlin: Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell* 64, 1057-1068 (1991)
51. Nakamura, T., T. Nishizawa, M. Hagiya, T. Seki, M. Shimonishi, A. Sugimura, K. Tashiro, & S. Shimizu: Molecular cloning and expression of human hepatocyte growth factor. *Nature* 342, 440-443 (1989)
52. Manfioletti, G., C. Brancolini, G. Avanzi, & C. Schneider: The protein encoded by a growth arrest-specific gene (gas6) is a new member of the vitamin K-dependent proteins related to protein S, a negative coregulator in the blood coagulation cascade. *Mol Cell Biol* 13, 4976-4985 (1993)
53. Schmidt, C., F. Bladt, S. Goedecke, V. Brinkmann, W. Zschiesche, M. Sharpe, E. Gherardi, & C. Birchmeier: Scatter factor/hepatocyte growth factor is essential for liver development. *Nature* 373, 699-702 (1995)
54. Uehara, Y., O. Minowa, C. Mori, K. Shiota, J. Kuno, T. Noda, & N. Kitamura: Placental defect and embryonic lethality in mice lacking hepatocyte growth factor/scatter factor. *Nature* 373, 702-705 (1995)
55. Beaton, A.H., I. Kiss, D. Fristrom, & J.W. Fristrom: Interaction of the Stubble-stubbloid locus and the Broad-complex of Drosophila melanogaster. *Genetics* 120, 453-464 (1988)

56. Perona, R.M. & P.M. Wassarman: Mouse blastocysts hatch in vitro by using a trypsin-like proteinase associated with cells of mural trophectoderm. *Dev Biol* 114, 42-52 (1986)
57. Yu, I.S., H.J. Chen, Y.S. Lee, P.H. Huang, S.R. Lin, T.W. Tsai, & S.W. Lin: Mice deficient in hepsin, a serine protease, exhibit normal embryogenesis and unchanged hepatocyte regeneration. *Thromb Haemost* 84, 865-870 (2000)
58. Yu, I.S., H.J. Chen, S.R. Lin, L.T. Li, T.W. Tsai, & S.W. Lin: The study of hepsin by transgenic technology. *Thromb Haemost* 82, 15 (1999)
59. Hoffmann, W.E., N. Everds, M. Pignatello, & P.F. Solter: Automated and semiautomated analysis of rat alkaline phosphatase isoenzymes. *Toxicol Pathol* 22, 633-638 (1994)

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