Plasminogen and plasminogen activators play important roles in liver regeneration. Previously, we found that plasminogen potentiates hepatocyte proliferation in the primary culture of rat hepatocytes. Here, we examined how exogenous plasminogen affects the downstream events leading to cell proliferation. The addition of plasminogen to hepatocytes increased urokinase-type plasminogen activator (uPA) activity, but did not affect matrix metalloproteinase (MMP)-9 or MMP-2 activities. To increase uPA activity, plasminogen was required to bind the hepatocyte surface through the lysine-binding site of plasminogen molecule, but neither uPA mRNA nor uPA receptor (uPAR) mRNA was affected by the exogenous plasminogen. In addition, treatment of hepatocytes with an uPA inhibitor, \(\alpha\)-benzamidine, inhibited the plasminogen-induced and even EGF-induced hepatocyte proliferation. These results suggest that plasminogen-related control of hepatocyte proliferation is exerted topically by producing a hyperfibrinolytic state on the cellular surface involving the activation of uPA.

**Key words:** hepatocyte; plasminogen; lysine-binding site; proliferation; urokinase-type plasminogen activator

Plasminogen is a zymogen of the fibrinolytic enzyme plasmin. The conversion of plasminogen to plasmin is catalyzed by plasminogen activators (PAs), such as tissue-type PA (tPA) and urokinase-type PA (uPA). Plasmin primarily degrades fibrin, but it can also degrade extracellular matrices and activate growth factors and matrix metalloproteinases (MMPs) directly or indirectly.\(^1\)\(^{-4}\)

Cell surface proteins with a lysine residue at their C-terminal play a role as a scaffold for the plasminogen molecule. The plasminogen bound on the surface via the lysine-binding site of its structure lures PAs, by which the plasminogen is cleaved and changed into plasmin. Such an activation mechanism acting between molecules localized on the surface is known to proceed far more efficiently than in the liquid phase.\(^5\) As one of the reasons the solid phase has an advantage for activation, the enzymes, plasmin and PAs, are sterically protected from inhibition with their specific inhibitors, \(\alpha\)-plasmin inhibitor and PA inhibitor (PAI).\(^6\)

Plasminogen is known to be activated in three steps: first, single chain-uPA (sc-uPA or pro-uPA) activates plasminogen to plasmin, then plasmin activates sc-uPA to two-chain uPA (tc-uPA; active-uPA), and then tc-uPA causes further activation of plasminogen to plasmin.\(^7\)

The plasmin/uPA system is also thought to be involved in pro-MMP activation, in which plasmin directly activates MMP-1, -3, -9, -10, and -13 in vitro.\(^8\) It has been found that with the use of uPA-deficient mice, the uPA/plasmin system is a pathophysiologically important system, with which aortic aneurysms are expanded through pro-MMP activation.\(^9\) These networks for protease activation have been found to operate generally as a cell-associated proteolytic event.

Liver regeneration after hepatic damage is terminated through multiple repair processes such as hepatocyte proliferation, hepatic tissue remodeling, and differentiation.\(^10\) Study methods using animal models, e.g., hepatectomy and CCl\(_4\) injection causing acute liver injury, have suggested that those matrix degrading enzymes, such as plasmin, uPA and MMPs may play an important role in these processes.\(^11,12\)

The possible participation of plasminogen and MMP in hepatocyte proliferation has been confirmed using primary cultures of rat hepatocyte in vitro. It has been reported that TIMP-1, a natural MMP inhibitor, suppressed DNA synthesis induced by HGF and TNF-\(\alpha\).\(^13\) We have also reported that synthetic plasminogen/plasmin inhibitors, tranexamic acid and PASI-535, suppressed DNA synthesis of primary rat hepatocytes.
Plasminogen Regulates Hepatocyte Proliferation

Materials and Methods

Reagents. Recombinant human EGF was kindly provided by Higeta Shoyu (Soy Sause) Co. (Chiba, Japan). Rat plasminogen was prepared from rat plasma by lysine-Sepharose column chromatography.14) Pancreatic carboxypeptidase B (CPB) and L-aminobenzamide were purchased from Sigma-Aldrich (St. Louis, MO). Tranexamic acid (TXA) was kindly provided by Daiichi Pharmaceutical Co. (Tokyo).

Isolation of hepatocytes from rat liver and primary culture. Hepatocytes were isolated from the livers of male Wistar strain rats (150–180 g) by the two-step collagenase perfusion method previously reported.15) The hepatocytes were cultured for 4 h at 37 °C in a type I collagen-coated dish containing 5% calf serum and Williams’ medium E supplemented with 10−3 M insulin and 10−8 M glucagon, then for 20 h at 37 °C in serum- and hormone-free Williams’ medium E, and subjected to experiment.

Measurement of PAs and MMPs activities. The activities of PAs and MMPs in conditioned media were measured by zymography,16) for which SDS–PAGE was performed on a 10% polyacrylamide gel containing either a 2 mg/ml plasminogen-rich fibrinogen or a 2 mg/ml gelatin for detection of PAs activity and MMP activity respectively. After electrophoresis, the gel was washed twice with 2.5% Triton X-100 for 30 min to remove SDS, and then incubated for 44 h at 37 °C either in a 50 mM Tris–HCl buffer, pH 8.3, containing 0.1 M glycine for PA activity, or in a 25 mM Tris–HCl buffer, pH 7.5, containing 5 mM CaCl2 and 0.9% NaCl for MMP activity. Each gel was stained with 0.075% Comassie blue R-250.

Analysis of mRNAs of uPA and uPAR. Total RNA was extracted from rat hepatocytes using Isogen (Nippon Gene, Tokyo). mRNAs of uPA, uPAR, and GAPDH were measured by RT-PCR, as previously described.17) PCR was performed with specific primers, as described as follows: Rat uPA primers, 5’-CGG AGA GAT GAA GTT TGA GTG GGT GGA GCA GCT-3’ (U) and 5’-CAC TCT GGG TCA GCA GCA CAC AGC ATT TTA-3’ (L), were used for 25-cycle amplification of uPA cDNA (annealing at 63 °C for 1 min, extension at 72 °C for 1 min, and denaturation at 94 °C for 1 min). Rat uPAR primers, 5’-GCA CCT TTT GAT GTT CCT A-3’ (U) and 5’-CCA TTA CAG GAG ATA GA-3’ (L), were used for 25-cycle amplification of uPAR cDNA (annealing at 55 °C for 1 min, extension at 72 °C for 1 min, and denaturation at 94 °C for 1 min). Rat GAPDH primers, 5’-AAC GAC CCC TTC ATT GAC-3’ (U) and 5’TCC ACG ACA TAC TCA GCA C-3’ (L) were used for 18-cycle amplification of GAPDH cDNA (annealing at 60 °C for 1 min, extension at 72 °C for 1 min, and denaturation at 94 °C for 1 min).

Measurement of hepatocyte proliferation. The rate of hepatocyte proliferation was estimated from the amount of proliferating cell nuclear antigen (PCNA) as measured by western blotting. Briefly, 10 µg of protein extracted from hepatocytes was subjected to SDS–polyacrylamide gel and transferred onto a nitrocellulose membrane. Mouse antibody against PCNA (PC-10, DakoCytomation, Glostrup, Denmark) was used as primary antibody, and horseradish peroxidase-conjugated anti-mouse IgG as secondary antibody. PCNA on the gel was visualized with a commercial immunostaining-kit HRP-1000 (Konica Minolta, Tokyo) and quantified with an Image analyzer (Bag2000).

Results

Plasminogen elevates uPA activity in primary culture of hepatocytes

Since we have found that plasminogen potentiates hepatocyte proliferation, it is highly possible that potentiation is dependent on the activity of fibrinolytic factors involving plasmin, tPA, and uPA as well as MMPs. First we examined how exogenous plasminogen and EGF, which is known to be an essential factor in hepatocyte proliferation, affect the PA and MMP activities of hepatocytes in primary culture. As can be seen in Fig. 1, the medium harvested at 24 h of culture had a certain level of tPA activity and a trace of uPA activity (see panel A, lane at left side). The addition of plasminogen to the hepatocytes increased uPA activity in a dose-dependent manner, although tPA activity remained unchanged. EGF, which is known to increase uPA activity in carcinoma cell lines,10) also increased tPA and uPA activities in primary-cultured rat hepatocytes. In this culture, increased uPA activity was elevated more by plasminogen than that by EGF alone, and the effect of plasminogen was exaggerated by increasing its concentration, and was synergistic with EGF (Fig. 1A). There was no effect of plasminogen on the activities of MMP-9 or MMP-2 (Fig. 1B). These results indicate that plasminogen-induced proliferation of hepatocyte is partially afforded by the uPA activity generated through the activation system involving pro-uPA and plasminogen/plasmin.

Cell surface-bound plasminogen is responsible for the elevation of uPA activity

The addition of plasminogen (5 µg/ml medium) to the co-cultured with nonparenchymal liver cells,14) but the detailed function of cell surface-bound plasminogen has not been clarified. In this study, we investigated to clarify whether plasminogen can regulate hepatocyte proliferation through the activation of uPA or MMPs. We report that surface-bound plasminogen potentiates hepatocyte proliferation via uPA activation, but not via MMP activation.
hepatocytes elevated uPA activity to 3 times higher than the control (Fig. 2A, lane 3 vs. lane 1). However, the co-addition of tranexamic acid (TXA, 1 mM), a synthetic inhibitor of plasminogen, almost completely abolished the effect of plasminogen (lane 4). The enhanced uPA activity due either to EGF or to EGF and plasminogen was also suppressed by TXA (Fig. 2A, lanes 5–8, or bars calculated).

A similar suppressive effect was obtained with an enzyme, carboxypeptidase B (CPB, 1–10 μg/ml medium) cleaving off C-terminal lysine from the cell surfaces. The uPA activity elevated by plasminogen was suppressed dose-dependently by co-addition of CPB to the culture (Fig. 2B, lanes 1–6), and the uPA activity enhanced by EGF or EGF and plasminogen was also suppressed by CPB (Fig. 2B, lanes 7–10, or bars calculated).

These results indicate that to elevate uPA activity, plasminogen is required to be localized on the hepatocyte surface, at which plasminogen binds with lysine residues of the surface protein via its lysine binding sites (LBS).

*Plasminogen-related cell surface structures do not affect EGF signaling*

As has been reported elsewhere, EGF increased both uPA mRNA and uPAR mRNA levels approximately 2-fold over the basal levels of these genes in rat hepatocytes in primary culture (Fig. 3A–C). However, plasminogen did not have any effect on these genes, even though it was loaded in increasing concentrations up to 5 μg/ml, an effective concentration for increasing uPA activity (see Fig. 2). The increase in uPA activity brought by the addition of plasminogen therefore occurs topically as an activating reaction operating on the cell surface. EGF-stimulated uPA and uPAR gene expression was unaffected by treatment either with TXA or with CPB (Fig. 4). In EGF signaling to induce uPA/uPAR gene expression of the hepatocytes, no molecule of plasminogen/plasmin or even of C-terminal lysine residues is involved.

**uPA activity is an effector of hepatocyte proliferation with plasminogen and EGF stimulation**

Finally, we attempted to determine the role of uPA activity in hepatocyte proliferation by introducing a competitive inhibitor of uPA, p-aminobenzamidine. As a marker of hepatocyte proliferation, proliferating cell nuclear antigen (PCNA), a cyclin, was determined for cells cultured for 24 h, as described in “Materials and Methods.”

The addition of plasminogen to the hepatocytes in primary culture doubled the amount of PCNA of the cells, whereas co-addition of p-aminobenzamidine decreased its level, i.e., with 250 μM of p-aminobenzamidine, the elevated PCNA level decreased to the basal level. The inhibitory effect of p-aminobenzamidine on the EGF-induced PCNA level was closely similar to its effect on the plasminogen-elevated PCNA level, i.e., the tripled PCNA level by EGF decreased to the basal with 250 μM p-aminobenzamidine (Fig. 5).

**Discussion**

Fibrinolytic and matrix-degrading enzymes, uPA/plasmin and MMPs, have been determined to be key enzymes for extracellular proteolysis that contribute to a variety of biological phenomena involving ovulation, cancer cell migration and invasion, and remodeling of vasculatures. These proteases are also known to...
play important roles in liver regeneration, and the evidence for this has been supported by animal studies using the knockout mice for these factors.\textsuperscript{22–24} Furthermore, an activation network connecting uPA/plasmin and MMPs has been found to be a mechanism in which they exhibit their respective functions efficiently; \textit{e.g.}, in an experiment with mice, inhibition of uPA by a specific inhibitor suppressed MMP-2 activity.\textsuperscript{25}
In a previous study, we found that plasminogen/plasmin system stimulates proliferation of hepatocytes in primary culture, but the relationship between this system and the actual enzymatic activity of fibrinolytic and proteolytic factors remains to be clarified. Here we studied plasminogen primarily as a stimulant of hepatocyte proliferation. It is inducible by certain factors relating to plasminogen, such as plasmin, the active form of plasminogen, and uPA or tPA, the activators of plasminogen. Treatment of hepatocytes with plasminogen induced hepatocyte proliferation (Fig. 5A). The fibrinolytic activity of a conditioned medium can be detected by zymography when serum-free medium is used for the culture. In a serum-free medium obtained by 24 h of culture of rat hepatocytes, we were able to obtain activities of tPA and uPA by zymography. The appearance of double lysis bands of tPA (see Fig. 1A) is derived from the difference in glycation rates of tPA protein, as has been reported elsewhere. Of the activities measured, only uPA activity increased more than control due to the addition of plasminogen. Therefore, the hepatocyte proliferating activity of plasminogen that we observed is exhibited substantially through uPA activity elevated by plasminogen. The fact that the uPA activity was upregulated in primary-cultured hepatocytes either by plasminogen or by EGF or by both in coordination with hepatocyte proliferation, indicates that hepatocytes require the active form of uPA as a growth factor.

Although EGF increased both tPA and uPA activities, the combined use of EGF and plasminogen was effective only for uPA activity, not for tPA or MMP-9 and MMP-2. Stable expression of tPA activity is known to be characteristic of hepatocytes in primary culture, so that elevated tPA activity due to EGF was not changed by plasminogen in this experiment.

Upregulation of uPA activity by plasminogen and/or EGF was found to be realized in a surface-touched fashion, since inhibition of binding between the cell surface and plasminogen by specific inhibitors largely abolished their effects (Fig. 2). The inhibitory effect of TXA and CPB observed in the cultures without exogenous plasminogen can be explained as follows: even in these cultures, some endogenous plasminogen is synthesized by hepatocytes, because hepatocytes are the major production site of plasminogen in vivo. The necessity
that plasminogen be placed on the surface to increase uPA cannot be explained fully as yet, but hepatocyte-bound plasmin, if any, activates scuPA (single chain uPA = pro-uPA) to its active form tcuPA (two chain uPA bearing uPA activity) on the cell surface. The system connecting between plasminogen and uPA on or in the hepatocytes should be clarified, but the fact that the exogenous plasminogen affected the plasminogen activator uPA of hepatocytes is of interest. This may be explained when the detailed relationships among plasminogen/plasmin and uPA/pro-uPA on the hepatocytes are determined.

Gene expression of uPA and uPAR was stimulated only by EGF, and not by the increasing amounts of plasminogen (Fig. 3). Therefore, the enhanced uPA activity due to plasminogen is produced topically as a result of the activating reaction taking place on the cell surface. In other words, the possible function of plasminogen as a putative signaling molecule for elevating uPA activity is deniable. Since EGF stimulated uPA and uPAR gene expression independently of the presence of plasminogen on the cell surface (Fig. 4), the role of plasminogen added to the cell is confined to the cell surface, not interfering with the function of EGF.

The slight change or no change in the activity of MMP-9 and MMP-2 observed after plasminogen or EGF stimulation was due to the quite small amount of these enzymes produced by the primary hepatocytes, which were harvested at 24 h of culture in this experiment. To demonstrate the pivotal role of MMPs in hepatocyte proliferation, we must improve the culture conditions, under which MMPs can be produced by hepatocytes as well as hepatocellular carcinoma cells.

The possible role of the uPA/plasminogen system in liver regeneration has been studied using knockout mice, but the regeneration profiles observed in them were different among the models, which were injured in different ways. Hepatocyte proliferation was impaired in uPA and plasminogen-deficient mice after two-thirds partial hepatectomy, and in those with anti-Fas antibody-induced hepatic apoptosis, but not in CCl4-induced acute liver injury. The liver regeneration model with CCl4-induced liver injury is more inflammatory than that with partial hepatectomy or anti-Fas antibody-induced apoptosis. In the CCl4-induced liver injury model, fibrinolytic factors might be primarily spent removing necrotic cells or degrade fibrin and other matrix components, such as fibronectin, in advance of hepatocyte proliferation. Rat hepatocyte in primary culture is a suitable culture system to clarify the direct effects of the uPA/plasminogen system on hepatocyte proliferation, because one can neglect the inflammatory conditions observed in CCl4-induced acute liver injury. Therefore, our results, obtained from the primary culture, can be thought to reflect the actual events occurring in partial hepatectomy and anti-Fas antibody-induced hepatic apoptosis.

uPA is present in a variety of cell, and is known to fulfill many functions through its biological activity. Among its functions, intracellular signaling from the uPA/uPAR interaction is said to be required for vascular smooth muscle cell migration via activation of phosphatidylinositol 3-phosphate kinase. On the other hand, uPA activity induces H-157 cell proliferation via tyrosine phosphorylation of a 78-kDa protein. We found that plasminogen as well as EGF is a potent inducer of hepatocyte proliferation, and that this function is abolished if the active site of uPA is blocked by an inhibitor, p-aminobenzamidine. These results suggest that uPA works as an effector of hepatocyte proliferation in stimulation with plasminogen and/or EGF. Recently, cell proliferation promoted by plasmin has been observed. Hence the investigation of cellular mecha-
nisms involving fibrinolytic factors will be crucial in every connection involving the promotion of regeneration of the liver, and in the prevention of metastasis of cancer cells.

Acknowledgments

We thank Dr. Akira Noguchi (Nihon University College of Bioresource Sciences) for helpful discussion.

This work was supported by a grant from the Japan Society for the Promotion of Science (JSPS), a Grant-in-Aid for Scientific Research (C) (to T.S.) and, in part, by grants from the following sources: (1) the Japan Health Sciences Foundation, a Research Grant for Health Sciences Focusing on Drug Innovation (to T.S.), (2) the Ministry of Education, Culture, Sports, Science, and Technology of Japan, an Academic Frontier Project (to T.S.), (3) Nihon University, an Individual Research Grant for 2005 (to T.S.), (4) JSPS, Japan-Belgium Research Cooperative Program (to T.S.).

N.O. was supported by a Fellowship from the COE Program of the 21st Century in Japan.

References


