HEPATOCYTE growth factor (HGF) has been first purified from serum of hepatectomized rats and found in rat platelets. The human HGF has been purified from the plasma of patients with fulminant hepatic failure. It is a powerful mitogen for both rat and human hepatocytes in vitro. The cDNAs of rat and human HGF have been cloned and characterized. Recently, it has been shown that HGF is identical with scatter factor (SF) and tumor cytotoxic factor. Furthermore, HGF has been shown to stimulate the growth of epithelial cells and endothelial cells and is angiogenic in vivo. HGF/SF is produced by stromal fibroblasts and is also involved in the local regulation of fibrinolysis and coagulation. One could speculate that HGF might modulate processes requiring matrix degradation by increasing the expression of the protease u-PA in one cell type and by upregulating the expression of the serine protease inhibitor PAI-1 in another cell type. Because u-PA has been shown to activate latent HGF to the active form, it could further more be speculated that by upregulating PAI-1, which in turn could inhibit u-PA, HGF might regulate its own activation.

MATERIALS AND METHODS

Tissue culture. Human umbilical vein endothelial cell (HUVEC) were isolated by mild collagenase treatment as described by Gim

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HGF is a powerful mitogen for both rat and human hepatocytes, epithelial cells and endothelial cells in vitro, and is angiogenic in vivo. It has considerable homology with plasminogen and has been shown to upregulate urokinase-type plasminogen activator (u-PA) in endothelial cells as well as u-PA and its receptor in kidney epithelial cells. In this study, we report that human recombinant HGF stimulates expression of plasminogen activator inhibitor type 1 (PAI-1) and tissue factor (TF) in the human hepatoma cell line HepG2. PAI-1 antigen as determined by a specific enzyme-linked immunosorbent assay increased up to threefold in conditioned media of HepG2. This increase was dose dependent with maximum stimulation achieved with a concentration of 50 ng/mL of hepatocyte growth factor (HGF). PAI-1 antigen also increased up to fourfold in the extracellular matrix in HGF-treated HepG2. The production of the PAI-1 binding protein vitronectin (Vn) was not affected by HGF. In contrast, TF activity in HepG2 treated with HGF increased up to twofold. As determined by Northern blotting, PAL1 and TF-specific mRNA were increased significantly in the presence of HGF, whereas Vn mRNA was not affected. The increase in PAI-1 and TF mRNA was also seen when HepG2 were incubated with HGF in the presence of cycloheximide, thereby indicating that de novo protein synthesis is not required to mediate the effect. u-PA could be detected neither in unstimulated or HGF-stimulated HepG2 cells on the antigen level nor on the mRNA level. In conclusion, our data give evidence that HGF, in addition to its proliferative effect for different cell types, is also involved in the local regulation of fibrinolysis and coagulation. One could speculate that HGF might modulate processes requiring matrix degradation by increasing the expression of the protease u-PA in one cell type and by upregulating the expression of the serine protease inhibitor PAI-1 in a different cell type. Because u-PA has been shown to activate latent HGF to the active form, it could furthermore be speculated that by upregulating PAI-1, which in turn could inhibit u-PA, HGF might regulate its own activation.

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The human hepatoma cell line HepG2 was obtained from American Type Culture Collection (ATCC; Rockville, MD), grown in Dulbecco’s modified Eagle’s medium (DMEM, Sigma) containing 10% FCS and subcultured at a split ratio of 1:6. The purity of rhHGF was more than 95%, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and protein staining. Such purified rhHGF was fully active as judged by overnight incubation at 4°C with protein A Sepharose (Pharmacia, Uppsala, Sweden). Unbound proteins were removed from the Sepharose beads by washing twice with 2 mL NaCl containing 0.1% Triton X-100 and once with distilled water. Bound proteins were released from the beads by boiling in Laemmli sample buffer for 5 minutes and fractionated by SDS-PAGE.Metabolically labeled CM and CL were collected and subjected to 8\% methionine (416 \mu C/mL) to the respective flasks. The cells were incubated for 6 hours at 37°C. Thereafter, cold methionine was added to the cells to achieve a final concentration of 150 mg/mL and the cells were incubated for 6 hours at 37°C. At the end of a total incubation time of 12 hours, CM and CL were collected as described above. Metabolically labeled CM and CL were each mixed with equal amounts of immunoprecipitation buffer (PBS, pH 7.4, containing 1% nonfat dry milk, 10 U/mL aprotinin (Trasyol; Bayer, Germany), 10 mmol/L benzamidinehydrochloride (Sigma), and 0.1% Triton X-100) and incubated with an excess of polyclonal rabbit antihuman Vn antibodies (Technoclone) for 2 hours at 37°C followed by overnight incubation at 4°C with protein A Sepharose (Pharmacia, Uppsala, Sweden). Unbound proteins were removed from the Sepharose beads by washing twice with 2 mL NaCl containing 0.1% Triton X-100 and once with distilled water. Bound proteins were released from the beads by boiling in Laemmli sample buffer for 5 minutes and fractionated by SDS-PAGE; subsequently, the gels were fixed (30% methanol, 10% acetic acid), soaked in Enlightning GF/A filters (Whatman, Maidstone, England) under light suction and washed twice with ice-cold 10% TCA, once with 70% ethanol, and once with distilled water. The filters were air dried and subjected to scintillation counting in Beckman Ready Scintillation fluid (Beckman, Fullerton, CA).
HGF STIMULATES EXPRESSION OF PAI-1 AND TF

Fig 1. Dose-dependent effect of rhHGF on PAI-1 antigen (A) and PAI-1 activity (B) in CM and CL of HepG2 cells. Confluent monolayers of HepG2 were incubated for 12 hours in the absence or presence of rhHGF at the indicated concentrations. CM (A) and CL (Δ) were collected and PAI-1 antigen and PAI-1 activity were determined as outlined in Materials and Methods. Values are given in ng/10⁶ cells/12 hours for PAI-1 antigen and in t-PA inhibiting units/10⁶ cells/12 hours for PAI-1 activity; they represent mean values ± SD of three independent determinations. Experiments were performed four times with a representative experiment shown. *P < .001, **P < .01.

Table 1. Effect of rhHGF on PAI-1 Activity Before and After Activation With Guanidinium

<table>
<thead>
<tr>
<th>PAI-1 Activity</th>
<th>Before Activation</th>
<th>After Activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.9 ± 0.2</td>
<td>8.6 ± 0.3</td>
</tr>
<tr>
<td>50 ng/mL HGF</td>
<td>6.3 ± 1.1*</td>
<td>55.6 ± 2.9†</td>
</tr>
</tbody>
</table>

Confluent monolayers of HepG2 were incubated for 12 hours in the absence or presence of 50 ng/mL rhHGF. CM were collected and PAI-1 activity before and after activation with 6 mol/L guanidinium hydrochloride was determined as outlined in the Materials and Methods section. Values are given in t-PA inhibiting units/10⁶ cells/12 hours and represent mean values ± SD of three independent determinations. Experiments were performed three times with a representative experiment shown.

* P < .005.
† P < .001.
Fig 3. Time-dependent effect of rhHGF on PAI-1 antigen in CM of HepG2 cells. Confluent monolayers of HepG2 were incubated for 4, 8, 12, or 24 hours in the absence (△) or presence of 50 ng/mL HGF (●). CM were collected and PAI-1 antigen was determined as outlined in the Materials and Methods section. Values are given in ng/10^6 cells and represent mean values ± SD of three independent determinations. Experiments were performed three times with a representative experiment shown. *P < .001, **P < .005.

mL of HGF or in control media only. The results of these experiments are shown in Fig 4. As can be seen, HGF did not affect Vn production by HepG2; also, total protein synthesis was not affected by HGF (data not shown).

No u-PA antigen could be detected in CM and CL of HGF-treated or untreated HepG2 cells with the ELISA used (lower limit of detection: 0.6 ng/mL).

When TF activity was determined in CL from HepG2 incubated for 4, 8, or 12 hours in the presence of 50 ng/mL of HGF or in control media only, a significant increase in TF activity in CL from cells treated with HGF was seen (Fig 5).

In contrast with HepG2, HGF affected neither PAI-1 production nor TF activity in HUVEC (Table 2).

The results obtained on the level of protein production were confirmed on the level of specific mRNA production as determined by Northern blotting experiments. As can be seen from Fig 6, PAI-1 as well as TF-specific mRNA increased significantly in HepG2 treated with 50 ng/mL of HGF for 4 hours as compared with the respective mRNA obtained from control cells. The increase in PAI-1 and TF-specific mRNA was also seen in the presence of 10 μg/mL cycloheximide. Vn-specific mRNA was not affected by HGF treatment. No u-PA- or u-PAR–specific mRNA could be detected in HepG2 cells under these conditions.

DISCUSSION

HGF has recently been implicated in the regulation of fibrinolytic components in different cell types. HGF stimulates production of u-PA by bovine capillary endothelial cells and expression of u-PA and u-PAR in kidney epithelial cells.12,25 We show in this paper that HGF stimulates the expression of PAI-1, the major physiological inhibitor of u-PA, in the human hepatoma cell line HepG2. This effect was dose- and time-dependent, with maximum responses achieved with 50 ng/mL of HGF. PAI-1 antigen increased significantly in CM, CL, and ECM of HGF-treated cells. The increase in PAI-1 antigen was paralleled by an increase in PAI-1 activity in CM and CL of such treated HepG2. It is noteworthy that little PAI-1 activity could be detected in native CM of HGF-treated cells as well as in CM of untreated controls. However, PAI-1 activity increased dramatically, when CM from such cells was treated with guanidinium hydrochloride, thereby changing latent PAI-1 to active PAI-1.55 In contrast with PAI-1 no u-PA could be detected in this cell line regardless of whether it was treated with HGF.

As determined by immunoprecipitation experiments, the increase in PAI-1 antigen brought about by HGF was not accompanied by an increase in the PAI-1 binding protein Vn.

When TF activity was determined in CL of HepG2, a significant increase of this activity was found in cells treated with HGF.

However, in HUVEC, HGF affected neither PAI-1 antigen production nor TF activity under the conditions tested.

These results were confirmed on the level of specific
HGF STIMULATES EXPRESSION OF PAI-1 AND TF

![Graph showing tissue factor activity over time](image.png)

**Fig 5.** Effect of rhHGF on TF activity in CL of HepG2 cells. Confluent monolayers of HepG2 were incubated for 4, 8, or 12 hours in the absence (○) or presence of 50 ng/mL HGF (▲). CL were collected and TF activity was determined as outlined in Materials and Methods. Values are given in mU/10^6 cells and represent mean values ± SD of three independent determinations. Experiments were performed three times with a representative experiment shown. *P < .001, **P < .002.

mRNA expression by Northern blotting. PAI-1–specific and TF-specific mRNA increased significantly in HepG2 cells treated for 4 hours with HGF, whereas Vn-specific mRNA did not change in the presence of HGF in these cells. Furthermore, HGF increased PAI-1–specific and TF-specific mRNA in HepG2 also in the presence of cycloheximide, suggesting that de novo protein synthesis is not required.

In conclusion, our data indicate that in the human hepatoma cell line HepG2, HGF induces an antifibrinolytic, procoagulatory profile by increasing the expression of PAI-1, a major inhibitor of fibrinolysis, and TF, a major activator of coagulation. This coregulation of PAI-1 and TF by HGF is consistent with the effects induced by endotoxin, interleukin-

Table 2. Effect of rhHGF on PAI-1 Antigen and TF Activity in HUVEC

<table>
<thead>
<tr>
<th>Condition</th>
<th>PAI-1 Antigen (ng/10^6 cells)</th>
<th>TF Activity (mU/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4,000 ± 345</td>
<td>290 ± 69</td>
</tr>
<tr>
<td>50 ng/mL HGF</td>
<td>3,854 ± 526</td>
<td>284 ± 56</td>
</tr>
</tbody>
</table>

Confluent monolayers of HUVEC were incubated for 12 hours (for determination of PAI-1 antigen) or 4 hours (for determination of TF activity), respectively, in the absence or presence of 50 ng/ml rhHGF. CM and CL were collected and PAI-1 antigen and TF activity was determined as outlined in the Materials and Methods section. Values are given in ng/10^6 cells/12 hours for PAI-1 antigen and in mU/10^6 cells/4 hours for TF activity and represent mean values ± SD of three independent determinations. Experiments were performed three times with a representative experiment shown.

![Image of Northern blot](image.png)

**Fig 6.** Effect of rhHGF on specific mRNA expression for PAI-1, TF, and Vn in HepG2 cells as determined by Northern blotting. Confluent monolayers of HepG2 were incubated for 4 hours in the presence of 10 µg/mL cycloheximide (CHX), 50 ng/mL HGF, a combination of CHX (10 µg/mL) and HGF (50 ng/mL), or without any addition, respectively. mRNA was prepared from such cells and Northern blotting performed using specific probes for PAI-1, TF, and Vn, and GAPDH as a control as outlined in Materials and Methods. Note uneven loading in the lanes labeled CHX and HGF+CHX as indicated by the different amounts of GAPDH. The respective mRNAs are indicated by arrows. Experiments were performed twice with a representative experiment shown.

1 (IL-1), and tumor necrosis factor (TNF) that have been shown also to stimulate PAI-1 and TF production in the same cell type. However, the effects of HGF on PAI-1 and TF expression were not seen in endothelial cells, which in vivo are a major source of PAI-1 and TF. This specificity in the action of HGF is different from cytokines such as IL-1 and TNF that increase PAI-1 expression both in endothelial cells and HepG2 cells.

Finally, it is tempting to speculate that HGF might be involved in the modulation of extracellular proteolysis by increasing the expression of the serine protease u-PA in one cell type and the expression of the serine protease inhibitor PAI-1 in a different cell type. This might be of importance in the regulation of processes involving matrix invasion by epithelial cells and endothelial cells and tube formation by kidney epithelial cells.
also be of interest that HGF modulates secretion of acute-phase proteins including protease inhibitors in HepG2 cells.26 Furthermore, it could be speculated that HGF might be involved in a feedback mechanism by which it stimulates u-PA expression that has been shown to cleave latent HGF to active HGF.26 Such an increase in active HGF could then induce PAI-1 expression that would inhibit u-PA and consequently inhibit the formation of more active HGF. In contrast, a positive feedback mechanism would be initiated within the coagulation pathway via HGF inducing TF expression, thereby generating thrombin, which in turn would indirectly activate more HGF via activating HGF activator.29 Therefore, the overall action of HGF on HepG2 cells would turn off fibrin dissolution and would favor fibrin formation and other thrombin-mediated effects.

Therefore, our data give more evidence that HGF, in addition to its proliferative effect for a variety of cell types, is also involved via increased expression of PAI-1 and TF in the local regulation of fibrinolysis and coagulation. A systemic regulation of the fibrinolytic system seems unlikely, given the fact that PAI-1 expression in human endothelial cells, which are the major in vivo source of PAI-1, is not affected by HGF. The implications of these effects in pathophysiologic processes have to be established by future investigations.

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Hepatocyte growth factor stimulates expression of plasminogen activator inhibitor type 1 and tissue factor in HepG2 cells

J Wojta, T Nakamura, A Fabry, P Hufnagl, R Beckmann, K McGrath and BR Binder