Plasminogen directs the pleiotropic effects of uPA in liver injury and repair

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Submitted 9 August 2002; accepted in final form 8 November 2002

Currier, Angela R., Gregg Sabla, Stephanie Locaputo, Hector Melin-Aldana, Jay L. Degen, and Jorge A. Bezerra. Plasminogen directs the pleiotropic effects of uPA in liver injury and repair. Am J Physiol Gastrointest Liver Physiol 284: G508–G515, 2003. First published November 13, 2002; 10.1152/ajpgi.00336.2002.—The urokinase-type plasminogen activator (uPA) plays a central role in liver repair. Nevertheless, the hepatic overexpression of uPA results in panlobular injury and neonatal mortality. Here, we define the molecular mechanisms of liver injury and explore whether uPA can regulate liver repair independently of plasminogen. To address the hypothesis that the liver injury in transgenic mice results from the intracellular activation of plasminogen by transgene-derived uPA (uPAT), we generated mice that overexpress uPAT and lack functional plasminogen (uPAT-Plg−). In these mice, loss of plasminogen abolished the hepatocyte-specific injury and prevented the formation of regenerative nodules displayed by uPAT littermates. Despite the increased expression of hepatic uPA, livers of uPAT-Plg− mice were unable to clear necrotic cells and restore normal lobular organization after an acute injury. Notably, high levels of circulating uPA in uPAT-Plg− mice did not prevent the long-term extrahepatic abnormalities previously associated with plasminogen deficiency. These data demonstrate that plasminogen directs the hepatocyte injury induced by uPAT and mediates the reparative properties of uPA in the liver.

regeneration; urokinase; proliferation; hepatocyte; tissue remodeling

REPARATIVE RESPONSE TO AN injury requires intact cellular proliferation and coordinated tissue remodeling. Shortly after injury, proliferation of undifferentiated and mature cells promptly increases to repopulate the injured tissue and later undergoes downregulation to prevent overgrowth and neoplasia. Meanwhile, tissue remodeling demands restructuring of the extracellular matrix to restore the architectural organization of the healthy tissue. In the liver, remodeling is directed by the combined forces of multiple cell-associated and extracellular proteases. Among serine proteases, plasminogen and plasminogen activators play a central role in liver repair after acute injury. In the absence of plasminogen or plasminogen activators, clearance of necrotic cell debris and matrix components is severely impaired, resulting in defective repair after an acute or a chronic injury (3, 4, 17, 19).

The plasminogen system includes the inactive proenzyme plasminogen, which is proteolytically converted to the active serine protease, plasmin, by either urokinase-type plasminogen activator (uPA) or tissue-type plasminogen activator (tPA). In addition to the traditional role in vascular hemostasis, plasmin and plasminogen activators regulate several biological processes involving proteolysis of the extracellular matrix, such as tissue remodeling, inflammation, tumor invasion, and metastasis (1, 2, 8, 9, 26). Although many of these processes are supported by plasmin-mediated fibrinolysis (6, 7), the defective remodeling of livers of mice lacking plasminogen is independent of fibrin deposition (3). An impairment in liver remodeling that is qualitatively similar is also produced when mice lack uPA or both uPA and tPA but not tPA alone (4).

Despite the key role of uPA in promoting liver repair, hepatic overexpression of uPA in transgenic mice causes severe liver injury with an early onset (12). Notably, the injury is specific to hepatocytes and is panlobular, leading to decreased survival of transgenic mice in the first few days after birth. In this paper, we explore the molecular mechanism(s) mediating these seemingly opposing effects of uPA in the liver. On the basis of the role of uPA as an activator of plasminogen, we hypothesized that the liver injury in transgenic mice is caused by the coexpression of uPA and plasminogen within hepatocytes. To address this hypothesis, we generated mutant mice that simultaneously express the uPA transgene (uPAT) and are homozygous for a null allele in the endogenous plasminogen gene. We report that the loss of plasminogen completely abolishes liver injury in mutant mice. Furthermore, by challenging these mice with a hepatotoxin, we demonstrate that the overproduction of uPA does not correct the defective remodeling in Plg− mice after

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acute liver injury. Together these data demonstrate that plasminogen mediates the pleiotropic effects of uPA in liver injury and repair.

MATERIALS AND METHODS

Reagents. Liver samples were fixed in 10% formalin from Accra Laboratory (Cleveland, OH), and hepatocyte proliferation was detected using a cell proliferation kit obtained from Amersham Pharmacia Biotech (Piscataway, NJ). CCl₄ was purchased from Aldrich (Milwaukee, WI), the sedatives ketamine and xylazine were obtained from Phoenix Pharmaceuticals (St. Joseph, MO), and acepromazine maleate was purchased from Fort Dodge Laboratories (Fort Dodge, IA). Protein concentrations were determined using the Bio-Rad protein assay from Bio-Rad Laboratories (Hercules, CA), and total RNA was isolated using TRIzol reagent (GIBCO-BRL; Manassas, VA). Specific signals were detected by Northern blot analysis and quantified using phosphor screens and an ImageQuant phosphoimager from Molecular Dynamics (Sunnyvale, CA). PCR was performed with a 9600 thermocycler (PerkinElmer, Norwalk, CT). Biochemical markers of liver function were determined by automated enzymatic assay using Vistros Chemistry Systems 950 (Johnson & Johnson, Rochester, NY).

Transgenic and gene-targeted mice. Heterozygous transgenic mice with albumin promoter/enhancer-driven overexpression of a uPAT were obtained from Jackson Laboratories (Bar Harbor, ME) (12). To generate mice overexpressing uPAT without plasminogen (uPAT-Plg⁻/⁻), we mated uPAT mice with Plg⁻/⁻ mice of a mixed 129 SvJ/CF-1/NIH Black background (6, 7). Offspring were genotyped by PCR using specific primers for endogenous and targeted alleles for plasminogen and uPAT by using tail biopsy DNA as the template (7, 13). Mice of all genotypes were housed in standard facilities and were fed food and water ad libitum. Survival analysis was performed by examining and weighing mice weekly for the entire duration of the study (58 wk). Animal protocols were approved by the Institutional Animal Care and Use Committee of the Children’s Hospital Research Foundation (Cincinnati, OH).

Liver injury and morphology. Urokinase transgene-induced liver injury was assessed visually, biochemically, and microscopically. At specific postnatal ages, mice were weighed and anesthetized intramuscularly with 0.1 ml of ketamine/xylazine/acepromazine (4:1:1) per 30 g body wt, weighed and anesthetized intramuscularly with 0.1 ml of ketamine/xylazine/acepromazine (4:1:1) per 30 g body wt, and blood samples were collected as described above. The anterior lobe of the liver was collected, fixed, and stained with hematoxylin and eosin as described above. Immunohistochemical analysis of fibrinogen was done in paraffin-embedded liver sections using rabbit antifibrinogen antiserum, the Vectastain ABC-AP detection system (Vector Laboratories, Burlingame, CA), and Fast Red TR/Naphthol AS-MX (Sigma, St. Louis, MO) to detect alkaline phosphatase activity in situ, as described previously (3).

Northern blot analysis. Total RNA was isolated from frozen liver tissue using TRIzol reagent, and the expression of uPAT and plasminogen was determined as described previously (10, 12, 13). Northern blots were hybridized with [³²P]cDNA probes for uPAT and plasminogen simultaneously. The specific signal was quantified using phosphor screens and an ImageQuant phosphoimager.

Zymographic analysis of plasminogen activator. Liver protein extracts were obtained from frozen liver tissue homogenized in buffer containing phosphate-buffered saline (in mM: 137 NaCl, 2.7 KCl, 4.3 Na₂HPO₄, 7H₂O, and 1.4 KH₂PO₄), 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, and 10% inhibitor cocktail (Calbiochem, La Jolla, CA). Homogenates were centrifuged at 12,000 g for 15 min at 4°C, and protein concentrations in liver lysates were measured using the Bradford method-based Bio-Rad assay. Plasma was obtained from the supernatants of heparinized blood samples after centrifugation at 9,000 rpm. Liver protein extracts (3.0 µg) and plasma samples (0.1 µl) were solubilized in nonreducing buffer and electrophoresed on SDS-polyacrylamide gel cast with 8% casein and 20 µg/ml plasminogen as described previously (12).

Hepatocyte proliferation. Hepatocellular proliferation after CCl₄ administration was measured by the incorporation of bromodeoxyuridine (BrDU) by hepatocytes with the use of the cell proliferation kit (Amersham Pharmacia Biotech) described previously (12). For each liver sample, the hepatocyte-labeling index (percentage of hepatocytes incorporating BrDU) was calculated by counting BrDU-labeled and unlabeled hepatocytes in 10 high-power fields (100 hepatocyte nuclei per field) by an investigator unaware of animal genotype.

Statistical analysis. Statistics for survival analysis were performed with Lifetest procedure in SAS for Windows version 8.01. The statistical package does not provide for multiple pairwise comparisons among groups. Such comparisons were carried out separately for each pair of groups using the Lifetest procedure, and the probability values were adjusted manually to account for the multiple comparisons. Values for additional experiments are shown as means ± SD, and statistical significance was assessed by Student’s unpaired t-test with the significance level at P < 0.05.

RESULTS

Plasminogen deficiency prevents urokinase-induced liver injury. The albumin promoter/enhancer-driven overexpression of uPAT causes liver injury that is apparent immediately after birth and increases neonatal mortality (12). In normal mice, endogenous uPA production in the liver is low and is derived from nonparenchymal cells, whereas plasminogen is expressed primarily by hepatocytes (28). To determine whether plasminogen activation by uPAT within hepatocytes is the mechanism of injury in transgenic mice, we mated uPAT mice with Plg⁻/⁻ mice. Visual inspection of livers from littermates revealed the typical pale,
Plasminogen deficiency prevents the development of urokinase-type plasminogen activator transgene (uPAT)-induced liver injury. A: visual appearance of uPAT livers showing a diffuse pale/lacy appearance at 3 wk of age, followed by the development of regenerative nodules that gradually expanded and repopulated the entire liver after 8 wk of age (Fig. 1A). In contrast, livers of uPAT-Plg− mice appeared normal and indistinguishable from Plg− mice and control littermates. To demonstrate that normal-appearing livers of uPAT-Plg− mice had expression of uPAT, we incubated Northern blots containing liver RNA from littermates with radiolabeled cDNA probes for plasminogen and uPAT transgene simultaneously. Using this approach, we documented ongoing expression of uPAT in normal-appearing livers of uPAT-Plg− mice (Fig. 1B). Consistent with the absence of injury, livers of uPAT-Plg− mice never developed regenerative nodules and displayed a normal appearance through adult life, suggesting that plasminogen deficiency prevented hepatotoxicity induced by uPAT.

To determine whether plasminogen deficiency completely abolishes the hepatocyte-specific injury in uPAT mice, we carried out microscopic analyses of liver sections from uPAT-Plg− mice and uPAT littermates. Hematoxylin and eosin staining of uPAT-Plg− livers revealed that plasminogen deficiency prevented the development of the panlobular injury typically seen in uPAT livers, as demonstrated by normal histological appearance at 3 and 8 wk and 7 mo of age (Fig. 2). In contrast, uPAT livers had easily identifiable hepatocyte injury at 3 wk of age and regenerative nodules surrounded by injured hepatocytes (Fig. 2). Cytoplasmic vacuolization and small nuclei were seen in hepatocytes of uPAT livers, whereas all other neighboring nonparenchymal cells appeared normal. At the ultrastructural level, electron microscopic analysis revealed that urokinase overexpression resulted in the accumulation of vacuoles and multisvesicular bodies surrounded by fragmented rough endoplasmic reticulum in mice with normal levels of plasminogen expression; such findings were completely absent in livers of uPAT-Plg− mice (Fig. 3) (25).

uPA overexpression does not correct the defective remodeling of Plg− livers. We previously demonstrated (4) that uPA deficiency results in a defect in liver repair after an acute toxic injury. The defect in removal of necrotic cells and reorganization of the hepatic architecture is qualitatively similar to but of a lesser degree than the one produced by plasminogen deficiency (3). To determine whether uPA-mediated pathway(s) directs liver repair independently of plasminogen activation, we administered CCl4 to mice of all genotypes at 2 mo of age, a time when uPAT-Plg− mice display a prominent overexpression of uPAT. A single dose of CCl4 causes necroinflammatory injury to centrilobular hepatocytes due to accumulation of CCl3 and other highly reactive byproducts (20). Visual inspection and microscopic analyses of livers demonstrated that the centrilobular injury induced by CCl4 was not affected by the animal genotype as supported by the similar centrilobular injury within 2 days (Fig. 4). At 7 and 14 days, the injury resolved, and the lobular organization was restored in normal and uPAT littermates. Although uPAT mice had some residual areas of transgene-induced injury, the additional toxic insult by CCl4 did not increase mortality or influence the ability of the liver to undergo a timely repair. More notable was the severe impairment in repair of livers from uPAT-Plg− and Plg− mice at 7 and 14 days, which displayed poor removal of necrotic cell debris and defective lobular organization. The appearance and extent of the centrilobular injury was similar in the livers of all uPAT-
Plg− and Plg− mice at 7 and 14 days. uPA overexpression in uPAT-Plg− mice did not influence liver function or the onset/degree of injury, indicated by similar plasma levels of albumin and aminotransferase in uPAT and uPAT-Plg− mice at different time points (data not shown). Notably, the BrdU labeling of hepatocytes was <0.5% for all genotypes before injection with CCl4, dramatically increased at 2 days after CCl4 in a similar fashion in all groups, and returned to low baseline levels at 7 and 14 days (Fig. 5).

In view of the persistent centrilobular injury and of the well-established role of uPA and plasminogen in fibrinolysis, we then determined the ability of uPAT-Plg− mice to clear fibrin from injured livers. We found a remarkable accumulation of fibrin within injured centrilobular areas at 2 days in mice of all genotypes, followed by effective fibrin clearance in livers of mice with functional plasminogen (Plg+) and uPAT mice at days 7 and 14 (Fig. 6). The fibrin clearance, however, was severely impaired in livers of Plg− and uPAT-Plg− mice as evidenced by intense fibrin immunostaining in areas of centrilobular injury throughout the duration of the study. Together, these data demonstrate that uPA overexpression does not overcome the defect in liver repair produced by plasminogen deficiency.

uPA overexpression does not correct extrahepatic consequences of plasminogen deficiency. We then explored whether uPA overexpression partially or completely corrects the extrahepatic phenotype displayed by plasminogen-deficient mice by examining littermates weekly and recording survival in littermates 17 mo of age. We found that long-term survival between uPAT-Plg− and Plg− mice decreased significantly compared with Plg+ and uPAT littermates (P < 0.01), with no uPAT-Plg− mice surviving beyond 13.2 mo (Fig. 7). There was no significant difference in the survival rate between Plg− and uPAT-Plg− mice. Furthermore, we found the extrahepatic manifestations of plasminogen deficiency in Plg− mice (rectal bleeding, rectal and penile prolapse, and wasting) in >90% of uPAT-Plg− mice, which became obvious by 10 mo of age (6). These data demonstrate that the overexpression of uPA does not reverse the extrahepatic effects of plasminogen deficiency.

**DISCUSSION**

Our studies demonstrate that plasminogen directs the uPA-induced liver injury and repair in vivo. The original findings of a panlobular liver injury in mice overexpressing uPA runs counter to expectations in
Fig. 4. uPA overexpression does not correct the persistent centrilobular injury of Plg− mice. Hematoxylin-eosin staining of liver sections 2 days after CCl₄ reveals a similar centrilobular injury in mice of all genotypes. The injury is resolved by 7 days in livers of control and uPAT mice, whereas the centrilobular injury persists in Plg− and uPAT-Plg− livers through 14 days in a similar fashion (magnification, ×200).

Fig. 5. uPAT-Plg− mice have adequate proliferation of hepatocytes after liver injury. A: bromodeoxyuridine (BrdU)-stained hepatocytes are easily found in the noninjured portions of hepatic lobule and in mice of all genotypes 2 days after CCl₄ administration. Between 7 and 14 days, the presence of BrdU-stained hepatocytes is uncommon in livers able to restore normal lobular organization (Plg+ and uPAT), as well as in those livers with persistent centrilobular injury (Plg− and uPAT-Plg−). B: quantification of BrdU-labeled hepatocytes reveals a similar increase in proliferating hepatocytes at 2 days after CCl₄ in mice of all genotypes, followed by a return to baseline levels within 7–14 days; n = 4–6 for each genotype at each time point; P > 0.05 for statistical comparison among subjects within each time point.
view of the growth-promoting properties of uPA (14, 15, 23). On the basis of the restriction of injury to hepatocytes and on the well-recognized role of uPA as a potent plasminogen activator, we hypothesized that intracellular production of the active protease plasmin by uPAT produced the liver injury. To address this hypothesis, we superimposed the genetic inactivation of the plasminogen gene in uPAT transgenic mice. In these mice, plasminogen deficiency completely abolished liver injury induced by uPAT. Despite high levels of uPA expression by hepatocytes, loss of plasminogen prevented the accumulation of multivesicular bodies and disruption of the rough endoplasmic reticulum within hepatocytes that are typically induced by uPAT (12). As a consequence, liver morphology remained normal and did not display formation of regenerative nodules during the neonatal period or early adulthood. Together, these data conclusively demonstrate that the intracellular availability of plasminogen to transgene-encoded uPA within hepatocytes leads to disruption of intracellular organelles and ultimate cellular injury/death. Moreover, the absence of nodular growth in uPAT-Plg−/H11002 livers formally rules out a direct growth-promoting role of uPA in the formation of regenerative nodules independent of plasminogen and the presence of injury. Instead, regenerative nodules in uPAT livers develop in response to the injury (only observed in uPAT livers with normal plasminogen expression), which creates a microenvironment permissive to growth of hepatocytes that escape the toxic effect of the transgene (13). Once the injury is established, uPA probably displays growth-promoting properties, as supported by the timely formation of regenerative nodules in uPAT livers and by defective regeneration after partial hepatectomy in uPA-deficient mice (23).

Timely cellular proliferation is central to the regenerative response of the liver to an injury. After partial hepatectomy, hepatocytes and nonparenchymal cells of liver remnants undergo well-orchestrated rounds of proliferation to restore liver mass (11, 16). With the use of this experimental model, uPA has been shown to be needed for the prompt entry of hepatocytes into the cell cycle, but there appear to be alternative mechanisms to gradually restore liver mass and maintain survival in the absence of uPA (23). Despite the apparent role of
uPA as a facilitator of hepatocyte proliferation after partial hepatectomy (23), we previously found that, after a toxic injury induced by CCl4, uPA deficiency had no detrimental effect on hepatocyte proliferation; instead, removal of necrotic cell debris and lobular reorganization were significantly impaired (4). The features of this defective repair were similar (but to a lesser degree) to the outcome in plasminogen-deficient livers. To directly explore the functional relationship between uPA and plasminogen in regulation of liver repair, we removed plasminogen from mice with hepatic overexpression of uPA and determined the ability of livers to undergo repair after CCl4 injury. Although uPA and the functional homologue tPA stimulate plasmin-dependent and -independent angiogenesis in vitro, no previous study directly explored whether uPA can direct proteolysis during tissue repair independently of plasminogen activation (5). In our studies using uPAT-Plg−/− mice challenged with CCl4, the hepatic overexpression of uPA did not correct the defective liver repair induced by plasminogen deficiency. The predominant phenotype of these mice was the inability to remove necrotic cellular debris in a fashion indistinguishable from the phenotype displayed by Plg−/− mice. Therefore, these data do not support an independent role of uPA in liver repair and place plasminogen as the central regulatory protease in the removal of necrotic cells and reorganization of the lobular architecture.

The genetic loss of uPA, tPA, or plasminogen leads to a gradual multisystem accumulation of fibrin. In extrahepatic tissues, fibrin deposits result in corneal ulcerations, gastrointestinal bleeding, rectal prolapse, and a progressive wasting syndrome with decreased life expectancy in the setting of plasminogen deficiency (6, 18, 21, 22). Notably, despite the increased levels of circulating uPA, similar multisystem abnormalities and abbreviated long-term survival developed in uPAT-Plg−/− mice. Together, these data demonstrate that the presence of plasminogen within intra- and extracellular environments is required for uPA to induce fibrinolysis and regulate tissue repair.

In summary, our studies reveal a central role for plasminogen in uPA-induced liver injury and repair. The ability of uPA to induce injury through intracellular activation of plasminogen is particularly important in view of the potential use of uPA in the treatment of hepatic fibrosis by the expression of a nonsecreted form of uPA in the liver (24). Although hepatocytes may be an ideal cellular source for high levels of expression of a uPA, the intracellular generation of plasmin by uPA may induce widespread injury. Whether activation of plasminogen in the extracellular milieu may efficiently decrease hepatic fibrosis through degradation of matrix components depends on whether plasmin directly or indirectly targets fibrin-unrelated substrates. The identification of these substrates will be critical to understanding the biological impact of plasmin in the clearance of hepatic and extrahepatic matrices.

We thank Dr. William Balisteri for insightful review of the manuscript.

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-55756 (to J. A. Bezerra) and National Heart, Lung, and Blood Institute Grant HL-47826 (to J. L. Degen).

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