Paradoxical pro-invasive effect of the serine proteinase inhibitor tissue factor pathway inhibitor-2 on human hepatocellular carcinoma cells*

Véronique Neaud§, Toru Hisaka§, Arnaud Monvoisin, Christiane Bedin, Charles Balabaud, Donald C. Foster**, Alexis Desmoulière, Walter Kisiel³, Jean Rosenbaum¶

From the Groupe de Recherches pour l’Etude du Foie, INSERM E9917, Université Victor Segalen Bordeaux 2, 33076 Bordeaux, France, ** ZymoGenetics Inc., Seattle, Washington, and ³ Department of Pathology, University of New Mexico School of Medicine, Albuquerque, New Mexico 87131

Running title : TFPI-2 induces invasion of hepatocellular carcinoma cells
Summary

We have previously shown that human liver myofibroblasts promote in vitro invasion of human hepatocellular carcinoma (HCC) cells through a hepatocyte growth factor (HGF)/urokinase/plasmin-dependent mechanism. In this study, we demonstrate that myofibroblasts synthesize the serine proteinase inhibitor tissue factor pathway inhibitor-2 (TFPI-2). Despite the fact that recombinant TFPI-2 readily inhibits plasmin, we show that it potentiates HGF-induced invasion of HCC cells and is capable of inducing invasion on its own. Furthermore, HCC cells stably transfected with a TFPI-2 expression vector became spontaneously invasive. HCC cells express tissue factor and specifically factor VII. Addition of an antibody to factor VII abolished the pro-invasive effect of TFPI-2. We suggest that TFPI-2 induces invasion following binding to a tissue factor/factor VIIa complex preformed on HCC cells. Our data thus demonstrate an original mechanism of cell invasion that may be specific for liver tumor cells.
Hepatocellular carcinoma (HCC) is one of the most frequent primary tumors in the world (1). It is a major complication of liver cirrhosis, although more rarely it will develop on a non-cirrhotic liver. HCC are characterized by a high rate of local, intra-hepatic invasion. HCC are infiltrated by myofibroblast-like cells, located around tumoral sinusoids and in fibrous septa and capsule, when present (2-4). We have previously shown that cultured human liver myofibroblasts strongly promoted *in vitro* invasion of human HCC cell lines through their secretion of hepatocyte growth factor (HGF) (5). In further studies, we showed that HGF induced invasion by increasing the expression of the urokinase-type plasminogen activator (uPA) by the cancer cells (6). Indeed, myofibroblasts- or HGF-induced invasion was dose-dependently blocked by a selective uPA antagonist (6). One of the main functions of uPA is to convert the inactive zymogen plasminogen into plasmin, a broad-spectrum proteinase able to degrade several components of the extracellular matrix and thus a likely effector of cancer cell invasion.

Tissue factor pathway inhibitor-2 (TFPI-2), also known as placental protein 5 is a serine proteinase inhibitor containing 3 tandemly arranged Kunitz-type proteinase inhibitor domains, homologous to tissue factor pathway inhibitor (7). TFPI-2 exists as 3 isoforms of 27, 31 and 33 kDa that are synthetic products of a single gene and arise from differential glycosylation. TFPI-2 is a strong inhibitor of plasmin as well as of trypsin, plasma kallikrein and factor Xla. It does not inhibit uPA (8). TFPI-2 synthesis has been described in dermal fibroblasts and endothelial cells (9-11). In these cell types, the major part of TFPI-2 is sequestered within the extracellular matrix (ECM), presumably bound to heparan sulfate. In the course of a systematic sequencing of a human liver
myofibroblasts cDNA library described elsewhere (12), we found that these cells expressed transcripts for TFPI-2. Given the ability of TFPI-2 to inhibit plasmin, we were interested in defining the contribution of myofibroblasts-derived TFPI-2 in regulating the pro-invasive effect of these cells towards HCC cells.
Experimental procedures

Recombinant proteins and antibodies—Human recombinant rTFPI-2 was expressed in BHK cells and purified as previously described by a series of chromatographic steps (7). Human recombinant HGF was a generous gift from Dr. George Vande Woude (NCI, Frederick, MD). An affinity-purified anti-human factor VII IgG was prepared as described (13) by applying a redissolved 50% ammonium sulfate pellet from rabbit anti-factor VII antiserum to a recombinant factor VIIa-Affi-Gel 15 column equilibrated with TBS and eluting with 0.1 M glycine-HCl (pH 2.5)/0.5 M NaCl into a 1/10 volume of 1M Tris-HCl (pH 8.8) to immediately neutralize the glycine.

Cells—HepG2, HuH7 and Hep3B human HCC lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM, GIBCO-BRL, Life Technologies, Cergy-Pontoise, France) containing 10% fetal calf serum (FCS, GIBCO-BRL). HT1080 cells, derived from a human fibrosarcoma, were obtained from the American Tissue Type Culture Collection and grown as the HCC cell lines. Human hepatic myofibroblasts were obtained from explants of non-tumoral liver resected during partial hepatectomy as previously described (14). Isolated cells were characterized as myofibroblasts as previously described in detail (14,15). Specifically, the procedure, that is based on the selective growth advantage of myofibroblasts in the culture conditions used, allowed for a 100% pure myofibroblast population, as shown by positive staining for smooth muscle alpha-actin and vimentin, and negative staining for CD 68 (a Kupffer cell marker), von Willebrand factor (an endothelial cell marker), or cytokeratin (an epithelial cell marker) (not shown). This procedure is in accordance with INSERM ethical regulations imposed by French legislation. Myofibroblasts were grown in DME containing 5% FCS, 5%
pooled human AB serum (Centre Régional de Transfusion Sanguine, Bordeaux, France) and 5 ng/ml recombinant human EGF (GIBCO-BRL).

**Northern blot**—Total RNA was prepared using the Qiagen RNeasy kit. Ten µg were analyzed by Northern blot as described (6). The probe used was the full length human TFPI-2 cDNA (7) and was labeled with [α³²P]dCTP by random priming using the Ready-to-go kit from Boehringer-Mannheim (Meylan, France).

**Reverse-transcription polymerase chain reaction**—1 µg total RNA was reverse-transcribed in a 50 µl volume using Moloney Murine Leukemia Virus Reverse Trancriptase (GIBCO-BRL), according to the manufacturer’s instructions. Three µl of the reaction were used for amplification with the following human TFPI-2 primers: 5'-GTC GAT TCT GCT GCT TTT CC -3', sense primer, corresponding to nucleotides 64-84 of the published sequence (7), and 5'-ATG GAA TTT TCT TTG GTG CG -3', antisense primer (nucleotides 484-504). Thirty five cycles were performed, each consisting of 94°C, 1min, 60°C, 1min and 72°C, 1 min. PCR was performed in 50 µl of a reaction buffer containing 50 mM KCl, 10 mM Tris HCl, pH 9.0, 1% Triton X-100, 2.4 mM MgCl₂, 0.4 mM dNTPs, 0.2 µM primers, and 1.25 U Taq polymerase (Promega, Madison, WI). An aliquot of the reaction was then analyzed by agarose gel electrophoresis. The size of the predicted product is 440 bp. For negative controls, reverse transcriptase was omitted during the RT procedure, or PCR was performed without cDNA. Similar conditions were used to amplify the cDNAs of human tissue factor and human factor VII, using the following primers: tissue factor sense (5'-TTC CTG ACC TCA GGT GAT CC-3'), tissue factor antisense (5'-GCA TAT TAG GAT GAA GGT
GCC CA-3’), factor VII sense (5’-GGA TGC ACA CAC AGA TGG TC-3’), factor VII antisense (5’-ACA GCA CAC ATG GAG TCA GC-3’). The size of the predicted product is 296 bp for tissue factor, and 295 bp for factor VII. β2-microglobulin was used as a positive control for RNA integrity and reverse transcription efficiency.

Detection of TFPI-2 protein expression—To prepare extracellular matrix (ECM) extracts for Western blotting, cells were grown to confluence, washed twice with serum-free DME, and incubated for 24 h in the same medium. The ECM was prepared as described by Rao et al (10). Briefly, cells were washed 3 times with phosphate buffered saline (PBS) and lysed with 0.5% (v/v) Triton X-100 in PBS for 20 min at room temperature. The remaining ECM was washed 3 times with PBS, and another 3 times with 20 mM Tris HCl, pH 7.4, containing 100 mM NaCl and 0.1% (v/v) Tween 20. ECM was then incubated for 2 h at room temperature with reducing Laemml buffer and collected by scraping. ECM or conditioned medium extracts were analyzed by SDS-PAGE on 15% gels and transferred to a PVDF membrane (Immobilon-P, Millipore) by semi-dry transfer (Transblot-SD, Biorad, Ivry s/Seine, France). The membrane was blocked with 4% skimmed milk in 10 mM Tris HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20 (TBS-Tween) for 2 h at room temperature, incubated overnight at 4°C with anti-TFPI-2 IgG (10 µg/ml) (10) in TBS-Tween containing 1% BSA, then 1h at room temperature with a peroxydase-conjugated anti-rabbit IgG antibody (Dako A/S, Glostrup, Denmark). Detection was achieved by enhanced chemiluminescence (Amersham, Les Ulis, France).

For metabolic labeling, cells were grown to confluence in 35-mm dishes. They were washed and incubated for 6 h in 1 ml methionine-cystine-free DME containing 0.25
mCi/ml Tran-35S label (ICN, Orsay, France). The labeled medium was briefly centrifuged and the supernatant was mixed with 1 volume of 2-fold concentrated immunoprecipitation buffer (RIPA buffer: 20 mM Tris-HCl, pH 7.5, 138 mM NaCl, 10% glycerol, 1% NP-40, 0.1% SDS, 10 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin A, 1 mM Pefabloc, final concentrations). The cell layer including ECM was directly lysed in warm 10 mM Tris-HCl, pH 7.5/1% SDS. Samples were precleared with normal rabbit IgG and protein A-Sepharose (Sigma, L’Isle d’Abeau, France) for 1 h, then incubated with 10 µg anti-TFPI-2 antibody with shaking overnight at 4°C. As a negative control, the anti-TFPI-2 antibody was replaced by normal rabbit IgG. Immunoprecipitates were collected with protein A-Sepharose. They were washed as described (16) and eluted by boiling in 2-fold concentrated reducing loading buffer. They were run on 10% polyacrylamide gels. Gels were stained, destained, dried and exposed for autoradiography.

Generation of stable transfected cell clones—The full-length human TFPI-2 cDNA was subcloned in the EcoRI site of the pcDNA 3.1 vector (Invitrogen, Groningen, Netherland). This plasmid, or the empty pcDNA 3.1 vector, was transfected into HepG2 cells with the Fugene™6 reagent (Roche, Meylan, France), according to the instructions of the manufacturer. Selection of transfected cells with G418 was initiated 24 h following transfection. Resistant colonies were individually picked, amplified and analyzed for TFPI-2 expression by RT-PCR and Western blot.

Cell proliferation assay—Tumor cells were resuspended in DMEM containing 2% FCS and cultured in plastic 24-well plates (5 x 10⁴ cells per well). After 15h, cultures were
washed twice with serum-free DMEM. Triplicates were counted after incubation with 0.25% trypsin and 0.02% EDTA (GIBCO-BRL) to determine the number of cells that had adhered to the plastic support (N₀). In other wells, tumor cells were incubated in triplicate in serum-free DMEM (control medium), with or without 50 nM rTFPI-2. After 2 d of culture, tumor cells were washed twice with serum-free DMEM and counted in a hemocytometer. Results were expressed as percentages of proliferation (P) as compared to proliferation in control medium. \[ P = \frac{(N_e - N_o)}{(N_c - N_o)} \times 100 \]

where \( N_o \) = number of cells that had adhered to the plastic support at the time of seeding; \( N_c \) = number of cells in the presence of control medium; \( N_e \) = number of cells in experimental samples.

**Cell invasion assay**—A Matrigel invasion assay was performed as described previously (6). Briefly, 8 µm polycarbonate pore size filters inserted in 24 well plates were coated with Matrigel basement membrane matrix (14 µg/filter). \( 45 \times 10^3 \) HepG2 cells were seeded onto the filters in 2% FCS/DMEM. Serum-free medium was added to the lower compartment so that the final concentration of FCS was 0.4%. In some experiments, rhHGF (100 ng/ml) was added in the lower compartment. After 48 hr, the cells on the upper surface of the filter were wiped with a cotton swab. Filters were fixed for 10 min with methanol and stained with hematoxylin. Cells that invaded the lower surface of the filter were counted under a photonic microscope at a final magnification of 320.

**Mitogen activated protein kinase (MAPK) assay**—HepG2 cells were seeded at a density of \( 1.5 \times 10^6 \) cells in 35 mm diameter dishes. After 6 h, they were washed 3 times...
with serum-free DME and incubated overnight in serum-free DMEM. They were then stimulated for various times with rTFPI-2 in 0.8 ml DMEM. At the end of the incubation, the dishes were put on ice, washed twice with ice-cold PBS containing 10 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin A, 1 mM Pefabloc, 1 mM sodium orthovanadate, 10 mM β-glycerophosphate, final concentrations. The cells were finally lysed in RIPA buffer with added 0.25% deoxycholate (w/v) and phosphatase inhibitors. Proteins were measured with a reagent from Biorad. Fifty µg of proteins were loaded on 10% gels and transferred to PVDF membranes. Equivalence of loading was assessed by staining the blots in Ponceau Red. The membranes were blocked with 2.5% BSA in 10 mM Tris HCl, pH 8, 100 mM NaCl, 0.1% Tween 20 and incubated with the anti-phospho-MAPK (ERK1 and ERK2) antibody (Promega), diluted 1/4000. The blots were washed with 10 mM Tris HCl, pH 7.5, 100 mM NaCl, 0.1% Tween 20. The peroxidase-conjugated secondary antibody was applied in the same buffer containing also 5% skimmed dry milk. The blots were subsequently rehybridized with an antibody to total MAPK (Promega).
Results

Expression of TFPI-2 by cultured human liver myofibroblasts and HepG2 cells

Northern blot analysis demonstrated a major 1.2 kb as well as a minor 1.8 kb transcripts in myofibroblasts. No TFPI-2 transcripts were detectable in HepG2 cells by either Northern blotting or RT-PCR (not shown). Western blot of myofibroblasts ECM extracts demonstrated the 27, 31 and 33 kD isoforms of TFPI-2 (Fig. 1). No TFPI-2 antigen could be detected in myofibroblast culture medium by this technique. However, using metabolic labeling and immunoprecipitation, TFPI-2 was observed in both cell/ECM extracts and in culture medium. Specificity was demonstrated by the absence of signal in samples precipitated with control IgG.

Recombinant TFPI-2 has pro-invasive properties

We have previously shown that myofibroblasts-induced invasivity was dependent on secretion of HGF by myofibroblasts, and could be reproduced by rhHGF through an uPA-dependent pathway (6). As HepG2 cells do not produce any detectable TFPI-2, we thus tested whether exogenous rTFPI-2 would counteract HGF-induced invasion of HepG2 cells. Surprisingly, TFPI-2 (50 nM) potentiated the effect of HGF on invasion (Fig. 2A). This effect was highly significant (p = 0.01, Wilcoxon paired test). The use of control plasmin inhibitors such as ε-aminocaproic acid or tranexamic acid resulted in a major inhibition of HGF-induced invasion, showing that the latter is indeed plasmin-dependent (Fig. 2B). Moreover, TFPI-2 alone induced invasion of Matrigel by HepG2 cells. This effect was dose-dependent (Fig. 2C). TFPI-2 also increased the invasive behavior of 2 other human HCC cell lines, HuH7 and Hep3B (not shown). As shown on Fig. 3A, the pro-invasive effect of TFPI-2 was not artefactually secondary to an increased proliferation of
the cells in the presence of TFPI-2. Additionally, as it had been reported that rTFPI-2 increased the activation of the MAPK ERK1 and ERK2 (17), we evaluated this activation, using immunoblotting with an anti-active MAPK. No increase in MAPK phosphorylation was observed in dose-response (5-75 nM) (Fig. 3B) or kinetics study (5 min-1 h) (not shown) whereas HGF used as positive control was highly active. Interestingly, TFPI-2 abolished the anti-proliferative effect of HGF, even at the lowest concentration tested (5 nM) (Fig. 3A).

To rule out the possibility that the pro-invasive effect of TFPI-2 was due to a contaminant carried over through the purification of the recombinant molecule, we repeated the experiments in the presence of an anti-TFPI-2 antibody. At a concentration of 50 µg/ml, the specific TFPI-2 antibody inhibited by 49.4 ± 11.3 % the invasion induced by 50 nM TFPI-2 (n = 5). Control IgG at the same concentration produced only a 6.5 ± 4.7 % inhibition. Furthermore, we established stable clones of HepG2, transfected with a TFPI-2 expression vector. Expression of TFPI-2 was verified by RT-PCR (not shown), Western blotting (Fig. 4A), and immunoprecipitation (Fig. 4B). One clone that expressed high levels of TFPI-2 was further studied, together with a clone transfected with an empty vector (Fig.4C). The TFPI-2-expressing clone was significantly more invasive than the clone containing the empty vector (p=0.018, Kruskal-Wallis test). The invasivity of this clone could be blocked with anti-TFPI-2 IgG (58 and 42 % inhibition with 50 µg/ml antibody in 2 separate experiments), but not control IgG (respectively 10.5 and 6 % inhibition). Invasive activity of the control clone could be enhanced by exogenous rTFPI-2, showing that this clone still retained its sensitivity to TFPI-2 (Fig 4C).
Factor VII is involved in the pro-invasive effect of TFPI-2. RT-PCR analysis showed that HepG2 cells expressed transcripts for factor VII, as well as for tissue factor. On the other hand, HT 1080 cells did not express detectable factor VII transcripts (Fig. 5A). An antibody to factor VII dose-dependently blocked the pro-invasive effect of TFPI-2 on HCC cells, whereas a non immune IgG was uneffective (Fig. 5B). The antibody effect was highly significant by ANOVA (p = 0.0002).
Discussion

Invasion is a characteristic feature of HCC. In previous studies, we have shown that tumor-associated myofibroblasts strongly promoted \textit{in vitro} invasion of HCC cells through a HGF/uPA mechanism (5,6). The proteolytic pathway of invasion is dependent on the balance of many components including among others serine proteinases such as uPA and plasmin, matrix metalloproteinases, and proteinase inhibitors (18-20). The latter include well-characterized proteins such as the tissue inhibitors of matrix metalloproteinases (TIMPs) and the plasminogen activator inhibitors (PAI) 1 and 2. TFPI-2 has been recently recognized as identical to placental protein 5, a serine proteinase inhibitor. We have shown that rTFPI-2 strongly inhibited the \textit{in vitro} invasion of the highly invasive HT1080 cell line, presumably through plasmin inhibition (21). In this study, we demonstrate that cultured human liver myofibroblasts synthesize TFPI-2 and secrete it, mainly in their ECM. We were thus interested to know whether TFPI-2 regulated the invasivity of HCC cells that involves uPA and plasmin. We tested the effect of exogenous, rTFPI-2, on the invasion of HCC cells that do not synthesize detectable TFPI-2. In our experimental conditions, human HCC cell lines are spontaneously poorly invasive. In these experiments, invasion was induced by addition of rhHGF that mimics the co-culture with myofibroblasts, as described previously (5). Surprisingly, rTFPI-2 did not decrease invasion in these conditions. On the contrary, it had an additive effect with HGF. Moreover, rTFPI-2 in the absence of HGF induced dose-dependently an invasive activity. These effects were observed with 3 different human HCC cell lines. Several arguments rule out the possibility that this effect was due to a contaminant carried over through the
purification of rTFPI-2: first, the effect of rTFPI-2 was blunted by an anti-TFPI-2 antibody; second, HCC cells stably transfected with a TFPI-2 expression vector are more invasive spontaneously than control-transfected cells. As transfected HepG2 cells express the 3 differently glycosylated isoforms, this latter result also argues against the hypothesis that the lack of anti-invasive effect of rTFPI-2 was due to the absence of the lower molecular weight species from the recombinant preparation. Altogether, these results suggested that TFPI-2 could have both an anti-invasive effect, probably indirect and mediated via plasmin inhibition, as well as a direct pro-invasive effect. Other proteinase inhibitors share such a dual effect. PAI-1, as an inhibitor of plasminogen activation, has anti-invasive properties. On the other hand, it can displace the binding of the uPA receptor to vitronectin, thus allowing cell migration and invasion (22). This has been nicely demonstrated in vivo using PAI-1-/- mice that show impaired tumor invasion (23). TIMP-2 inhibits MMP-2 activity and can thus reduce ECM breakdown and invasion. However, it also participates in the ternary activation complex of pro-MMP-2, together with MT1-MMP and can thus increase MMP-2 activity (24).

Our data also suggest a mechanism for the pro-invasive effect of TFPI-2. It has recently been shown that the TFPI-2 homolog, TFPI-1, could induce the migration of tissue-factor expressing bladder tumor cells in the presence of exogenous factor VIIa (25). We reasoned that TFPI-2 could have the same property and that HCC cells, being derived from hepatocytes, were the only tumor cell type with the ability to synthesize factor VII themselves. We have confirmed this expression, as well as that of tissue factor by RT-PCR. On the other hand, HT 1080 cells, which invasion is inhibited by TFPI-2 (21), do not express factor VII. Finally, we demonstrated that TFPI-2-induced invasion
could be dose-dependently inhibited by an antibody to human factor VII. These data suggest that TFPI-2 could induce invasion following binding to a tissue factor/factor VIIa complex, preformed on the surface of HCC cells. Further work is still needed to identify the transduction mechanisms involved in the pro-invasive effect. Altogether, our data identify a new mechanism of invasion, that could be specific for HCC cells.

Acknowledgments We thank Dr. Yu-ichi Kamikubo and Dr. George Vande Woude for their generous gifts of rTFPI-1 and rhHGF, respectively.
References


23. Bajou, K., Noël, A., Gérard, R. D., Masson, V., Brunner, N., Holst-Hansen, C.,

24. Strongin, A. Y., Collier, I., Bannikov, G., Marmer, B. L., Grant, G. A., and

25. Fisher, E. G., Riewald, M., Huang, H. Y., Miyagi, Y., Kubota, Y., Mueller, B. M.,
Footnotes

*This work was supported by grants from Comité de la Dordogne from the Ligue Nationale Contre le Cancer, Association pour la Recherche sur la Cancer, and Conseil Régional d’Aquitaine. AM is a recipient of a fellowship from Comité de la Dordogne from the Ligue Nationale Contre le Cancer. WK was supported by a grant (HL-35246) from the National Institutes of Health.

§These authors contributed equally to this work.

¶ To whom correspondence should be addressed: Groupe de Recherches pour l’Etude du Foie, INSERM E9917, Université Victor Segalen Bordeaux 2, 33076 Bordeaux, France. Tel: 33 5 57 57 17 71; Fax: 33 5 56 51 40 77; E-mail: jean.rosenbaum@gref.u-bordeaux.2.fr

1 The abbreviations used are: HCC, hepatocellular carcinoma; HGF, hepatocyte growth factor; uPA, urokinase-type plasminogen activator; TFPI-2, tissue factor pathway inhibitor-2; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; ECM, extracellular matrix; MAPK, mitogen activated protein kinase.
Figure legends

Fig. 1 Human liver myofibroblasts express TFPI-2
A- Northern blot
B- Western blot. Extracellular matrix extracts from cultured myofibroblasts were analyzed by Western blot with an anti-TFPI-2 antibody.
C- Myofibroblasts were labeled with [35S]methionine and cysteine. Cell lysates (1, 2) and supernatants (3, 4) were immunoprecipitated with an antibody to TFPI-2 (1, 3) or a non specific IgG (2, 4).

Fig. 2 Recombinant TFPI-2 is pro-invasive. HepG2 cells were seeded in invasion chambers coated with Matrigel as described in Material and Methods. A-rhHGF was used at 100 ng/ml and TFPI-2 at 50 nM (n = 8 in triplicate). Values indicate the number of invasive cells/filter and are expressed as mean ± 1 SEM. Lane 1 : control ; lane 2 : rhHGF ; lane 3 : rhHGF with TFPI-2 ; lane 4 : TFPI-2 alone.
B- Effect of the plasmin inhibitors ε-aminocaproic acid and tranexamic acid, on HGF-induced invasion of HepG2 cells. The results are expressed as percentage of the number of invasive cells in the presence of HGF (100 ng/ml). Lane 1, no addition ; lane 2, ε-aminocaproic acid 3 mM ; lane 3, ε-aminocaproic acid 6 mM ; lane 4, tranexamic acid 1 mM, lane 5, tranexamic acid 3 mM. The results are the mean of 2 experiments conducted in triplicate.
C- Dose-response of the pro-invasive effects of rTFPI-2. The results are expressed as fold increase over baseline values (n = 3).
Fig. 3 Effect of TFPI-2 on HepG2 proliferation and on ERK phosphorylation.

A- Cell proliferation. Cells were seeded in 24 wells and cultured for 2 d in the absence (o) or in the presence of rhHGF at 100 ng/ml (n). TFPI-2 was used at the indicated concentrations. At the end of the experiment, the cells were counted and the percentage of proliferation calculated as described in Materials and Methods. The results are the mean of 2-4 experiments conducted in triplicate.

B- Effect of TFPI-2 on the phosphorylation of the MAPK ERK1 and ERK2 in HepG2 cells. HepG2 cells were incubated for 10 minutes with the indicated concentrations of rTFPI-2 or with rhHGF (100 ng/ml). Cell lysates were analyzed by Western blot with an anti-phospho-MAPK antibody. The blot was rehybridized with an anti-total MAPK.

Fig. 4 Transfection of HepG2 cells with a TFPI-2 expression vector increases their invasivity.

A- Extracellular matrix extracts from cultured HepG2 cells were analyzed by Western blot with an anti-TFPI-2 antibody. 1 : cells transfected with an empty vector, 2 : cells transfected with a TFPI-2 expression vector.

B- Cell lysates (Top panel) and supernatants (Bottom panel) from labeled cells were immunoprecipitated with an antibody to TFPI-2 (+) or a non specific IgG (-). 1 : cells transfected with a TFPI-2 expression vector, 2 : cells transfected with an empty vector. Bars indicate the specifically immunoprecipitated bands.

C- Invasion assay. The experimental design is the same as in Fig. 2. Lane 1, mock-transfected cells ; lane 2, mock-transfected cells with added TFPI-2 (50 nM) ; lane 3, cells stably transfected with a TFPI-2 expression vector. Values indicate the
number of invasive cells/filter and are expressed as mean ± 1 SEM (n = 4).

Fig. 5. Role of factor VII in TFPI-2-induced invasivity.

A- HepG2 cells express factor VII and tissue factor transcripts. RNA from HepG2 cells (2-3), or HT1080 cells (4-5) was reverse transcribed and amplified with primers for factor VII, tissue factor or β2-microglobulin. Lanes 2, and 4 are controls where reverse transcriptase was omitted. Lane 1 is a PCR negative control where H2O was used instead of cDNA.

B- Invasion assay. The experimental design is the same as in Fig. 2. Invasion was induced with 75 nM TFPI-2 together with the indicated concentrations of non immune IgG or anti-VII antibody (in µg/ml). Results are expressed as mean ± SEM of 4 experiments in triplicate.
Paradoxical pro-invasive effect of the serine proteinase inhibitor tissue factor pathway inhibitor-2 on human hepatocellular carcinoma cells

Véronique Neaud, Toru Hisaka, Arnaud Monvoisin, Christiane Bedin, Charles Balabaud, Donald C Foster, Alexis Desmoulière, Walter Kisiel and Jean Rosenbaum

J. Biol. Chem. published online August 22, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M006101200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts