Presence of Urokinase in Serum-free Primary Rat Hepatocyte Cultures and Its Role in Activating Hepatocyte Growth Factor

Wendy M. Mars, Tae-Hyoung Kim, Donna Beer Stolz, Meng-Lun Liu, and George K. Michalopoulos

Department of Pathology, University of Pittsburgh, Pittsburgh, Pennsylvania 15261

ABSTRACT

Serum-free rat hepatocyte cultures can be stimulated to divide by the inactive, single-chain form of hepatocyte growth factor (scHGF), suggesting that hepatocytes contain a protein that can cleave scHGF to its biologically active, two-chain (tcHGF) form. We added radiolabeled scHGF to serum-free cultures and confirmed that tcHGF was being generated. Because scHGF can be cleaved to tcHGF by plasminogen activators (PAs), we next tested the cultures for active PA. Although little PA activity was initially present, the majority was of the urokinase type (u-PA) as determined by neutralization studies using either a polyclonal antibody against u-PA or, since u-PA functions in the context of its receptor (u-PAR), a monoclonal antibody against u-PAR. Considerable PA activity developed within 24 h, which was also neutralizable with antibody. To test whether the active, receptor-bound u-PA from the cell cultures was cleaving scHGF, iodinated scHGF was added to intact cells in the presence of the antibody against u-PAR. Comparison to control cultures determined that the antibody prevented scHGF cleavage. Analysis of cultures treated with HGF, epidermal growth factor, and transforming growth factor-α (TGF-α) showed that these growth factors increased the hepatocyte PA activity in parallel with the mRNA for u-PA. TGF-β had the opposite effect, and when TGF-β was added to the culture system, conversion of scHGF to tcHGF was prevented in concert with the production of the type 1 PA inhibitor. When liver remnants from hepatectomized animals were assayed for active TGF-β, elevated protein was found just prior to the appearance of PA inhibitor 1 message and protein. Collectively, our data show that in culture, active u-PA is present and cleaves scHGF to tcHGF in the context of its receptor. It also suggests that modulation of u-PA activity by various growth factors is relevant for regulating cleavage of scHGF to tcHGF both in vitro and in vivo.

INTRODUCTION

HGF, also known as scatter factor, is synthesized as a single chain which must be cleaved to its heterodimeric form to become active (1). Although it is generally accepted that activated HGF induces pleiotropic effects through its receptor (Ref. 2; including mitogenesis, motility, and morphogenesis), there is some confusion regarding the activation of HGF. Both FXIIa and a novel FXIIa-1 enzyme (sometimes referred to as HGF activator) have been shown to cleave inactive HGF (scHGF) to active HGF (tcHGF) in vitro (3); however, FXIIa-1, rather than FXIIa, seems to be the more relevant of the two converting enzymes (3). Like many of the coagulation proteins, FXIIa-1 is synthesized in the liver, as determined by Northern blot analysis (4). The precursor protein is found circulating in plasma, whereas the activated form is present in serum. Thrombin is the most likely candidate for initiating the serum activation of FXIIa-1 (5), and it is probable that FXIIa-1 is generated under procoagulation, rather than fibrinolytic, conditions.

We (6, 7) and others (8) have shown that t-PA and u-PA can also cleave scHGF to tcHGF both in vitro and for u-PA in vivo. Surprisingly, the activation of HGF by u-PA occurs in a stoichiometric rather than enzymatic fashion (9). Furthermore, there is evidence that a stable complex forms between u-PA and HGF on the surface of cells after HGF binds to its receptor (9). In the process of liver regeneration, active u-PA is found in remnant livers as early as 1 min after PHx. Antibody-blocking experiments show the presence of active u-PA results in cleavage of scHGF to tcHGF (7). Interestingly, the enhanced u-PA activity probably results primarily from an increase in the u-PAR rather than from u-PA. Simultaneous measurements of the quantity of u-PAR, tcHGF, and the activity of u-PA show a positive correlation between the three parameters. Additionally, loss of u-PA activity in the PHx liver remnants correlates with the induction of RNA and protein for the u-PA inhibitor known as PAI-1 (10), suggesting that PAI-1 is inhibiting u-PA activity and preventing activation of scHGF to tcHGF.

There is ample evidence to indicate that u-PA is involved in cancer metastasis and the liver is a common site where secondary tumors develop. Still, little is known about the function of u-PA in primary hepatic disease processes, although u-PA levels are reportedly elevated in the plasma from patients with severely compromised livers due to entities such as cirrhosis and hepatocellular carcinoma (11, 12). It is probable that these high levels result from a decrease in liver function since the values correlate positively with total serum bilirubin (12). Elevated HGF levels which correlate with the total bilirubin values have also been reported in the plasma from patients with impaired liver function (13). The importance of these observations are unclear; however, since u-PA is involved in activation of HGF and since HGF is relevant for normal liver repair, the increased plasma values may reflect a decrease in hepatic HGF/u-PA complexes which would lead to impaired tissue restoration.

Because preparations of HGF which are primarily in the inactive, single-chain form can mitotically stimulate hepatocytes in culture (4), we hypothesized that serum-free hepatocyte cultures would possess a mechanism for cleaving scHGF to tcHGF and provide an in vitro model system for studying HGF activation relevant to the liver. Active u-PA protein was detected in hepatocytes immediately following isolation by collagenase perfusion. Active TGF-β was found to be elevated just prior to the appearance of PAI-1 message and protein, suggesting that modulation of PAI-1 by TGF-β is also relevant in vivo. Thus, this study shows that in serum-free hepatocyte cultures, regulation of HGF cleavage occurs via a mechanism involving both u-PA and u-PAR.

The additional finding that various growth factors can either posi-
tively or negatively regulate u-PA activity in the hepatocytes along with the correlation to in vivo observations provides a model explaining the interactive effects of HGF with other growth factors in liver regeneration.

MATERIALS AND METHODS

Hepatocyte Isolation and Culture. Primary hepatocytes were isolated using our standard two-step collagenase perfusion technique which is completely serum free (14). Cell populations with 80% or higher viability were diluted in serum-free MEM (catalogue no. 41600-016: Life Technologies, Inc., Grand Island, NY) containing 50.0 μg/ml gentamicin, either with or without 0.5 ng/ml bovine insulin and 5.0 ng/ml BSA, and dispersed onto either plain plastic (insulin/BSA-containing) or collagen-coated (insulin/BSA-free) plates. After 2 h the media (containing nonadherent cells) was removed and replaced with fresh media, either with or without growth factors. In some experiments involving collagen-coated plates, after removing the nonadherent cells the cultures were incubated an additional 18 h before changing to media with growth factors. On the collagen-coated dishes, growth factors were used at the following concentrations: HGF, EGF, and TGF-α, 50 ng/ml and TGF-β1, 1 ng/ml. For the insulin/BSA-containing cultures, the HGF concentration was 10 ng/ml. Cultures were seeded at 1.5 × 10^5 cells/25-mm dish, 2 or 3 × 10^5 cells/80-mm dish, or 2.4 × 10^6 cells/80-mm dish and maintained in 0.5, 1.0, or 5.0 ml medium, respectively. Human recombinant TGF-β1 was purchased from R&D Systems (Minneapolis, MN), whereas both murine submaxillary gland EGF and human recombinant TGF-α were purchased from Collaborative Research (Waltham, MA). Human recombinant scHGF, used for general cell culture, was a gift from Snow Brand Milk Products (Tochigi, Japan) and corresponded to the naturally occurring five amino acid deleted form.

RNA Isolation and Northern Blot Analysis. RNA was isolated from hepatocytes plated on 80-mm collagen-coated plates using RNAzol B reagent (Biotex Laboratories, Inc., Houston, TX) as indicated by the manufacturer. Integrity was assessed using ethidium bromide staining of formaldehyde gels. For Northern blot analyses, equal amounts (20 μg) of total RNA were electrophoresed through formaldehyde gels (15) and transferred to the bloting membranes Genescreen Plus (New England Nuclear, Boston, MA) or BioTrans (ICN, Costa Mesa, CA) as recommended by the manufacturer. Membranes were prehybridized and then hybridized using either a rat u-PA 360-bp cDNA fragment (a gift from Dr. Jay Degen, University of Cincinnati, Cincinnati, OH) or a rat PAI-1 760-bp cDNA fragment (a gift from Dr. Paul Higgins, Albany Medical College, Albany, NY) that was labeled with [32P]dCTP using the multiprime labeling kit (Amersham, Arlington Heights, IL). After washing, filters were subjected to autoradiography using Kodak X-OMAT film.

Protein Lysate Preparation and Western Blot Analysis. Lysates were prepared from hepatocytes plated on 80-mm collagen-coated plates using a cold 10 mM Tris-buffered (pH 7.6) salt solution (50 mM NaCl) containing 1% Triton X-100 in the presence of multiple protease inhibitors (100 μg/ml BSA, 5 mM EDTA, 100 μM sodium o-vanadate, 1 mM phenylmethylsulfonyl fluoride, 50 mM sodium fluoride, 30 mM sodium PPi, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 5 μg/ml trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane, 10 μg/ml aprotinin, and 50 μg 1,10-phenanthroline). By separating the Triton-X-100-soluble and Triton-X-100-insoluble proteins with centrifugation, nucleus-extracellular matrix-cytoketolysed-associated (insoluble) and nonsolubilated (soluble) fractions were obtained (16). Triton X-100-insoluble fractions were dispersed with 1% SDS in the same lysate solution, sonicated, and the protein concentrations were determined using the bicinchoninic acid assay (Sigma, St. Louis, MO). For lysates from hepatocyte-m trimester and sham-operated animals, the livers were homogenized in the buffer described above except that the NaCl was eliminated, the Tris was buffered to pH 8.0, and 1% SDS was substituted for the Triton X-100. Insoluble material was removed by centrifugation at 14,000 × g before assaying for protein concentration.

For Western blot analysis, equal amounts of protein were subjected to SDS-PAGE and electrotransferred to Immobilon-P membranes (Millipore, Bedford, MA) using a solution containing 50 mM Tris base, 95 mM glycine, and 0.05% SDS. Membranes were blocked with 5% powdered milk in a solution of 20 mM Tris (buffered to pH 7.5 with HCl), 150 mM NaCl, and 0.1% Tween 20 (blotto base), and then primary antibodies were either applied for 1 h in the blocking buffer at room temperature or overnight at 4°C. After extensive washing with 1% powdered milk in blotto base, horseradish peroxidase-conjugated secondary antibody was applied for 1 h in the 1% milk solution. Membranes were then washed extensively in blotto base, and signal was visualized using the ECL detection system (Amersham). The primary antibodies used were as follows: rabbit anti-mouse u-PA polyclonal, mouse anti-human u-PAR monoclonal, rabbit anti-rat PAI-1 polyclonal at 2 μg/ml (1189, 3936, and 1062, respectively; American Diagnostica, Inc., Greenwich, CT), and rabbit anti-human TGF-β polyclonal at 0.1 μg/ml (sc-146; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Secondary antibodies, used at 0.5 μg/ml, were goat anti-rabbit and goat anti-mouse (A6164 and A4416, respectively; Sigma).

HGF Metabolism Studies. scHGF, corresponding to the naturally occurring five amino acid deleted form (gifts from Genentech, South San Francisco, CA and Toyobo, Shiga, Japan), was iodinated as previously described using chloramine T (6). Radiolabeled scHGF was added to the hepatocyte cultures, and protein lysates were prepared after various periods of time. Sample radioactivity was assessed, and equivalent quantities of radioactive counts were subjected to SDS-PAGE. Gels were dried onto Whatman 3MM paper and subjected to autoradiography using Kodak X-Omat film. Lysates from the insulin-containing cultures were prepared as described above; however, in the collagen-coated experiments, 1% SDS was substituted for the Triton-X-100. In experiments where antibody against u-PAR was used to block the reaction, the medium was replaced, antibody was bound to the cells for 30 min, the medium was replaced, and iodinated scHGF was added to the cultures. The concentration of the antibody was 0.5 mg/ml, and the volume was either 50 or 100 μl for 22- or 35-mm wells, respectively.

PA Studies. We utilized a modification of a previously published casein clearance assay (17). Essentially, medium was removed from the cells and replaced with 0.7% agarose and 2% powdered milk in insulin/BSA-containing MEM, either with or without plasminogen (40 μg/ml, 410A; American Diagnostica, Inc.). The solution was first made without plasminogen, heated sufficiently for the agarose to go into solution, and then maintained at 50°C. After adding the plasminogen, 400 or 800 μl solution were added to 22- or 35-mm wells, respectively, and allowed to harden. Plates were then placed in the tissue culture incubator for various time periods. In blocking experiments, antibody was applied as described for the HGF metabolism studies before adding the agarose solution. Anti-u-PA and anti-u-PAR were used at concentrations of 0.34 and 0.5 mg/ml, respectively.

RESULTS

Cleavage of scHGF to tcHGF Occurs in Serum-free Hepatocyte Cultures. Primary hepatocytes were seeded directly onto culture plates immediately after isolation by collagenase perfusion of the liver. After 2 h, nonadherent cells were removed, and radiolabeled scHGF was added to the cultures. Hepatocytes were harvested immediately and at various time points thereafter to assess the form of HGF present in the cultures. As seen in Fig. 1A, a substantial portion of the HGF was already in the two-chain form as early as 1 h after addition of the iodinated scHGF. Pulse-chase experiments using Triton X-100-soluble and Triton X-100-insoluble fractions indicated that cleavage occurred in the Triton X-100-insoluble fraction (data not shown). By pulsing the cells with scHGF and then observing them over several days, it could be shown that initially cleavage occurred rapidly, and thereafter the reaction proceeded very slowly (Fig. 1B).

Active u-PA Is Present in Serum-free Hepatocyte Cultures. u-PA and t-PA can cleave scHGF to active tCHGF in vitro (6, 8). Additionally, u-PA activity is present in liver remnants as early as 1 min after PHx, and the endogenous u-PA is capable of cleaving scHGF to the two-chain form (7). Since scHGF was being cleaved to the two-chain form very early in serum-free hepatocyte cultures, we tested for the presence of PA activity in these cells. Serum-free hepatocyte cultures were initially set up and tested for PA activity at various times after seeding using a casein clearance assay (17).
Although relatively little activity was present at the earliest time points, by 24 h the PA activity had risen substantially (data not shown). Addition of a polyclonal antibody against u-PA at 2 h after plating the cells blocked the majority of clearance (Fig. 2A), suggesting that u-PA was responsible for most of the early PA activity.

To confirm our findings, we next examined whether u-PA protein and/or the mRNA was present in the hepatocyte cultures at various times after seeding the cells. To help localize the u-PA within the hepatocytes, protein lysates were divided into Triton X-100-soluble and -insoluble fractions. Western blot analyses showed that a substantial quantity of single-chain u-PA was initially present in the Triton X-100-soluble fraction, and it remained unchanged for over 48 h. In contrast, the quantity of single-chain u-PA in the Triton X-100-insoluble fraction was initially high and began to lessen with time (Fig. 2B). Using Northern blot analysis, the u-PA mRNA was undetectable at 2 h; however, by 24 h the mRNA reached a peak and then declined (Fig. 2C). Northern blot analysis for t-PA was negative (data not shown).

**Antibody against u-PAR Prevents Cleavage of scHGF to tcHGF.** To test whether the u-PA present in the cell cultures was cleaving scHGF to tcHGF, an experiment was designed to inhibit u-PA activity and determine whether cleavage occurred. Cultures were set up in the presence of a monoclonal antibody against u-PAR and then pulsed with radiolabeled scHGF. We preferred this antibody because a monoclonal such as anti-u-PAR is less likely to cross-react with unrelated proteins and because of long-term cellular toxicity induced by the u-PA antibody. Furthermore, u-PA is known to function in vivo in the context of its receptor; therefore, the results using this antibody should be biologically more relevant. Casein overlay assays showed that the monoclonal antibody against u-PA was able to inhibit PA activity in the cell cultures, reaffirming our results with the polyclonal antibody against u-PA (Fig. 3A). Additionally, thymidine incorporation into control cells and cells incubated for 48 h with the antibody indicated little or no toxicity. When cells were incubated with antibody against u-PAR and then pulsed with scHGF, the generation of tcHGF was inhibited (Fig. 3B). This indicates that cleavage of scHGF is occurring in a u-PA/u-PAR complex. In various experiments performed under similar conditions, the reduction in tcHGF that was generated in the presence of antibody against u-PAR ranged from 18 to 50%, with an average reduction of 34% as determined by densitometric analyses. It should be noted that the variation in these values depends on the quantity of radiolabeled tcHGF generated by the cell surface u-PA from the cultures rather than on the amount of inhibition by the antibody (which is constant). Since various preparations of primary rat hepatocytes were utilized, this amount differed in each experiment.

**Growth Factors Affect u-PA Activity in Hepatocyte Cultures.** It is known that many growth factors, including HGF, can affect the synthesis of u-PA in cell cultures (18, 19). Additionally, several growth factors are known to act in an additive manner with HGF on hepatocytes (14, 20, 21). Therefore, we decided to assess u-PA mRNA, protein, and activity in cultures treated with various factors known to be important in regulating the growth of hepatocytes. Total RNA was isolated from cells after 24 h of treatment with HGF, EGF, TGF-α, or TGF-β and compared to untreated cultures using Northern blot analysis. u-PA mRNA was enhanced in the growth factor-treated hepatocytes, whereas the cells treated with TGF-β (a negative growth regulator) displayed a decrease in mRNA (Fig. 4A). Analysis of the protein showed no change in the Triton X-100-soluble fraction and minor changes in the Triton X-100-insoluble single-chain u-PA (data not shown). The PA activity mimicked the RNA results, with a diminished response particularly evident in the TGF-β-treated cultures. Since substantial amounts of u-PA protein were still present in the TGF-β-treated cells, we decided to assess whether PAI-1 might be up-regulated and thus turning the PA activity off. Analysis of the cultures for PAI-1 mRNA and protein determined that PAI-1 mRNA was significantly increased in cultures treated with TGF-β (Fig. 4B). This was also reflected in the Western blot analyses where PAI-1 protein was found to be associated with both the Triton X-100-soluble and -insoluble fractions in the TGF-β-treated cultures (data not shown).

Since TGF-β-treated cultures contained u-PA that was biologically inactive, presumably due to the presence of its natural inhibitor PAI-1, we assessed the cleavage of scHGF in TGF-β-treated cultures. Cultures were treated overnight with TGF-β and then pulsed with radiolabeled scHGF. Similar to the cultures treated with antibody against u-PAR, densitometric scanning analysis of autoradiography results from TGF-β-treated cultures revealed that under these conditions, treated hepatocyte cultures also contained less tcHGF than the control cultures did (Fig. 5). In experiments in which cells from the same preparation were treated with either antibody against u-PAR or TGF-β, the reduction in generated tcHGF was similar, indicating that the effect of TGF-β was probably a result of the inhibition of u-PA activity induced by PAI-1. The effect was not due to a loss of cell viability induced by the TGF-β since tritiated thymidine incorporation...
Fig. 2. u-PA is present in serum-free hepatocyte cultures. In the experiments shown, hepatocytes were plated onto collagen-coated dishes. A, after removing nonadherent cells, antibody against u-PA was added to selected wells, and the cells were immediately analyzed for PA activity using a casein clearance assay. Dark areas in the wells, regions of PA activity where the exogenous plasminogen has been activated to plasmin and digested the casein. To control for casein digestion by enzymes other than the generated plasmin, wells without plasminogen were also included. Wells shown in this experiment were incubated overnight. B, proteins from Triton X-100-insoluble and Triton X-100-soluble fractions of hepatocytes obtained at 2, 24, or 48 h after plating were separated using SDS-PAGE and processed for Western blot analysis using a polyclonal antibody against u-PA. The bands shown represent single-chain u-PA. C, RNAs were isolated from hepatocytes obtained at 2, 24, or 48 h after plating, separated under denaturing conditions using formaldehyde gel electrophoresis, and processed for Northern blot analysis using a rat cDNA fragment as the probe (a gift from Jay Degen). Top panel, 2.3-kb u-PA transcript; bottom panel, ethidium bromide stain of the gel before transfer.

DISCUSSION

Previously, we and others determined that u-PA and t-PA can activate scHGF in vitro (6, 8) and that u-PA activity corresponds to the appearance of activated HGF in the PHx model of regenerating liver (7). We now show that completely serum-free hepatocyte cultures contain active u-PA and that inhibition of the activity with antibody against u-PAR prevents cleavage of scHGF. Furthermore, we show that u-PA activity can be modulated in serum-free hepatocyte cultures by exogenous growth factors and that one of these factors, TGF-β, may be pertinent in regulating the activity of u-PA both in vitro and in vivo.

Several points are worth noting about our results. First, it was an unexpected finding that serum-free hepatocyte cultures contain u-PA activity at such an early time point. In regenerating liver induced by PHx, we and others have shown that u-PA mRNA is not synthesized in large quantities by the regenerating tissue (7, 10), although active u-PA is present in remnant livers shortly after surgery (7). Rather, activity seems to result because the protein is taken up from the...
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Fig. 3. Cleavage of sHGF to tHGF is prevented by antibody against u-PAR. Hepatocytes were plated on collagen-coated dishes. A, cells were treated as in Fig. 2A, except that the antibody was against u-PAR, the assay for PA activity was done the following day (rather than immediately), and the incubation period was 4 h. -, control cells; +, antibody treated. B, cells from the same experiment as shown in A were incubated overnight in the presence or absence of anti-u-PAR. The following day, the hepatocytes were pulsed with iodinated sHGF for 2.5 h before harvesting and assessing the relative quantity of tHGF and sHGF. At the conclusion of this particular experiment, scanning densitometry indicated the tHGF was 15.7 and 26.0% in the antibody-treated and control cells, respectively, reflecting 40% less tHGF generated by the antibody-treated cells. Lanes, two different exposures of the same gel; however, scanning was done on a single autoradiographic exposure for accuracy. sc, hc, and lc represent sHGF, the heavy chain of tHGF, and the light chains of tHGF, respectively.

Peripheral blood by increasing amounts of u-PAR (7). Still, it was surprising to find that both u-PA protein and activity were initially present in these cells, despite the absence of u-PA mRNA. The most likely explanation is that the u-PA adhered to the hepatocytes and was carried along after the collagenase perfusion. In support of this hypothesis is the fact that u-PA protein is stable for several days (22).

Most of the cleavage-blocking experiments using antibody against u-PAR were set up using cells that had been plated for 24 h rather than 2 h since we were simultaneously assessing the effects of overnight treatment with TGF-β. While analyzing the data, we noted that cleavage of sHGF to tHGF occurred much more effectively with cells plated for 2 h on plastic in the presence of insulin as opposed to 24 h on collagen-coated plates without insulin (compare Figs. 1A and 3B). Although surprising, this is not totally unexpected because either differences in the culture media or in the state of the cells could affect cleavage. As shown by the experiment in Fig. 1B, even with prolonged incubation on plastic the cleavage of sHGF slows significantly after 6 h, and it is probable that the biological state of the cells after prolonged incubation is less amenable to cleavage of sHGF. In this regard, it is worth noting that despite an increase in u-PA mRNA after 24 h, the quantity of single-chain u-PA protein in the Triton X-100-insoluble fraction (where cleavage of sHGF occurs) is comparable to the level of sHGF activation; both are initially elevated and then decline (Fig. 2B). Thus, although u-PA mRNA becomes elevated at 24 h, the increase does not reflect either the protein levels of Triton X-100-insoluble, single-chain u-PA or the activation rate of sHGF. Instead, the mRNA correlates with the rapidity of casein cleavage in the plasmin-generating assay. It is unclear what the role of the u-PA in the Triton X-100-soluble fraction is, although it does

Fig. 4. Effect of growth factors on u-PA and PAI-1 expression. Hepatocytes were cultured for 24 h on collagen-coated plates and then treated for 24 h with various growth factors. Thus, control cells correspond to a 48-h incubation with media only. RNAs were isolated, separated under denaturing conditions using formaldehyde gel electrophoresis, and processed for Northern blot analysis. Samples 1-5 correspond to control, HGF-treated, EGF-treated, TGF-α-treated, and TGF-β-treated hepatocytes, respectively. Autoradiography signals were corrected for differences in the loading, as determined by the intensity of the ethidium bromide staining. A, samples were probed with the rat u-PA cDNA used in Fig. 2C. Top panel, 2.3-kb u-PA transcript; bottom panel, ethidium bromide stain of the gel before transfer. B, samples were probed with a rat PAI-1 cDNA (a gift from Dr. Paul Higgins). Top panel, 3.2-kb PAI-1 transcript; bottom panel, ethidium bromide stain.

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not appear to be involved in cleavage of scHGF. One possibility is that it reflects intracellular u-PA generated by the endogenous mRNA. If so, it would represent the u-PA which correlates plasminogen activation. Alternatively, the Triton X-100-soluble fraction may act as a reservoir for the Triton X-100-insoluble u-PA, exchanging u-PA between the two locations as needed.

Although the hepatocyte cultures contained a great deal of u-PA mRNA when examined 24 h after plating, by 48 h the u-PA mRNA decreased significantly. When the growth factors HGF, EGF, or TGF-α were added, the mRNA became elevated; however, addition of TGF-β had the opposite effect. The addition of TGF-β to cultures has been reported to result in a decrease of u-PA mRNA (23); however, recently an elevation of u-PA mRNA (rather than a decrease) was reported in serum-free human hepatocyte cultures after 48 h of TGF-β treatment (24). The difference in hepatocyte results may stem from the fact that we assayed for mRNA 24 h, rather than 48 h, after addition of the TGF-β. Also, there were some differences in the culture systems which might account for the results. For example, their media contained hydrocortisone whereas ours did not. Hydrocortisone can inhibit u-PA production when added to cells (25), and it is possible that TGF-β overcomes this suppression. Conversely, our cells were plated on collagen-coated plates whereas theirs were not, and it is possible that the matrix is modulating the u-PA mRNA production.

Still, it is important to note that despite the differences there are also similarities in the effects that TGF-β has on the hepatocytes under the two culture conditions. Most important, there was an elevation of both mRNA and protein production of PAI-1 in their system (24) as well as ours.

Cleavage of scHGF was prevented by the addition of exogenous antibody against u-PAR. Given our previous results (6, 7), this is not a totally unexpected finding. Still, it is worth reemphasizing that the present studies were performed on intact cells, whereas the earlier studies were performed in vitro or using protein extracts from liver homogenates. Furthermore, both the finding that cleavage occurred in the Triton X-100-insoluble fraction and the usage of intact cells indicates that cleavage is a cell surface phenomenon. Recently, a complex of scHGF, high molecular weight u-PA, and the HGF receptor (c-met) was shown to occur on the cell surface of A549 cells (9). Our results suggest that this same complex occurs on hepatocytes, resulting in cleavage of scHGF and activation of c-met.

The fact that selected growth factors could alter the levels of u-PA and PAI-1 mRNAs and u-PA activity in this system is intriguing. Although it has long been known that many different growth factors can modulate the PA system (18, 19), the reason for this is not known. It has long been hypothesized that regulation of u-PA is important for cleavage of extracellular matrix by plasmin (26). Our results would suggest that regulation of u-PA could also be important for altering the mitotic response via HGF. For example, TGF-β causes hepatocytes to cease growing (27). We have now shown that hepatocytes exposed to TGF-β down-regulate the activity of the cell surface u-PA through generation of PAI-1, and concomitantly cleavage of scHGF is prevented. Furthermore, we have found elevated levels of active TGF-β levels in lysates from liver remnants of animals with PHx with a time frame that immediately precedes the reported production of PAI-1 message and protein (10). The appearance of PAI-1 protein
corresponds with the abrogation of u-PA activity and detection of tchGF (7, 10). Thus, it seems plausible to hypothesize that altered u-PA activity, by HGF itself or other factors such as EGF, TGF-α, and TGF-β, plays a role in regulation of the growth response via a HGF-mediated pathway. Interestingly, Lieber et al. (28) recently reported that the introduction of u-PA into intact livers via a retroviral vector induces an enhanced mitotic response. The mechanism for the retrovirally induced response is unknown; however, the presence of active u-PA seems to be an important component of events leading to DNA synthesis and mitosis in hepatocytes.

Our results show that cleavage of scHGF to tchGF in hepatocytes occurs via a u-PA/α2-MP-mediated mechanism in the Triton X-100-insoluble fraction of cells. Although cleavage corresponds to the quantity of u-PA in the Triton X-100-insoluble fraction, it does not correspond to the plasmin-generating levels detected using a casein clearance assay. This suggests that u-PA can have two different functions, possibly depending on its cellular location (see model in Fig. 7). Regulation of the plasmin-generating activity can be manipulated by multiple growth factors in either a positive (HGF, EGF, and TGF-α) or negative (TGF-β) manner. It is presently unclear as to whether HGF, EGF, and TGF-α also enhance the generation of tchGF, although preliminary data suggest that at least EGF does. With TGF-β, abrogation of both the cleavage of scHGF and the plasmin-generating activity corresponds with production of PAI-1 mRNA and protein (which localizes to both the Triton X-100-soluble and -insoluble fractions). Lysates from regenerating livers contain elevated levels of active TGF-β just prior to the reported induction of PAI-1 (10). Not surprisingly, in vivo generation of PAI-1 protein corresponds with the abrogation of both u-PA activity and the appearance of tchGF (7, 10). As active TGF-β is generated from its latent form by plasmin, induction of PAI-1 by TGF-β provides a mechanism for down-regulating its own activity simultaneously with the cessation of cell growth (Fig. 7). Thus, stimulation of u-PA activity in normal hepatocytes by positive growth factors also begins the process for the down-regulation of their growth via a TGF-β-mediated mechanism. Understanding where this process goes awry should ultimately lead to a better understanding of events contributing to the malignant process.

REFERENCES

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