Hepatocyte Growth Factor Stimulates Expression of Plasminogen Activator Inhibitor Type 1 and Tissue Factor in HepG2 Cells

By Johann Wojta, Toshikazu Nakamura, Astrid Fabry, Peter Hufnagl, Renate Beckmann, Katherine McGrath, and Bernd R. Binder

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, PAI-1 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 nor 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 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. © 1994 by The American Society of Hematology.

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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 smooth muscle cells. It promotes motility and matrix invasion of epithelial cells and endothelial cells and tube formation of kidney epithelial cells into collagen or fibrin matrices. HGF/SF is the ligand for the tyrosine kinase receptor encoded by the met proto-oncogene.

HGF/SF shows a 38% primary sequence homology with plasminogen. The protein is a heterodimer consisting of an α chain containing four kringles and a putative hairpin loop, and a β chain with homology to the catalytic domain of serine proteases. However, the histidine and serine residues of the active site are replaced by glutamine and tyrosine, respectively.

Recently, several reports have implicated HGF in the regulation of components of the fibrinolytic system. It has been shown that HGF increases the expression of urokinase-type plasminogen activator (u-PA) and its receptor, u-PAR, in kidney epithelial cells. Furthermore, it has been shown that SF/HGF stimulates the production of u-PA in bovine capillary endothelial cells. On the other hand, u-PA has been identified as one of the putative serine proteases responsible for the activation of HGF from its single-chain precursor to the active two-chain form. In addition, a serine protease, the so-called HGF activator, with homology to blood coagulation factor XII has been cloned and shown to activate HGF by proteolytic cleavage. There is recent evidence that the zymogen of this protease is activated by thrombin. Because the activation of HGF by u-PA and the stimulatory effect of HGF on u-PA expression suggests a feedback mechanism of ongoing activation of HGF by u-PA, we asked whether recombinant human HGF (rHGF) might also regulate the expression of components of the fibrinolytic system responsible for inhibition of the proteolytic activity of u-PA. Therefore, we studied the effect of HGF on the production of the major physiological inhibitor of u-PA, plasminogen activator inhibitor type 1 (PAI-1), by human endothelial cells, which are a major source of PAI-1 in vivo, and the human hepatoma cell line HepG2, which has been used extensively as a model to study PAI-1 regulation in liver cells. To analyze a possible regulatory feedback within the coagulation cascade, we also investigated the effect of HGF on the production of tissue factor (TF), which is very often coregulated with PAI-1.

MATERIALS AND METHODS

Tissue culture. Human umbilical vein endothelial cell (HUVEC) were isolated by mild collagenase treatment as described by Gimble et al. From the Department of Medical Physiology, Laboratory of Clinical Experimental Physiology, University of Vienna, Austria; the Department of Biochemistry, Osaka University, Japan; and the Department of Diagnostic Haematology, The Royal Melbourne Hospital, Melbourne, Australia.

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brane et al.19 The cells were confirmed as endothelial by their “cobblestone” morphology, positive staining with von Willebrand factor antibodies, and uptake of immunofluorescence-labeled acetylated low-density lipoprotein.40,41 HUVEC were grown in gelatine-coated (BioRad Laboratories, Richmond, CA) Petri dishes (Costar, Cambridge, MA) using M199 (Sigma Chemical Co, St Louis, MO) containing 20% supplemented calf serum (SCS; Hyclone, Logan, UT), 50 μg/mL endothelial growth supplement,42 and 5 μL/mL heparin (Hoffmann LaRoche, Basel, Switzerland). After 5 to 8 days, HUVEC were subcultured using a split ratio of 1:3.

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% SCS and subcultured at a split ratio of 1:6.

Preparation of rhHGF. The full-size human HGF cDNA was inserted into expression vector CM3 and the constructed expression vector was transfected into COS-1 cells by the diethyl aminoethyl (DEAE)-dextran method.43 rhHGF was purified from the conditioned medium of COS-1 cells according to the method described previously.43 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 determined by amino acid analysis and enzyme-linked immunosorbent assay (ELISA). The method for the measurement of HGF by ELISA was described previously.44

HGF treatment of HUVEC and HepG2. HUVEC and HepG2 grown to confluence in 24-well plates (Costar) under conditions described above were incubated before the experiments for 24 hours in M199 containing 1% SCS or DMEM containing 0.1% bovine serum albumin (BSA; Sigma), respectively. Thereafter, the respective cell monolayers were rinsed three times with Flanks’ Balanced Salt Solution (HBSS) and M199 or DMEM containing 0.1% BSA, respectively, with and without different concentrations of rhHGF added. After the indicated time periods, the respective conditioned media (CM) were collected, centrifuged at 300g and stored at -70°C. At the same time points the respective monolayers were rinsed with HBSS and lysed with phosphate buffered saline (PBS) pH 7.4, containing 0.1% Triton x 100. Cell lysates (CL) were processed as described above for CM.

Assays for PAI-1 antigen and u-PA antigen. PAI-1 antigen in CM and CL of HUVEC and HepG2, respectively, was quantified by a specific ELISA based on monoclonal antibodies (MoAbs), which allows determination of active and latent PAI-1 as well as PAI-1 in complex with tissue-type PA (t-PA)46 (Technoclone, Uppsala, Sweden). Purified melanoma PAI-1 was used as a standard. In some of the samples, PAI-I reference reagent for PAI-I (87-512), agreed to have a unitage of 25 U per mg protein. A Sepharose (Pharmacia, Uppsala, Sweden), Unbound proteins were removed from the Sepharose beads by washing twice with 2 mol/L 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-PAGE52; subsequently, the gels were fixed (30% methanol, 10% acetic acid), soaked in Enlightning GF/A filters (Whatman, Maidstone, England) under light suction and intensifying screens.

Assay for PAI-1 activity. PAI-1 activity in the samples was measured by a modified ELISA as described recently (Technoclone). Briefly, an excess of t-PA was bound to ELISA plates by an anti-t-PA MoAb (MPW3VPA).44 Thereafter, the samples were added. Only the active PAI-1 present would bind to the immobilized t-PA by forming a complex. PAI-1 bound to t-PA was then quantified by a peroxidase-labeled anti-PAI-1 MoAb (3PAI5).46 The reference reagent for PAI-1 (87-512), agreed to have a unitage of 25 U per mg per sample, was used as a standard. In some of the samples, PAI-1 activity was determined after activation of latent PAI-1 in these samples by guanidinium hydrochloride. Briefly, guanidinium hydrochloride was added to the samples to achieve a final concentration of 6 mol/L. Such samples were incubated for 4 hours at 4°C. Thereafter the samples were dialyzed against PBS overnight at 4°C.

Preparation of extracellular matrix (ECM) of HepG2. ECM of HepG2 was prepared as described previously for HUVEC.45 Briefly, ECM of HepG2, which had been exposed to DMEM without and with 50 ng/mL rhHGF for 12 hours, respectively, was prepared after the method of Knudsen et al.45 Confluent monolayers of HepG2 grown in 24-well plates (Costar) were lysed with 0.1% Triton X 100 in PBS (pH 7.4) followed by a 10-minute incubation with 25 mmol/L NH₄OH to remove cytoskeletal elements. Remaining ECM was washed three times with 1 mL/well of PBS (pH 7.4). ECM was extracted by scraping into 1 mL of PBS containing 0.1% SDS after incubation for 30 minutes at 37°C. The extracts were dialyzed overnight at 4°C against PBS. PAI-1 antigen in such samples was determined by an ELISA as described above.

Metabolic labeling and immunoprecipitation of vitronectin (Vn) in HepG2. Metabolic labeling was performed as previously described.51 Briefly, HepG2 were grown to confluence in 25-cm² culture flasks (Costar) in DMEM containing 10% SCS. Thereafter, cells were rinsed with HBSS and incubated for 24 hours with DMEM containing 0.1% BSA. The cells were rinsed three times with RPMI 1640 without methionine (Sigma) and 3 mL methionine-free RPMI 1640 without or with 50 ng/mL of rhHGF was added together with ³⁵S-methionine (416 μCi/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 (Trasylol; 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 mol/L 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 intensifying screens.

Total protein synthesis was quantified by trichloroacetic acid (TCA) precipitation of CM and CL. Equal amounts of sample and TCA (100%) were mixed, diluted 10-fold in PBS pH 7.4 and incubated for 1 hour at 4°C. The precipitate was collected on Whatman GF/A filters (Whatman, Maidstone, England) under light suction and intensifying screens.

Preparation of RNA and Northern blots. Total mRNA was prepared from cells grown to confluence in 25-cm² flasks. HUVEC were incubated in M199 containing 1% SCS and HepG2 were incubated in DMEM containing 0.1% BSA for 24 hours before the experiments, respectively. Thereafter, the respective cells were washed once with HBSS and M199 containing 0.1% BSA (HUVEC) or DMEM containing 0.1% BSA (HepG2).
Results

As can be seen from Fig 1, rhHGF increased PAI-1 production by HepG2 in a dose-dependent manner. This increase was up to fourfold in the CM of HepG2 and up to twofold in the CL. Maximum effects were achieved with 50 ng/mL of HGF. The increase in PAI-1 antigen in CM and CL of HepG2 treated with HGF was paralleled by an increase of PAI-1 activity in CM and CL of such cells.

When samples were treated with guanidinium hydrochloride to activate latent PAI-1, an up to ninefold increase in active PAI-1 was seen in CM from HGF-treated as well as from untreated HepG2 (Table 1). PAI-1 antigen increased also in the ECM of HepG2 treated with HGF (Fig 2).

To investigate the possible effect of HGF on Vn, which has been identified as a PAI-1 binding protein, immunoprecipitation studies were performed using CM and CL of HepG2 cultivated for 12 hours in the presence of 50 ng/mL rhHGF with or without rhHGF was added. After 4 hours, RNA was prepared by the guanidinium thiocyanate-phenolchloroform extraction. RNA was electrophoretically separated on 1.2% agarose gels containing 6% formaldehyde, transferred to nitrocellulose (BioRad) and hybridized [10^6 RPM (Cerenkov)/mL] for 16 hours at 42°C with random-primed (32P) deoxycytidine triphosphate probes (Boehringer Mannheim, Mannheim, Germany) for either human PAI-1 (a 1.4-kb EcoRI-BglII fragment of polymerase chain reaction-amplified cDNA), mouse Vn (a 1.5-kb PstI-PstI fragment), human TF (a 0.64-kb EcoRI-EcoRI fragment, kindly provided by E. Hofer, Vienna, Austria), human u-PA (a 1.5-kb PstI-PstI fragment; ATCC), human u-PAR (a 1.1-kb XhoI-EcoRI fragment, kindly provided by E.K.O. Kruithof, Lausanne) or rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH, a 1.3-kb PstI cDNA fragment, kindly provided by M. Busslinger, Vienna, Austria).

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>
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<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>
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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/10^6 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.
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 (A) or presence of 50 ng/mL HGF (A). 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.

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.3,25 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-1.59

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
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-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. In that respect, it might...
also be of interest that HGF modulates secretion of acute-phase proteins including protease inhibitors in HepG2 cells.\textsuperscript{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.\textsuperscript{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.\textsuperscript{29} Therefore, the overall action of HGF on HepG2 cells would turn off fibrin dissolution and 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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