Plasmin decreases the BH3-only protein BimEL via the ERK1/2 signaling pathway in hepatocytes

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Abstract

Since the signal transduction mechanisms responsible for liver regeneration mediated by the plasminogen/plasmin system remain largely undetermined, we have investigated whether plasmin regulates the pro-apoptotic protein BimEL in primary hepatocytes. Plasmin bound to hepatocytes in part via its lysine binding sites (LBS). Plasmin also triggered phosphorylation of ERK1/2 without cell detachment. The plasmin-induced phosphorylation of ERK1/2 was inhibited by the LBS inhibitor epsilon-aminocaproic acid (EACA), the serine protease inhibitor aprotinin, and the MEK inhibitor PD98059. DFP-inactivated plasmin failed to phosphorylate ERK1/2. Plasmin temporally decreased the starvation-induced expression of BimEL and activation of caspase-3 via the ERK1/2 signaling pathway, resulting in an enhancement of cell survival. The amount of mRNA for Bim increased 1 day after the injection of CCl4 in livers of plasminogen knockout (Plg-KO) and the wild-type (WT) mice. The increase in BimEL protein persisted for at least 7 days post-injection in livers of Plg-KO mice, whereas WT mice showed an increase in BimEL protein 1 day after the injection. Plg-KO and WT mice showed notable phosphorylation of ERK1/2 7 and 3 days after the injection of CCl4, respectively. Our data suggest that the plasminogen/plasmin system could decrease BimEL expression via the ERK1/2 signaling pathway during liver regeneration.

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1. Introduction

Plasmin is generated from its precursor plasminogen which is synthesized by normal hepatocytes and converted to the two-chain serine protease plasmin by endogenous tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA) [1]. Plasminogen has five kringle domains at the N terminus and a serine-protease domain at the C-terminal end. Lysine binding sites (LBS) present in kringle 1 and 4 display affinity for lysine residues, physiologically relevant for binding to fibrin and cells, an essential step in fibrinolysis and pericellular proteolysis [2]. The plasminogen/plasmin system has multiple physiological/pathophysiological roles, being involved in thrombolysis, fibrosis, neuronal death, migration, wound healing, and tissue remodeling, in a variety of tissues and cells [3,4]. It has been demonstrated that plasmin can activate several intracellular signaling pathways, including the phosphatidylinositol 3′-kinase (PI3K) and ERK1/2 pathways [5–9]. Of note is that plasmin induces the expression of the growth factor-like gene Cyr61, primary response gene c-fos, and early growth response gene egr-1 and causes the proliferation of smooth muscle cells via the ERK1/2 signaling pathway [5–7,9].

Previous studies have shown that the plasminogen/plasmin system has extensive roles in the liver. Plasmin may directly potentiate the proliferation of hepatocytes in primary culture [10]. In addition, a deficiency in plasminogen led to impaired hepatic remodeling in mice with carbon tetrachloride (CCl4)-induced liver injury [11]. Recently, we have shown that regeneration of the liver was impaired in plasminogen knockout (Plg-KO) mice whereas it progressed remarkably in alpha2-
antiplasmin knockout mice, suggesting that the plasmin/alpha2-antiplasmin system plays a key role in hepatic regeneration through the proteolysis of matrix elements and clearance of cellular debris after liver injury [12]. Thus, plasmin might contribute to the liver regeneration after injury. Nevertheless, the signal transduction mechanisms underlying the plasminogen/plasmin system-mediated regeneration remain largely open to question.

The BH3-only protein Bim, a member of the Bcl-2 family, promotes apoptosis in several cell types, including lymphocytes, neurons, epithelial cells and hepatocytes [13]. In healthy cells, Bim is bound to cytoplasmic dynein light chain (LC8) and thereby sequestered to the microtubule-associated dynein motor complex. Certain apoptotic stimuli disrupt the interaction between LC8 and the dynein motor complex. Therefore, Bim localizes to the mitochondria, where it initiates the mitochondrial cell death pathway through either the activation of Bax protein or binding to the pro-survival Bcl-2 family member [14]. Bim is also rapidly and substantially expressed de novo following the withdrawal of survival-promoting factors [13,15]. There are three major isoforms of Bim created by alternative splicing, BimEL, BimL and BimS. Interestingly, BimEL is phosphorylated by pro-survival stimuli, such as serum and IL-3, and then degraded by the ubiquitin/proteasome system [16]. Because the phosphorylation of BimEL could be blocked by MEK inhibitors or a PI3K inhibitor, activation of the MEK-ERK and PI3K-Akt pathways attenuates the pro-apoptotic function of BimEL [16–18].

To clarify further the roles that the plasminogen/plasmin system plays in liver regeneration, we thus examined if plasmin could regulate BimEL, expression through phosphorylation of either ERK1/2 or Akt in primary hepatocytes. Here, we provide evidence that plasmin decreases BimEL levels via activation of the ERK1/2, but not Akt, signaling pathway in hepatocytes, resulting in an enhancement of cell survival, during liver regeneration.

2. Materials and methods

2.1. Animals

The plasminogen knockout (Plg-KO) and wild-type (WT) mice were kindly provided by Prof. D. Collen (University of Leuven, Belgium) [19]. All experiments were approved by the Institutional Animal Care and Use Committee at the Kinki University School of Medicine.

2.2. Materials

The human plasmin was purchased from Enzyme Research Laboratories (South Bend, IN, USA). Collagenase, P90859, and cycloheximide were from Sigma (St. Louis, MO, USA). Polyclonal rabbit anti-Bim antibody was obtained from BD Biosciences Pharrmingen (San Jose, CA, USA). p44/42 MAP kinase antibody, phospho-p44/42 MAP kinase (Thr202/Tyr204) antibody, cleaved caspase-3 (Asp175) antibody, Akt antibody, phospho-Akt (Ser473) antibody, and Bad antibody were from Cell Signaling Technology (Beverly, MA, USA). Aprotinin, diisopropyl fluorophosphate (DFP), carbon tetrachloride (CCl4), corn oil and protease inhibitor cocktail were purchased from Wako Pure Chemicals (Osaka, Japan). e-Aminocaproic acid (EACA) was obtained from Kyowa Hakko Kogyo (Tokyo, Japan). MG-132 was purchased from Calbiochem (Darmstadt, Germany). Inhibition of the proteolytic activity of plasmin by DFP was achieved according to a previously described method [20]. Excess DFP was removed by gel filtration and then plasmin’s inactivation was confirmed by fibrin zymography.

2.3. Binding assays using the IAsys biosensor

An IAsys resonant mirror biosensor (Affinity Sensors, Cambridge, UK) was used to measure the binding of hepatocytes to immobilized plasmin in real time in vitro. Hepatocytes were isolated from the WT mouse by a method using liver perfusion with collagenase as described previously [10], and then the cells were suspended in PBS. Immobilization of proteins and blocking of the free sites on the cuvette were performed according to previously described methods [21,22]. Briefly, the surface of the carboxylate cuvette was activated using EDC/NHS [400 mM, N-ethyl-N’-(3-dimethylaminopropyl)carbodiimide and 100 mM, N-hydroxysuccinimide] and thereafter plasmin, plasminogen, or BSA at 200 μg/ml was added to the activated carboxylate cuvette. The amount of immobilized plasmin was about 1.667 ng/mm2. After immobilization, the surface was blocked with 1 M ethanolamine (pH 8.5) and then washed with 0.1 M glycine–HCl (pH 3.0) to remove non-coupled protein. All binding experiments were performed in PBS at 25 °C. The contents of the reaction cuvette were stirred continuously with the aid of a propeller. The reactions were routinely followed for 10 min. In inhibition experiments, the cuvette was pre-incubated with EACA, aprotinin, or DFP for 5 min and then the isolated hepatocytes were added to the cuvette. After each binding experiment, the cuvette was regenerated by repeated washes with 0.1 M glycine–HCl (pH 3.0). The data readout from the biosensor was measured in units of arc-seconds.

2.4. Primary hepatocyte culture

Hepatocytes were isolated from Plg-KO and WT mice by a method using liver perfusion with collagenase as described above. The isolated hepatocytes were plated on collagen-coated culture dishes (Iwaki Glass, Chiba, Japan). All cultures were incubated in a humidified atmosphere of 5% CO2: 95% air at 37 °C. The plated cells were cultivated in Williams’ medium E (Sigma, MO, USA) supplemented with 20% FBS (Gibco, NY, USA), 10 nM insulin, 10 nM glucagon, and 1.6 nM human recombinant epidermal growth factor for 24 h. To avoid the effect of plasminogen included in the FBS, it was removed by lysinensepharose 4B (Amersham Biosciences, Buckinghamshire, UK) before use.

2.5. Detection of BimEL, cleaved caspase-3, and phosphorylated ERK1/2 and Akt by Western blot analysis in primary hepatocytes

After a pre-culture for 24 h, the medium was replaced with fresh medium with or without serum and growth factors, and hepatocytes were cultured for 24 h. Then, the hepatocytes cultured in the serum and growth factor-free medium were stimulated with plasmin at 5 to 50 nM for 5, 15, 30, 60, 120 or 240 min. The cells were also treated with EGF at 50 nM for 15 min. In inhibition experiments, EACA, aprotinin, and P90859 were added to the culture medium 24 h, 10 h, 30 min before the plasmin, respectively. In experiments on BimEL turnover, hepatocytes were cultured in the serum and growth factor-free medium for 24 h, and then cycloheximide and MG-132 were added to the medium 30 min before the plasmin. After aspiration of the medium at scheduled time points, cells were rinsed with PBS, and lysed in a lysis buffer containing 10 mM sodium phosphate buffer, 150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 0.2% sodium azide, and 1% protease inhibitor cocktail (pH 7.2). The cell lysate was harvested and exposed to freeze–thawing. After vortexing and sonication, the samples were centrifuged at 15,000 rpm for 15 min at 4 °C. The concentration of protein in the supernatant was then determined using a BCA protein assay kit (Pierce, Rockford, IL, USA). The samples were denatured by heating at 95 °C for 5 min. Protein samples were separated by electrophoresis on a 10 to 20% SDS-polyacrylamide gradient gel (Duolichu Pure Chemicals, Tokyo, Japan) and transferred onto a polyvinylidene difluoride membrane (Immobilon-P, Millipore Corporation, Billerica, MA). The membrane was blocked with a blocking solution containing 5% BSA for ERK1/2, phospho-ERK1/2, Akt, and phospho-Akt or 5% skim milk for Bim and cleaved caspase-3, 137 mM NaCl, 0.1% Tween 20, and 20 mM Tris–HCl (pH 7.6) for 1 h at room temperature.
being washed three times with Tris-buffered saline (TBS) containing 0.1% Tween 20, the membrane was incubated with the primary polyclonal antibodies at appropriate dilutions with gentle agitation overnight at 4 °C. The primary antibodies employed were: rabbit anti-p44/42 MAPK antibody (dilution 1:2000), rabbit anti-phospho-p44/42 MAPK (Thr202/Tyr204) antibody (1:1000), rabbit anti-Akt antibody (1:1000), rabbit anti-phospho-Akt (Ser 473) antibody (1:1000), rabbit anti-Bim antibody (1:1000), and rabbit anti-cleavage caspase-3 (Asg 175) antibody (1:1000). After incubation with the primary antibody, the membrane was washed three times with the above-mentioned TBS solution and then incubated with a horseradish peroxidase-conjugated anti-rabbit IgG antibody (Zymed Laboratories Inc., South San Francisco, CA) for 1 h at room temperature. The membrane was washed three times with the TBS solution again, and positive bands were detected using ECL Plus Western Blotting Detection Reagents (Amersham Biosciences, Buckinghamshire, UK). The densities of positive bands were quantified by using a Luminescent image analyzer (Fuji Photo Film Co., Ltd., Tokyo, Japan). Data on the phosphorylation of ERK1 are shown relative to ERK1. Data on dephosphorylated BimEL and cleaved caspase-3 are expressed as a ratio of the density before plasmn treatment.

2.6. Quantitative measurement of apoptosis in primary hepatocytes

After a pre-culture for 24 h, primary hepatocytes were cultured in fresh medium with or without serum and growth factors in the presence or absence of plasmin at 50 nM for 3, 6, 12, 24, or 48 h. Then, the APOPercentage Apoptosis Assay (Biocolor Ltd., Belfast, Northern Ireland) was used to quantify apoptosis, according to the manufacturer’s instructions [23,24]. The assay uses a dye that stains red the apoptotic cells undergoing the membrane “flip-flop” event when phosphatidyserine is translocated to the outer leaflet. Detection of apoptosis can be readily observed by microscopy. For the quantification of apoptosis, the dye within the labeled cells can subsequently be released into solution and the concentration measured at a wavelength of 550 nm, using a color filter microplate colorimeter.

2.7. Cell viability assay

The viability of primary hepatocytes isolated from Pig-KO and WT mice was reflected by the activity of mitochondrial dehydrogenases to cleave tetrazolium salts to produce formazan, using the tetrazolium salt (WST-1) assay. After a pre-culture for 24 h, hepatocytes were cultured in fresh medium with or without serum and growth factors in the presence or absence of plasmin at 50 nM for 24 or 48 h. Then, the culture medium was carefully decanted and hepatocytes were incubated with the premix WST-1 solution (Takara Bio Inc., Shiga, Japan) diluted in Hanks’ balanced salt solution (1:10, v/v) for 4 h. Absorbances of the samples were measured against a blank using a microplate reader at 450 nm with a reference wavelength of 630 nm. In inhibition experiments, after a change of medium to fresh medium without serum and growth factors, EACA, aprotinin, or PD98059 was added 60, 10, and 30 min before plasmn at 50 nM, respectively. Data are expressed as a ratio of cell viability measured before plasmn treatment.

2.8. Carbon tetrachloride-induced liver injury in mice

Pig-KO and WT mice (18–23 g) received an intraperitoneal administration of CCl4 solution (25% vol/vol in corn oil) at 2 ml/kg [12]. Under anesthesia with pentobarbital (50 mg/kg, i.p.), mice were sacrified by exsanguination, and then livers were collected before and 1, 3, and 7 days after the injection of CCl4 [12]. The liver samples were stored at −80 °C prior to use.

2.9. Detection of BimEL, Bad, and phosphorylation of ERK1/2 by Western blot analysis in the mouse liver

The frozen liver was minced in 500 μl of the lysis buffer, using the same solution described above, and the lysate was sonicated for 10 s on ice. After centrifugation, the protein concentration of the supernatant was then determined using the BCA protein assay kit. Western blot analysis of BimEL, Bad, ERK1/2, and phospho-ERK1/2 was performed as described above. Data on BimEL are expressed as a ratio of the density before the injection of CCl4. Data on the phosphorylation of ERK1 are expressed relative to ERK1.

2.10. Analysis of mRNA for Bim by quantitative real time RT-PCR in mouse liver

Total RNA was isolated from the frozen liver using a Qiagen RNeasy mini kit (QIAGEN, Hilden, Germany). Then, mRNA was reverse-transcribed with a RT reaction kit (Perfect Real Time, Takara Bio Inc., Shiga, Japan) according to the manufacturer’s instructions. Quantitative real-time RT-PCR was assessed by incorporation of SYBR Green into double-stranded DNA and performed with an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster, CA). The PCR primers targeted at mouse Bim were 5′-CGACAGTCTCTACGGAG-GAAC-3′ (forward) and 5′-CCCTCTCCAAGAGACAGGA-3′ (reverse) [25]. The primers for mouse GAPDH were 5′-ATCAGTGCAAGCAGAGGAC-GAC-3′ (forward) and 5′-CACATTGCGAGTGAACGACGCA-3′ (reverse) [25]. The PCRs for Bim and GAPDH were allowed to proceed for 40 cycles, starting with a 15-s denaturation period at 95 °C followed by a 60-s annealing and extending period at 60 °C. The specific mRNA amplification for Bim was determined as the Ct value followed by normalization with the GAPDH level.

2.11. Statistical analysis

Data are shown as the mean with SEM. Statistical significance was evaluated by ANOVA followed by Tukey’s multiple comparison test or Student’s t-test for two-group data, and was set at a P<0.05 level.

3. Results

3.1. Interaction of plasmin and primary hepatocytes

An IAsys resonant mirror biosensor was used to examine the interaction between plasmin and primary hepatocytes isolated from WT mice. Hepatocytes bound to immobilized plasmin in a cell number-dependent manner (Fig. 1A). The binding was partially blocked by the lysine binding sites (LBS) inhibitor EACA at 10 mM, implying that the active site of plasmin was bound to immobilized plasminogen, and the binding was inhibited by DFP. However, since the effect of aprotinin, a highly basic protein, might be non-specific, we examined the effect of the active site inhibitor DFP on the binding. DFP did not block the binding (Fig. 1D) although it bound to immobilized plasmin. Hepatocytes also bound to immobilized plasminogen, and the binding was inhibited by EACA at 10 mM, indicating partial involvement of the LBS in the binding of plasmin and hepatocytes (Fig. 1B). Surprisingly, aprotinin, a serine protease inhibitor, at 400 KIU/ml augmented the binding (Fig. 1C). However, since the effect of aprotinin, a highly basic protein, might be non-specific, we examined the effect of the active site inhibitor DFP on the binding. DFP did not block the binding (Fig. 1D) although it bound to immobilized plasmin. Hepatocytes also bound to immobilized plasminogen, and the binding was inhibited by EACA at 10 mM, implying that the active site of plasmin was not involved in the binding between plasmin and hepatocytes (Fig. 1E). Although hepatocytes bound to immobilized BSA, as a control protein, EACA at 10 mM failed to inhibit the binding (data not shown). These results suggested that plasmin could bind to hepatocytes in part via LBS, and its active site did not contribute to the binding.

3.2. Phosphorylation of ERK1/2, but not Akt, by plasmin in primary hepatocytes

Plasmin at 50 nM caused a marked phosphorylation of ERK1/2 at 15 min, which then notably decreased but still remained at 60 min (Fig. 2A). The plasmin-induced phosphorylation of ERK1/2 was concentration-dependent at 5–50 nM.
increased mobility and expression in a Western blot analysis, indicating an increase in the amount of dephosphorylated BimEL (Fig. 4A and B, left). Plasmin at 50 nM for 30 to 60 min significantly decreased the starvation-induced dephosphorylation of BimEL, following phosphorylation of ERK1/2, although the vehicle had no effect (Fig. 4A and B, right). Although starvation for 3, 6 and 12 h induced phosphorylation of BimEL, plasmin decreased the level of phosphorylated BimEL independent of the period of starvation (data not shown). Starvation for 24 h also increased the amount of cleaved caspase-3, an active form of caspase-3, following an increase

3.3. Down-regulation of BimEL and caspase-3 by plasmin via the ERK1/2 signaling pathway in primary hepatocytes

Because plasmin could activate the ERK1/2 signaling pathway, known to promote phosphorylation and proteasome-dependent degradation of BimEL, a pro-apoptotic protein, we next examined if plasmin could regulate the expression of BimEL induced by starvation in primary hepatocytes isolated from WT mice. When primary hepatocytes were cultured in serum and growth factor-free medium for 24 h, BimEL exhibited

Fig. 1. (A–D) Binding analysis of plasmin and primary hepatocytes using the IAsys biosensor. (A) Hepatocytes were added to the cuvette in the indicated number. (B–D) Effect of the lysine binding site inhibitor EACA (B), serine protease inhibitor aprotinin (C), and active site inhibitor DFP (D) on the interaction of immobilized plasmin with primary hepatocytes. Hepatocytes (2500 cells) were added to the cuvette 5 min after each inhibitor or vehicle. (E) Binding analysis of plasminogen and hepatocytes using the IAsys biosensor. Hepatocytes (2500 cells) were added to the cuvette 5 min after EACA or vehicle.

Fig. 2. Phosphorylation of ERK1/2 after stimulation with plasmin in primary hepatocytes isolated from WT mice. Hepatocytes were cultured in serum and growth factor-free medium for 24 h. (A and B) Time-related (A) and concentration-dependent (B) phosphorylation of ERK1/2 caused by plasmin. Plasmin was added to the medium at 50 nM for 5–60 min (Fig. 2C, D). The plasmin-induced phosphorylation of ERK1/2 was also significantly inhibited by either EACA at 10 mM or aprotinin at 400 KIU/ml (Fig. 2C, D). Plasmin with its active site blocked by DFP could not trigger phosphorylation of ERK1/2 (Fig. 2E). Plasmin at 50 nM failed to phosphorylate Akt at 5–60 min (Fig. 3A), although EGF, as a positive control, at 50 nM for 15 min caused a marked phosphorylation of Akt (Fig. 3B). Phosphorylated Akt was also detected in hepatocytes cultured with complete medium (Fig. 3B). These results suggested that plasmin could activate the ERK1/2, but not Akt, signaling pathway in a manner dependent on its proteolytic activity and LBS in primary hepatocytes.
in the amount of dephosphorylated BimEL in primary hepatocytes (Fig. 4A and C, left). The increase in the amount of cleaved caspase-3 was significantly inhibited by plasmin at 50 nM at 30–120 min (Fig. 4A and C, right). These effects of plasmin were abolished by PD98059 at 50 μM, implying involvement of the ERK1/2 signaling pathway (Fig. 5).

Since BimEL protein is phosphorylated by pro-survival stimuli via the ERK1/2 signaling pathway, and then degraded by the ubiquitin/proteasome system [16], we investigated the effect of MG-132, a proteasome inhibitor, on the plasmin-induced decrease in dephosphorylated BimEL. Plasmin at 50 nM for 3 to 6 h significantly facilitated degradation of BimEL in primary hepatocytes pretreated with cycloheximide, a protein synthesis inhibitor (Fig. 6). The effect of plasmin was completely prevented by MG-132 at 5 μM (Fig. 6). Cycloheximide at 500 ng/ml did not affect the starvation-induced expression of BimEL (data not shown). These results suggested that plasmin phosphorylated BimEL via the ERK1/2 signaling pathway, and then the phosphorylated BimEL was degraded by the ubiquitin/proteasome system.

3.4. Plasmin protects against the starvation-induced apoptosis in primary hepatocytes

Since plasmin decreased the expression of BimEL via ERK1/2 signaling, a pro-survival signaling pathway, we investigated whether it could enhance cell viability in primary hepatocytes. When the primary hepatocytes, isolated from WT mice, were cultured in complete medium, plasmin at 50 nM had no effect on the viability (Fig. 8A, left). Plasmin at 50 nM also failed to enhance cell survival when hepatocytes isolated from WT mice were cultured in the serum and growth factor free-medium for 3–48 h when apoptosis was quantified by measurement of the amount of dye within the stained cells (Fig. 7D).

3.5. Effect of plasmin on cell survival in primary hepatocytes

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24 h (Fig. 8B, left). Then, we next examined the viability and survival of hepatocytes isolated from Plg-KO mice because DNA synthesis in primary hepatocytes was inhibited by tranexamic acid, a LBS inhibitor, or PASI-535, an active site inhibitor, indicating that plasminogen generated from hepatocytes might contribute to cell proliferation [10]. The viability and survival of hepatocytes isolated from Plg-KO mice tended to decrease when vehicle was added to the complete medium (Fig. 8A) or serum and growth factor-free medium (Fig. 8B). Plasmin at 50 nM did not affect the viability when hepatocytes isolated from Plg-KO mice were cultured in complete medium (Fig. 8A, right). Of note is that plasmin at 50 nM significantly enhanced cell survival when hepatocytes isolated from Plg-KO mice were cultured in the serum and growth factor-free medium for 24 h (Fig. 8B). The enhancement of cell survival by plasmin was abolished by EACA at 10 mM and aprotinin at 400 KIU/ml, implying involvement of the LBS and proteolytic activity of plasmin (Fig. 8C). The MEK inhibitor PD98059 at 50 μM also prevented the plasmin-induced enhancement of cell survival (Fig. 8C).

### 3.6. Persistent expression of BimEL after CCl4-induced liver injury in the plasminogen knockout mouse

We previously reported that WT mice had almost recovered from liver injury 7 days after the injection of CCl4, although Plg-KO mice remained in a damaged state until day 14 [12].
Because the intraperitoneal administration of CCl4 is known to induce apoptosis in hepatocytes [26,27], we next investigated the expression of BimEL after the injection of CCl4 in livers of Plg-KO and WT mice. The Western blot analysis revealed that the expression of dephosphorylated BimEL notably increased 1 day post-injection in WT mice, then decreased to the basal level 7 days post-injection (Fig. 9A). On the other hand, although the expression of dephosphorylated BimEL in Plg-KO mice expressed almost the same amount of Bad after the injection of CCl4 (Fig. 9A). Collectively, degradation of BimEL would seem to be decreased in Plg-KO mice after CCl4 induced-liver injury, as compared with that in WT mice.

3.7. Delayed phosphorylation of ERK1/2 after CCl4-induced liver injury in the plasminogen knockout mouse

Since plasmin could decrease the starvation-induced expression of BimEL via activation of the ERK1/2 signaling pathway (Figs. 4–6), we examined the phosphorylation of ERK1/2 in
livers of Plg-KO and WT mice after the injection of CCl₄. Phosphorylation of ERK1/2 was observed 3 days after the injection in WT mice (Fig. 10A). In contrast, Plg-KO mice showed phosphorylation of ERK1/2 7 days, but not 3 days, post-injection (Fig. 10A). Significant differences were detected in the phosphorylation of ERK1 between the Plg-KO and WT mice 3 and 7 days after the injection of CCl₄ (Fig. 10B).

4. Discussion

The present study demonstrates that plasmin caused the phosphorylation of ERK1/2, but not Akt, in a manner dependent on its proteolytic activity and LBS in primary hepatocytes, and that the starvation-induced expression of BimEL was inhibited by plasmin through activation of the ERK1/2 signaling pathway accompanied by cytoprotection and enhancement of cell survival. We also found that Plg-KO mice showed persistent expression of BimEL, and delayed phosphorylation of ERK1/2 in liver tissue after the injection of CCl₄, as compared with WT mice.

Plasmin has LBS, consisting of kringle domains, through which it is able to bind several cell surface proteins, resulting in the distribution of its proteolytic activity on the pericellular surface. In the present study, plasmin directly bound to hepatocytes and caused the phosphorylation of ERK1/2, known to mediate pro-survival signaling, in primary hepatocytes and enhanced the viability of hepatocytes during starvation (Figs. 1, 2, 8). These effects of plasmin were abolished by the lysine binding sites inhibitor EACA (Figs. 1, 2, 8). A previous study demonstrated that tranexamic acid, a lysine binding sites inhibitor, suppressed DNA synthesis in primary hepatocytes [10]. Moreover, EACA also inhibited the plasmin-induced release of leukotriene B₄ in peripheral monocytes [28], proliferation of smooth muscle cells [7], and gene expression in fibroblasts [9]. Collectively, it is likely that the plasmin-induced phosphorylation of ERK1/2 and enhancement of cell survival require binding to certain proteins expressed on the cell surface via LBS. A detailed analysis is now in progress in our laboratory. Majumdar et al. showed that plasmin binds to integrin αiiβ1 via kringle domains and simultaneously activates PAR-1, protease-activated receptor-1, resulting in a facilitation of migration [29]. Since it is known to be constitutively expressed in hepatocytes [30], integrin αiiβ1 might function as a receptor for plasmin, leading to the activation of GPCRs [5,9,29,31] and matrix metalloproteases [8,32] and release of growth factors, such as heparin-binding epidermal growth factor and basic fibroblast growth factor, from the extracellular matrix (ECM) [8,33].

It is well established that production of the pro-apoptotic protein BimEL is induced by serum withdrawal [13,15]. BimEL is also phosphorylated by either the MEK-ERK1/2 or PI3K-Akt signaling pathway, and then the phosphorylated BimEL is degraded by ubiquitin/proteasome systems [16–18]. Of special interest is that plasmin decreased the starvation-induced dephosphorylation of BimEL in the present study (Fig. 4). In addition, the effect of plasmin was completely abolished by the MEK inhibitor PD98059 (Fig. 5). Taken together with the finding that the plasmin-induced degradation of BimEL was completely blocked by the proteasome inhibitor MG-132 (Fig. 6), plasmin-induced ERK1/2 signaling contributes to the down-regulation of BimEL expression via the ubiquitin/proteasome system in hepatocytes. Several reports demonstrated that PAR-1 triggers activation of the MAP kinase cascades in various mammalian cells [34,35]. Interestingly, Chalmers et al. showed that a synthesized PAR-1-activating peptide and the endogenous PAR-1 activator thrombin inhibit expression of Bim via activation of ERK1/2 signaling in the fibroblast cell line CCl39 [36]. Collectively, the PAR-1/ERK1/2 signaling system might be involved in the plasmin-mediated down-regulation of BimEL expression in hepatocytes although plasmin could exert its effect in many ways, such as degradation of the ECM and activation of latent growth factors and matrix metalloproteases. However, the significant effect of plasmin on BimEL disappeared after 120 min (Fig. 4A and B, right). Since plasmin is degraded by its proteolytic activity, the down-regulation of BimEL expression by plasmin was temporary. In fact, plasmin triggered a marked phosphorylation of ERK1/2 at 15 min, which then notably decreased but still remained at 180 min (Figs. 2A and 4A). Plasmin also partially decreased the starvation-induced activation of caspase-3 via the ERK1/2 signaling pathway (Figs. 4, 5). In addition to increasing cell survival (Fig. 8), plasmin could protect against apoptosis in hepatocytes, in part, inhibiting the down-regulation of BimEL (Figs. 4, 7). This notion is consistent with evidence that plasminogen inhibits TNF-alpha-induced apoptosis in monocytes and the cytoprotective effect requires proteolytic activity [37]. Although at a concentration of more than 250 nM, plasmin is known to cause anoikis, a subtype of apoptosis induced by the detachment of adherent cells from the
ECM [38], at 50 nM it had a cytoprotective effect without causing the detachment of hepatocytes in the present study (Figs. 7, 8). Plasmin would thus appear to play a different role at the pericellular surface, being dependent on its local concentration.

The present study demonstrated that starvation caused apoptotic events, such as expression of BimEL, activation of caspase-3 (Fig. 4), and alteration of membrane composition (Fig. 7). However, starvation for 48 h did not decrease cell survival in hepatocytes isolated from WT mice as evaluated with the WTS-1 assay (Fig. 8B). The APOPercentage Dye uptake was observed 48 h after starvation although the amount taken up tended to decrease (Fig. 7), most hepatocytes might still possess cellular metabolic activity.

CCL4 is known to cause sinusoidal microcirculatory disturbance accompanied by fibrosis in liver tissue, and CCL4-induced liver injury is widely used as a model in studies of hepatic regeneration [11,12,39]. CCL4 is activated by cytochrome P-450 to form the trichloromethyl radical that reacts with oxygen to form the trichloromethylperoxy radical, a highly reactive species. These radicals trigger lipid peroxidation, damage to DNA, and the release of nitric oxide and several cytokines, resulting in acute liver injury following necrotic and apoptotic cell death in hepatocytes [26,27]. BimEL is considered to be involved in apoptosis caused by serum withdrawal and DNA damage [40]. Therefore, in the present study, it is likely that the up-regulation of BimEL expression is caused by the CCL4-induced sinusoidal microcirculatory disturbance and DNA damage in hepatocytes. The Western blot and quantitative real-time RT-PCR analyses showed that the mRNA for BimEL was degraded more rapidly than the protein in Plg-KO mice, as compared with WT mice (Fig. 9). Since plasmin could activate ERK1/2 signaling in primary hepatocytes (Fig. 2), these results most likely suggest that the signaling pathway for the degradation of BimEL is diminished in Plg-KO mice. Indeed, delayed phosphorylation of ERK1/2 was detected by Western blotting 7 days after the injection of CCL4 in livers of Plg-KO mice (Fig. 10). Thus, ERK1/2 signaling seems to be important to the stability of BimEL protein in vivo because BimEL is phosphorylated by pro-survival stimuli via the ERK1/2 signaling pathway, and then degraded by the ubiquitin proteasome system in vitro. We have been shown that the ubiquitin proteasome system regulates c-fos and egr-1 expression via the MEK/ERK pathway, Biochem. Biophys. Res. Commun. 329 (2005) 237–245.

In conclusion, we propose that plasmin could bind to hepatocytes, at least in part, via LBS and trigger the activation of ERK1/2 signaling, requiring proteolytic activity. Plasmin also would decrease the starvation-induced increase in BimEL and cleaved caspase-3 through activation of ERK1/2 signaling, in addition to protecting against apoptosis and enhancing cell survival. Although we found that the expression of BimEL was temporally induced by toxic injury to the liver, the plasminogen/plasmin system seems to be involved in the regulation of BimEL levels in the injured liver. The present study thus provides a novel mechanism for liver regeneration mediated by the plasminogen/plasmin system.

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