Enhanced migration of tissue inhibitor of metalloproteinase overexpressing hepatoma cells is attributed to gelatinases: Relevance to intracellular signaling pathways

Elke Roeb, Anja-Katrin Bosserhoff, Sabine Hamacher, Bettina Jansen, Judith Dahmen, Sandra Wagner, Siegfried Matern

© 2005 The WJG Press and Elsevier Inc. All rights reserved.

Abstract

AIM: To study the effect of gelatinases (especially MMP-9) on migration of tissue inhibitor of metalloproteinase (TIMP-1) overexpressing hepatoma cells.

METHODS: Wild type HepG2 cells, cells stably transfected with TIMP-1 and TIMP-1 antagonist (MMP-9-H401A), a catalytically inactive matrix metalloproteinase (MMP) which still binds and neutralizes TIMP-1 were incubated in Boyden chambers either with or without Galardin (a synthetic inhibitor of MMP-1, -2, -3, -8, -9) or a specific inhibitor of gelatinases.

RESULTS: Compared to wild type HepG2 cells, the cells overexpressing TIMP-1 showed 115% migration (P<0.05) and the cells overexpressing MMP-9-H401A showed 62% migration (P<0.01). Galardin reduced cell migration dose dependently in all cases. The gelatinase inhibitor reduced migration in TIMP-1 overexpressing cells predominantly. Furthermore, we examined intracellular signal transduction pathways of TIMP-1-dependent HepG2 cells. TIMP-1 deactivates cell signaling pathways of MMP-2 and MMP-9 involving p38 mitogen-activated protein kinase. Specific blockade of the ERK pathway suppresses gelatinase expression either in the presence or absence of TIMP-1.

CONCLUSION: Overexpressing functional TIMP-1-enhanced migration of HepG2-TIMP-1 cells depends on enhanced MMP-activity, especially MMP-9.

© 2005 The WJG Press and Elsevier Inc. All rights reserved.

Key words: HepG2; MMP-2; MMP-9; p38MAPK; Galardin; Genistein

INTRODUCTION

Proteins of the matrix metalloproteinases (MMPs), a family of structurally related zinc-dependent enzymes, are involved in the breakdown of extracellular matrix (ECM) in normal physiological processes, such as embryonic development, reproduction, and tissue remodeling as well as in disease processes, such as arthritis, tumor cell invasion, migration, and metastasis[11,12]. Most MMPs are secreted as inactive proteins, which are activated when cleaved by extracellular proteinases. MMPs may be expressed on the surface of cells, thus allowing a precise and localized proteolysis[11]. The gelatinases MMP-2 and MMP-9 are necessary for the migration of Langerhans cells and dermal dendritic cells from human and murine skin[11]. MMP-2 (gene ID 4313 on chromosome 16q13-q21) e.g., is involved in cell migration of human lung carcinoma cells[9]. MMP-9 (gene ID 4318, chromosome 20, q11.2-q13.1) is a major secretion product of macrophages and a component of cytoplasmic granules of neutrophils[9]. The enzyme is also secreted by lymphocytes and stromal cells upon stimulation by inflammatory cytokines, or upon delivery of bidirectional activation signals following integrin-mediated cell-cell or cell-matrix contacts[9]. MMP-9 plays an important role in tumor invasion and angiogenesis. Secretion has been reported in various cancer types including lung, colon, and breast cancer. The activity of MMPs is tightly controlled by endogenous specific inhibitors, the tissue inhibitors of metalloproteinases (TIMPs). The balance between activated MMPs and their free inhibitors determines the net MMP activity and seems to be of great importance for matrix protein turnover associated with tumor cell invasion and metastasis. A striking phenotypic characteristic of fibrotic liver is a dramatic up-regulation of TIMP-1 (GenBank number NM 003254.1, chromosome X, Xp11.3-p11.23), which seems to be a functional promoter of hepatic fibrosis[9,10]. But there is also evidence that TIMPs are associated with suppression of programed cell death, tissue remodeling during tumor progression and mitogenic activity[11,12]. TIMP-1 has been shown to suppress apoptosis in a series of Burkitt's lymphoma
blockade of the ERK pathway suppresses the expression especially inhibition of gelatinases. Following this discovery, TIMP-1 overexpressing cells exhibit increased migration with clear and defined conditions. We showed here that hepatoma cells by using an established cell motility assay MMPs in the migration and signal transduction of human MMP inhibitory activity) have complicated the understanding suppression of programmed cell death independent of the recent developments in the field of MMP-9 gene expression in different cell types, from the triggering of cell-surface receptors to the activation of cytoplasmic mediators and transcription factors responsible for the activation of MMP-9 promoter. Fibronectin up-regulates MMP-9 and induces coordinated expression of MMP-2 and its activator, the membrane-type MT1-MMP (MMP-14) by human T-lymphocyte cell lines, a process repressed through Ras/MAP kinase signaling pathways[23]. Accordingly, transfection of T-cell lines with dominant negative mutant of Ha-Ras strongly increases fibronectin-induced expression of MMP-2 and MMP-9[23]. In human glioma cells, angiopoietin-2 induces tumor cell infiltration by activation of MMP-2. MMP-2 is associated with tumor size, invasiveness, and survival in breast cancer[24]. SB203580, a p38 MAP kinase inhibitor, reduces MMP-2 expression in melanoma cells[25]. Non-steroidal anti-inflammatory drugs inhibit MMP-2 via suppression of ERK/Sp1-mediated transcription[26].

Recently, we demonstrated an increased expression of MMP-2 and MMP-9 protein as a result of increased TIMP-1 expression in human hepatoma cells[27]. Beyond that TIMP-1 overexpressing HepG2 cells could grow in nests and keep in contact with each other[27,28]. This striking phenotype could be abrogated by a TIMP-1 antagonist, the mutant MMP-9-H401A, PCR-aided mutagenesis was performed using the following primers: 5'-TGG CAG CGG CCG AGT TCG GCC ATG-3' sense and 5'-CGA ACT CGG CCG CTG CCA CCA GG-3' antisense.

A specific blockade of the ERK pathway suppresses the expression of MMP-9 and inhibits markedly the invasiveness of tumor cells[29]. The PKC-dependent NFK kappa B activation is absolute for MMP-9 induction and PKC-dependent ERC activation devotes to increase the expression level of MMP-9 in hepatocellular carcinoma cells[21,22]. St-Pierre et al[30] have given an overview of recent developments in the field of MMP-9 expression independent of the growth of human melanoma cells[23]. This striking phenotype could be abrogated by a TIMP-1 antagonist, the mutant MMP-9-H401A, PCR-aided mutagenesis was performed using the following primers: 5'-TGG CAG CGG CCG AGT TCG GCC ATG-3' sense and 5'-CGA ACT CGG CCG CTG CCA CCA GG-3' antisense.

**Construction of a TIMP-1 antagonist**

The MMP-9 mutant MMP-9-H401A was cloned as previously described[30]. To introduce a point mutation at amino acid 401 of murine MMP-9 (MMP-9-H401A), PCR-aided mutagenesis was performed using the following primers: 5'-TGG CAG CGG CCG AGT TCG GCC ATG-3' sense and 5'-CGA ACT CGG CCG CTG CCA CCA GG-3' antisense.

**Cells and cell treatment**

Human hepatoma cells (HepG2) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with fetal calf serum (FCS), 100 µg/mL streptomycin and 60 µg/mL penicillin. Cells were grown at 37 °C in a water-saturated atmosphere in air/CO2 (19:1). Cells were assessed for the expression of TIMP-1 by Western blotting after incubation under serum-free conditions for 24 h. Selection of HepG2 clones stably transfected with murine TIMP-1 cDNA or murine MMP-9-H401A cDNA was previously described[27,28]. For stable expression, HepG2 cells were transfected with the expression vector pECDNA3.1-mTIMP-1 or pECDNA3.1-MMP-9-mut containing the neomycin resistance gene. Colonies resistant to 500 µg/mL neomycin were isolated, several clones were established and assessed for the expression of murine TIMP-1 or mutant MMP-9 by Northern and Western blotting. Expression by these cells was maintained for more than 20 passages. Three positive clones for each plasmid were used for further analyses.

**Western blot**

Serum-free cell supernatants of HepG2 cells and HepG2
cell clones were separated by SDS-polyacrylamide gel electrophoresis separating gels of 12.5% polyacrylamide and stacking gels of 3% polyacrylamide. Lanes were loaded with 2 μg of total protein each. Following electrophoresis at 30 °C the proteins from serum-free cell supernatants were transferred to nitrocellulose membrane. Protein bands were localized by staining Ponceau S. Blots were blocked with TBS-N (pH 7.6) containing 10% BSA, 20 mM/L Tris, 137 mM/L NaCl, 0.1% Nonidet P40, washed and incubated with antibodies against TIMP-1 (dilution 1:1000). Signals were visualized by enhanced chemiluminescence according to the manufacturer’s instructions (Amersham). Experiments were done in triplicate.

Boydner chamber experiments
Transwell 0.64-cm² filter inserts (8-μm pores) coated with a thin layer of gelatin (125 μg/cm²) were re-hydrated according to the customer's instructions essentially as described previously [30]. Prior to seeding into the transwell inserts, cells were released using trypsin-EDTA and sequentially rinsed with DMEM containing 10% FCS and serum-free DMEM. The rinsed cells were re-suspended in serum-free DMEM with DMEM containing 10% FCS and serum-free DMEM. Prior to seeding into the transwell inserts, cells were harvested by trypsin-EDTA and sequentially rinsed with DMEM containing 10% FCS and serum-free DMEM. The rinsed cells were re-suspended in serum-free DMEM (2.5×10⁶ cells/mL) and 800 μL was added to the upper chambers. Galardin (2 μmol/L), MMP-2/MMP-9 inhibitor I (20 μmol/L) or 0.1% DMSO as control were added. In the lower compartment, fetal calf serum was used as chemoattractant. Chambers were incubated in a humified tissue incubator, containing 50 mL/L CO₂ for 4 h at 37 °C. The cells on the upper surface of the filters were then removed using a cotton swab and those remaining on the lower surface of the filter were fixed with 10% methanol and stained with hematoxylin and eosin. The filters were rinsed with deionized water, dried and examined using light microscopy. The number of cells in five random optical fields (×400 magnification) from triplicate filters was averaged to obtain the number of migrating cells.

Attachment assay
Attachment assays were performed in 96-well plates. The wells were coated with gelatin solutions at a concentration of 10 µg/mL at 4 °C for 18 h. Unspecific binding of cells was blocked by pre-incubation of the wells with 3% BSA/PBS for 2 h. Hepatoma cell clones were harvested by trypsin incubation for 2 min, resuspended in DMEM without FCS for 2 h. Hepatoma cell clones were harvested by trypsin incubation for 2 min, resuspended in DMEM without FCS for 2 h. The rinsed cells were re-suspended in serum-free DMEM with DMEM containing 10% FCS and serum-free DMEM. Prior to seeding into the transwell inserts, cells were harvested by trypsin-EDTA and sequentially rinsed with DMEM containing 10% FCS and serum-free DMEM. The rinsed cells were re-suspended in serum-free DMEM (2.5×10⁶ cells/mL) and 800 μL was added to the upper chambers. Galardin (2 μmol/L), MMP-2/MMP-9 inhibitor I (20 μmol/L) or 0.1% DMSO as control were added. In the lower compartment, fetal calf serum was used as chemoattractant. Chambers were incubated in a humified tissue incubator, containing 50 mL/L CO₂ for 4 h at 37 °C. The cells on the upper surface of the filters were then removed using a cotton swab and those remaining on the lower surface of the filter were fixed with 10% methanol and stained with hematoxylin and eosin. The filters were rinsed with deionized water, dried and examined using light microscopy. The number of cells in five random optical fields (×400 magnification) from triplicate filters was averaged to obtain the number of migrating cells.

RT-PCR
RNA was isolated from whole cells by Ultraspec™ phenol chloroform extraction procedure (Biotex Laboratories Inc., Houston, TX) according to the instruction manual, quantified spectrophotometrically at 260 nm and stored at -70 °C. Five micrograms of RNA were heated to 65 °C for 10 min in 50% formamide, 20 mM/L 3-[N-morpholino] propane sulfonic acid (MOPS), 5 mM/L sodium acetate, 1 mM/L EDTA, and 2.2 mM/L formaldehyde before gel electrophoresis in 1% agarose containing 2.2 mol/L formaldehyde, 20 mM/L MOPS, 5 mM/L sodium acetate, and 1 mM/L EDTA. After electrophoresis, integrity of the RNA was controlled by ethidium bromide staining of 18S and 28S ribosomal RNA. First-strand complementary DNA (cDNA) was synthesized from 1 μg of DNA-free total RNA with a T-primed first-strand kit (Pharmacia Biotech, Freiburg, Germany) according to the manufacturer’s instructions. A 1:100 final dilution of the reaction was used as template for PCR. In preliminary experiments, we determined the optimal number of cycles within the linearity of reactions for each PCR product. The cycle number was 35 cycles for MMP-9, MMP-14, and human TIMP-1, and 25 cycles for MMP-2 and GAPDH. The primers for PCR were as follows: human MMP-2 (756-1682 nt, GenBankTM accession no. AL832088), forward (5’-CCA CGC TGC GGC ACC CCA GAT GTG GGC AAC-3’) and backward (5’-GGC GTG GGC AAC CCA GAT GTG GGC AAC-3’); human TIMP-1 (292-885 nt, GenBankTM J04038), forward (5’-CCC ATC ACC TTC TCC CAG) and backward (5’-CCT GCT TCA CCA CCT TCT); human MMP-9 (1512-2120 nt, GenBankTM BC006093), forward (5’-ACC GCC ACT ACT CTG CCT TTG-3’) and backward (5’-AGG GTC ATG GGC ACT ACT CTG CCT TTG-3’); human TIMP-1 (169-494 nt, GenBankTM NM003254), forward (5’-GCC CAT TGG CCA GTT CTG GCG GG-3’) and backward (5’-AGT GTT GCT TCG CTG GCG GG-3’); human MMP-14 (1247-1766 nt, GenBankTM NM010566), forward (5’-GCC CAT TGG CCA GTT CTG GCG GG-3’) and backward (5’-AGT GTT GCT TCG CTG GCG GG-3’); human TIMP-1 (169-494 nt, GenBankTM NM003254), forward (5’-GCC CAT TGG CCA GTT CTG GCG GG-3’) and backward (5’-AGT GTT GCT TCG CTG GCG GG-3’); human TIMP-1 (169-494 nt, GenBankTM NM003254), forward (5’-GCC CAT TGG CCA GTT CTG GCG GG-3’) and backward (5’-AGT GTT GCT TCG CTG GCG GG-3’); human TIMP-1 (169-494 nt, GenBankTM NM003254), forward (5’-GCC CAT TGG CCA GTT CTG GCG GG-3’) and backward (5’-AGT GTT GCT TCG CTG GCG GG-3’). Using these primers, PCR was performed by the expand high fidelity PCR system (Roche Diagnostics) at 94 °C for 4 min followed by individual cycles at 94 °C for 30 s, at 58 °C for 30 s, and at 72 °C for 1 min with an extension step at 72 °C for 7 min at the end of the last cycle. The products were resolved in agarose gel (1.8%), followed by staining with ethidium bromide, and recorded by digital camera. The relative density of the products was quantitated by the Bio-Rad Gel-Doc system. The predicted PCR product size for MMP-2 and MMP-9 was 926 and 608 bp, respectively. For glyceraldehyde 3-phosphate dehydrogenase (GAPDH) the product size is 1098. Buffers used were formulated to decrease amplification of longer DNA fragments during RT-PCR. Follow the manufacturer’s instructions. Buffers used were formulated to decrease amplification of longer DNA fragments during RT-PCR. Follow the manufacturer’s instructions. Buffers used were formulated to decrease amplification of longer DNA fragments during RT-PCR. Follow the manufacturer’s instructions. Buffers used were formulated to decrease amplification of longer DNA fragments during RT-PCR. Follow the manufacturer’s instructions. Buffers used were formulated to decrease amplification of longer DNA fragments during RT-PCR. Follow the manufacturer’s instructions. Buffers used were formulated to decrease amplification of longer DNA fragments during RT-PCR. Follow the manufacturer’s instructions. Buffers used were formulated to decrease amplification of longer DNA fragments during RT-PCR. Follow the manufacturer’s instructions. Buffers used were formulated to decrease amplification of longer DNA fragments during RT-PCR. Follow the manufacturer’s instructions. Buffers used were formulated to decrease amplification of longer DNA fragments during RT-PCR. Follow the manufacturer’s instructions. Buffers used were formulated to decrease amplification of longer DNA fragments during RT-PCR. Follow the manufacturer’s instructions. Buffers used were formulated to decrease amplification of longer DNA fragments during RT-PCR. Follow the manufacturer’s instructions. Buffers used were formulated to decrease amplification of longer DNA fragments during RT-PCR. Follow the manufacturer’s instructions. Buffers used were formulated to decrease amplification of longer DNA fragments during RT-PCR. Following PCR, the amplicons were analyzed by gel electrophoresis. Since the amplification efficiency of each primer pair was equivalent, the intensity of each
amplicon band was proportional to their relative concentration in the cDNA sample. Agarose gel electrophoresis bands were scanned by computer analysis, and the optical density was plotted by histogram. The Bio-Rad Gel-Doc system was used to quantify PCR data. Data from MMP-cDNA were normalized to the respective content of GAPDH.

RESULTS

Effect of TIMP-1 and TIMP-1 antagonist on TIMP-1 expression

HepG2 wild type cells were compared with HepG2 cell clones overexpressing murine TIMP-1 (#T1, #T2, #T3) and HepG2 cells overexpressing the TIMP-1 antagonist MMP-9-H401A (#M1, #M2, #M3). Western blot analysis was performed for serum-free supernatants from HepG2 cells, HepG2-TIMP-1, (Figure 1, Lane 3) and HepG2-MMP-9-H401A. Using a specific polyclonal antiserum against murine TIMP-1 that did not detect human TIMP-1, amounts of murine TIMP-1 could be detected in HepG2-TIMP-1 cells. The supernatants of three different clones for HepG2-TIMP-1 (Figure 1, lane 2) and three different clones for MMP-9-H401A (Figure 1, lane 1), respectively, were pooled.

Figure 1 TIMP-1 protein expression of hepatoma cells overexpressing murine TIMP-1 and those overexpressing the TIMP-1 antagonist MMP-9-H401A. The arrow indicates the position of murine TIMP-1. TIMP-1 protein could be detected in HepG2-TIMP-1 cells only.

Effect of TIMP-1 and TIMP-1 antagonist on expression of MMP-1, -3, -7, -2, -9, and MMP-13

Semi-quantitative multiplexPCR was used to investigate the expression of MMP-1, -3, -2, -9, and -14 simultaneously in HepG2 cells, HepG2-TIMP-1 cells and HepG2 cells overexpressing MMP-9-H401A. The average values of three different clones for each cell line are indicated in Figure 2. Expression values of HepG2 cells were set to 100%. There were no significant changes in the expression levels of MMP-1, MMP-3, and MMP-14 between wild type HepG2 cells, TIMP-1 overexpressing HepG2 cells (black columns), and HepG2 overexpressing MMP-9-H401A (halftone columns). We observed a significant increase in the expression of MMP-9 (HepG2-TIMP-1: \( p = 0.003 \); HepG2-MMP-9-H401A: \( p = 0.05 \)) in both HepG2-TIMP-1 and HepG2-MMP-9-H401A cells (Figure 2). MMP-9 primers did not differ between endogenous human MMP-9 and the transgene MMP-9-H401A with a single point mutation on DNA level. MMP-2 expression increased in HepG2-TIMP-1 in comparison to HepG2 cells but values did not reach the level of significance. MMP-2 expression levels in HepG2-MMP-9-H401A, however, decreased (Figure 2). There were no changes in the expression of the endogenous TIMP-1 between all cell clones examined (Figure 2, right panel).

Migration of human hepatoma cells was influenced by endogenous TIMP-1

To investigate whether TIMP-1 or TIMP-1 antagonism contributed to differences in cell migration, HepG2-TIMP-1 and HepG2-MMP-9-H401A cells were incubated in Boyden chambers. Fetal calf serum was used as chemoattractant. After incubated for 4 h, migrated cells were numbered on the underside of the permeable membrane coated with gelatin. Data were normalized by setting the number of HepG2 wild type cells to 100% (Figure 3, white column, lane 1) and were presented as mean±SD. Compared with wild type HepG2 cells, the cells overexpressing TIMP-1 showed 114.6±10.7% (\( p = 0.039 \)) migration (Figure 3, black column, lane 2) and the cells overexpressing the TIMP-1 antagonist showed 62.2±16% (\( p = 0.0196 \)) migration (Figure 3, halftone column, lane 3). The difference between TIMP-1 clones and clones overexpressing the TIMP-1 antagonist was highly significant (\( p = 0.0057 \)). Thus, TIMP-1 overexpression associated with increased expression of MMP-9 and MMP-2 favors migration in contrast to TIMP-1 inhibition by MMP-9-H401A.

Figure 3 TIMP-1 and TIMP-1 antagonist contribute to differences in cell migration. \( a^P < 0.05; b^P < 0.01. \)
Migration of hepatoma cells overexpressing TIMP-1 was inhibited by a broad spectrum inhibitor of MMPs and a specific inhibitor of MMP-2 and MMP-9

To investigate whether MMPs were responsible for the different migration rates of HepG2 cells overexpressing TIMP-1 or MMP-9-H401A, cells were incubated with the broad spectrum MMP-inhibitor Galardin (Figure 4, lanes 2, 5, 8, 11, 14, 17, 20; few points) and a specific inhibitor of MMP-2/MMP-9 (Figure 4, lanes 3, 6, 9, 12, 15, 18, 21; many points). HepG2, HepG2-TIMP-1, and HepG2-MMP-9-H401A cells were incubated in Boyden chambers either with or without 2 µmol/L Galardin (GM 6001, inhibitor of MMP-1, -2, -3, -8, -9) and 20 µmol/L MMP-2/MMP-9 inhibitor. Fetal calf serum was used as chemoattractant. After 4 h of incubation migrated cells were numbered on the bottom of the permeable membrane coated with gelatin. Incubation with 2 µmol/L Galardin and 20 µmol/L gelatinase inhibitor reduced cell migration from 27.7±15.6 to 22.2±10.3 (P = 0.038) and 25.1±15.7 in HepG2 wild type cells (Figure 4, column 1-3). In the case of HepG2-MMP-9-H401A, incubation with Galardin reduced cell migration significantly from 21.5 to 16.1 (P = 0.02, #M1), 17.6 to 4.8 (P = 0.0016, #M2), and 12.6 to 6.9 (P = 0.03, #M3) for all clones analyzed separately. Gelatinase inhibitor was not able to reduce cell migration significantly in wild type cells and two of three cell clones overexpressing the TIMP-1 antagonist MMP-9-H401A (Figure 4, lanes 3, 6, and 12; many points). In the case of HepG2-TIMP-1, incubation with Galardin reduced cell migration from 33.9 to 25.8 (P = 0.02, #T1), 30.8 to 24.6 (P = 0.018, #T2), and 28.1 to 14.5 (P<0.0001, #T3). In the presence of MMP-2/-9 inhibitor a reduced migration was observed in all TIMP-1 overexpressing cell clones (Figure 4, lane 15, 18, and 21; many points), although reduction did not achieve significance in all cases (Figure 4, lane 18). Incubation of HepG2-TIMP-1 (#T1) with the specific MMP-2/-9 inhibitor reduced cell migration from 33.9±18.9 to 15.4±10.7 (P = 0.000003) beyond the number achieved by incubation with Galardin.

Signaling pathways required for expression of MMP-2 and MMP-9 in TIMP-1 overexpressing cells

To test which signaling pathways were involved in the expression of MMP-2 and MMP-9 in wild type HepG2 and TIMP-1 overexpressing HepG2, cells were incubated with different kinase inhibitors. Ras proteins are key molecular switches that mediate transmembrane signaling through the activation of multiple downstream pathways, which include the MAPK pathways[31]. A series of pharmacological inhibitors were used to test the potential contribution of one or more signaling pathways that might regulate the steady-state transcription of the MMP-2 and MMP-9 gene in wild-type or TIMP-1 overexpressing HepG2 cells: genistein (25 and 50 µmol/L; tyrosine kinase inhibitor), SB 202190 (2.5 µmol/L; potent inhibitor of p38 MAP kinase), and UO126 (1 µmol/L; potent and specific inhibitor of MEK1 and MEK2).

UO126 and genistein treatment at a dose of 50 µmol/L inhibited the secretion of MMP-2 in wild type and TIMP-1 overexpressing hepatoma cells (Figures 6A, B and columns 3, and 5). There was also a less pronounced but reproducible effect using the general tyrosin kinase inhibitor genistein at a dose of 25 µmol/L (Figure 6A, B and column 2). The specific p38 MAP kinase inhibitor SB 202190 was effective in blocking MMP-2 expression in HepG2 wild-type cells only, suggesting that the MMP-2 expression was not regulated through p38 MAPK in the case of TIMP-1 overexpression. These data all supported the hypothesis that constitutive MMP-2 expression is regulated through a steady-state signaling event, passing through Ras, MEK1/2, and p38 MAP kinases (Figure 6A). All TIMP-1 overexpressing cell clones (#T1, #T2, #T3) behaved similar (Figure 6 B).

Genistein at a dose of 25 and 50 µmol/L inhibited the secretion of MMP-9 in wild-type and TIMP-1 overexpressing hepatoma cells (Figure 6C, D and columns 2, 3). There was also a less-pronounced but reproducible effect using the specific MEK1/2 inhibitor UO126 at a dose of 1 µmol/L.
The specific p38 MAP kinase inhibitor SB 202190 was effective in blocking MMP-9 expression in HepG2 wild-type cells and less pronounced in one (#T1) of the TIMP-1 overexpressing clones (individual data not shown) whereas MMP-9 expression could not be influenced by p38 MAK inhibition in other TIMP-1 overexpressing clones (#T2, #T3) (individual data not shown). These data suggested that TIMP-1 overexpression attenuates MMP-9 expression through p38 MAPK. Similar to MMP-2 constitutive MMP-9 expression is regulated through a steady-state signaling event passing through Ras, MEK1/2, and p38 MAP kinases (Figure 6C).

DISCUSSION
A critical involvement of TIMP-1 and MMPs, in the extent of migration of human hepatoma cells, is shown here. It has previously been reported that TIMP-1 may play an important role in growth and migration, especially of hepatocellular and cholangiocellular carcinomas[32]. In this report, we extend these data in several ways. The migration of human hepatoma cells is characterized by the presence of TIMP-1 and a novel TIMP-1 antagonist (Roeb et al. TIMP-1 enhanced migration 1101). This TIMP-1 antagonist is effective in blocking MMP-9 expression in HepG2 wild-type cells and less pronounced in one (#T1) of the TIMP-1 overexpressing clones (individual data not shown) whereas MMP-9 expression could not be influenced by p38 MAK inhibition in other TIMP-1 overexpressing clones (#T2, #T3) (individual data not shown). These data suggested that TIMP-1 overexpression attenuates MMP-9 expression through p38 MAPK. Similar to MMP-2 constitutive MMP-9 expression is regulated through a steady-state signaling event passing through Ras, MEK1/2, and p38 MAP kinases (Figure 6C).

The specific p38 MAP kinase inhibitor SB 202190 was effective in blocking MMP-9 expression in HepG2 wild-type cells and less pronounced in one (#T1) of the TIMP-1 overexpressing clones (individual data not shown) whereas MMP-9 expression could not be influenced by p38 MAK inhibition in other TIMP-1 overexpressing clones (#T2, #T3) (individual data not shown). These data suggested that TIMP-1 overexpression attenuates MMP-9 expression through p38 MAPK. Similar to MMP-2 constitutive MMP-9 expression is regulated through a steady-state signaling event passing through Ras, MEK1/2, and p38 MAP kinases (Figure 6C).

DISCUSSION
A critical involvement of TIMP-1 and MMPs, in the extent of migration of human hepatoma cells, is shown here. It has previously been reported that TIMP-1 may play an important role in growth and migration, especially of hepatocellular and cholangiocellular carcinomas[32]. In this report, we extend these data in several ways. The migration of human hepatoma cells is characterized by the presence of TIMP-1 and a novel TIMP-1 antagonist (Roeb et al. TIMP-1 enhanced migration 1101). This TIMP-1 antagonist is effective in blocking MMP-9 expression in HepG2 wild-type cells and less pronounced in one (#T1) of the TIMP-1 overexpressing clones (individual data not shown) whereas MMP-9 expression could not be influenced by p38 MAK inhibition in other TIMP-1 overexpressing clones (#T2, #T3) (individual data not shown). These data suggested that TIMP-1 overexpression attenuates MMP-9 expression through p38 MAPK.

TIMP-1 overexpression leads to increased migration of hepatoma cells (Figure 2). This phenomenon linked to TIMP-1 is confirmed by the use of the novel TIMP-1 antagonist MMP-9-H401-A[28]. This MMP-9 mutant has the opposite effect of TIMP-1 and reduces migration in our system. Although both TIMP-1 and the synthetic MMP-inhibitor GM6001 are able to inhibit MMPs, TIMP-1 increases and GM6001 decreases migration. Thus, the action of TIMP-1 on migration of hepatoma cells is functionally different from GM6001. Since TIMP-1 enhances migration in hepatoma cells, the influence of TIMP-1 on migration seems to be at least partly independent of its MMP inhibitory activity and indicates other functions of TIMP-1 besides its MMP inhibitory capacity. Recent studies showed that apoptotic effects of TIMP-1, especially the inhibition of apoptosis in tumor cells, are independent of MMP inhibition[11,33]. However, apoptosis would hardly be the reason for increased migration in our experiments because we measured migration over 4 h, too short for significant effects of programmed cell death. Furthermore, we measured apoptosis in all cell clones used as HepG2 wild-type cells by a cell death detection (nuclear matrix protein) but ELISA and apoptotic DNA Ladder kit could not detect any significant differences (data not shown). Further biological functions of TIMP-1, mitogenic activity, and cell-growth promoting activity, can be mainly excluded for the short period of time selected[11,34-36]. The effect of TIMP-1 on hepatoma cell migration might be cell tissue specific because in B16-F10 melanoma cells a specific up-regulation of murine TIMP-1 expression directly suppresses the invasive
ability in a matrigel transwell invasion assay\cite{17}. Recently the modulation of cell morphology has been reported to have a function for TIMP-1\cite{11,38}. In smooth muscle cells a minimum expression of the contractile proteins and a maximal proliferation rate are correlated with the highest levels of both MMP-1 and TIMP-1\cite{38}. TIMP-1 overexpressing HepG2 cells actually have a characteristic phenotype; they grow in nests and lie on top of each other\cite{27}. It might be possible that this specific phenotype enables the cells to enhance migration through the gelatin-coated transwell filters. Whether the modulation of cell morphology by TIMP-1 takes place by changing the expression pattern of contractile proteins is the subject of our current investigation.

MMP-2 and -9 are necessary for the migration of many cell types and tumor cells, e.g., Langerhans cells and dermal dendritic cells from human and murine skin\cite{9}. The migration of the latter cells, however, is inhibited by TIMP-1 and TIMP-2. We also demonstrated that MMPs are critical for the migration of hepatoma cells either in the presence or absence of TIMP-1 (Figure 4). Overexpressing functional TIMP-2 and -9, however, reduction of migration did not achieve significance.

Migration of cells through a coated membrane involves not only ECM degradation but also requires the ability of adhesive interactions between cells and matrix. Cell migration can be viewed as a process regulated by counter-balanced forces or activities. Strength of cell adhesion and inhibitory degrees of cell adhesion can regulate the speed of motility\cite{39}. In the present study, however, decreased and increased migration did not correlate with different adhesion (Figure 5).

MAPK activities exert a crucial role in cellular migration, and their implication in this process has been highlighted by the use of specific inhibitors (indicated in Figure 7).

Several reports have shown that SAPK2/p38 is involved in chemokine-induced chemotaxis of cells\cite{40-42}. For this reason, we examined the migration potential of TIMP-1 overexpressing cells in the presence of specific signal transduction inhibitors (Figure 7) and our study presents evidence that p38 but not ERK, intervenes in the control of hepatoma cell migration. Signal transduction has traditionally been examined by short-term stimulation of cells with agents that induce mitogenesis, differentiation, or apoptosis\cite{43}. Recent work showed that maintenance of basal cell function also requires “steady-state” signal transduction\cite{44}. In this specific instance, the steady-state production of MMP-2 and MMP-9 involved in cell attachment, detachment, and migration was examined in our study. Constitutive MMP-2 and MMP-9 expression is regulated through a steady-state signaling event passing through tyrosine kinases, MEK1/2, and p38 MAP kinases (Figures 6, 7). Our results are in accordance with others\cite{45-48}. For head and neck cancer cells, it has already been reported that the general tyrosine kinase inhibitor genistein induces several specific molecular changes, such as down-regulation of c-erbB-2 expression, down-regulation of MMP-2 and MMP-9 secretion, inhibition of tumor cell invasion and down-regulation of nuclear factor-kappaB DNA binding activity\cite{49}. In human keratinocytes, the inhibition of MAPK pathway is correlated with down-regulation of MMP-9 secretion induced by TNF-alpha\cite{50}. In brain cells, ERK and p38 MAP kinases are up-regulated after mechanical injury, and mediate the secretion of MMP-9\cite{47}. Recently, it has been shown that inhibition of monocyte migration occurs via inhibiting crucial signaling pathways, like SAPK2/p38 (Figure 7) and MMP-2 activities\cite{48}.

Given the data presented in this manuscript, we could now predict that expression of MMP-2 and MMP-9 in

![Figure 7 Signal transduction pathways and MAP kinase signaling cascades in mammalian cells shown in schematic form.](image-url)
liver tumors containing high TIMP-1 activity cannot be influenced by inhibition of p38 MAK (Figure 7). This should result in a higher metastatic potential and probably a poorer overall prognosis.

Our data add new insights in the complex function of TIMP-1 and control of MMP activity in human liver cells. Thus, the suggested role of TIMP-1 in tumor cell migration has substantially changed from the initial focus on MMP inhibition to a broader focus including modulation of MMP secretion, cell morphology, and cell migration. With regard to the treatment of inflammatory disorders and metastasis, it might be useful to target the signaling pathways regulating these MMPs in more detail.

ACKNOWLEDGEMENTS

A part of the data has been presented as a plenary talk on the Single Topic Conference Liver Fibrosis of the EASL in Florence, 2001.

REFERENCES

1. Stetler-Stevenson WG, Azzavoorian S, Liotta LA. Tumor cell interactions with the extracellular matrix during invasion and metastasis. Annu Rev Cell Biol 1993; 9: 541–573
2. Coussens LM, Werb Z. Matrix metalloproteinases and the development of cancer. Cancer Chem 1996; 8: 895–904
3. Shapiro SD. Matrix metalloproteinase degradation of extracellular matrix: biological consequences. Curr Opin Cell Biol 1998; 10: 602–608
4. Yu Q, Stamenovic I. Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. Genes Dev 2000; 14: 163–176
5. Ratzinger G, Stoitznzer P, Ebnner S, Lutz MB, Layton GT, Rainer C, Senior RM, Shipley JM, Fritsch P, Schuler G, Romani G, Mani N. Matrix metalloproteinases 9 and 2 are necessary for the development of cancer. J Clin Invest 1996; 98: 11069–11076
6. Coussens LM, Werb Z. Matrix metalloproteinases and the development of cancer. Annu Rev Pathol 2010; 5: 201–205

ACKNOWLEDGEMENTS

A part of the data has been presented as a plenary talk on the Single Topic Conference Liver Fibrosis of the EASL in Florence, 2001.

REFERENCES

1. Stetler-Stevenson WG, Azzavoorian S, Liotta LA. Tumor cell interactions with the extracellular matrix during invasion and metastasis. Annu Rev Cell Biol 1993; 9: 541–573
2. Coussens LM, Werb Z. Matrix metalloproteinases and the development of cancer. Cancer Chem 1996; 8: 895–904
3. Shapiro SD. Matrix metalloproteinase degradation of extracellular matrix: biological consequences. Curr Opin Cell Biol 1998; 10: 602–608
4. Yu Q, Stamenovic I. Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. Genes Dev 2000; 14: 163–176
5. Ratzinger G, Stoitznzer P, Ebnner S, Lutz MB, Layton GT, Rainer C, Senior RM, Shipley JM, Fritsch P, Schuler G, Romani G, Mani N. Matrix metalloproteinases 9 and 2 are necessary for the development of cancer. J Clin Invest 1996; 98: 11069–11076
6. Coussens LM, Werb Z. Matrix metalloproteinases and the development of cancer. Annu Rev Pathol 2010; 5: 201–205

References: 1. Stetler-Stevenson WG, Azzavoorian S, Liotta LA. Tumor cell interactions with the extracellular matrix during invasion and metastasis. Annu Rev Cell Biol 1993; 9: 541–573
2. Coussens LM, Werb Z. Matrix metalloproteinases and the development of cancer. Cancer Chem 1996; 8: 895–904
3. Shapiro SD. Matrix metalloproteinase degradation of extracellular matrix: biological consequences. Curr Opin Cell Biol 1998; 10: 602–608
4. Yu Q, Stamenovic I. Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. Genes Dev 2000; 14: 163–176
5. Ratzinger G, Stoitznzer P, Ebnner S, Lutz MB, Layton GT, Rainer C, Senior RM, Shipley JM, Fritsch P, Schuler G, Romani G, Mani N. Matrix metalloproteinases 9 and 2 are necessary for the development of cancer. J Clin Invest 1996; 98: 11069–11076
6. Coussens LM, Werb Z. Matrix metalloproteinases and the development of cancer. Annu Rev Pathol 2010; 5: 201–205

References: 1. Stetler-Stevenson WG, Azzavoorian S, Liotta LA. Tumor cell interactions with the extracellular matrix during invasion and metastasis. Annu Rev Cell Biol 1993; 9: 541–573
2. Coussens LM, Werb Z. Matrix metalloproteinases and the development of cancer. Cancer Chem 1996; 8: 895–904
3. Shapiro SD. Matrix metalloproteinase degradation of extracellular matrix: biological consequences. Curr Opin Cell Biol 1998; 10: 602–608
4. Yu Q, Stamenovic I. Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. Genes Dev 2000; 14: 163–176
5. Ratzinger G, Stoitznzer P, Ebnner S, Lutz MB, Layton GT, Rainer C, Senior RM, Shipley JM, Fritsch P, Schuler G, Romani G, Mani N. Matrix metalloproteinases 9 and 2 are necessary for the development of cancer. J Clin Invest 1996; 98: 11069–11076
6. Coussens LM, Werb Z. Matrix metalloproteinases and the development of cancer. Annu Rev Pathol 2010; 5: 201–205

References: 1. Stetler-Stevenson WG, Azzavoorian S, Liotta LA. Tumor cell interactions with the extracellular matrix during invasion and metastasis. Annu Rev Cell Biol 1993; 9: 541–573
2. Coussens LM, Werb Z. Matrix metalloproteinases and the development of cancer. Cancer Chem 1996; 8: 895–904
3. Shapiro SD. Matrix metalloproteinase degradation of extracellular matrix: biological consequences. Curr Opin Cell Biol 1998; 10: 602–608
4. Yu Q, Stamenovic I. Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. Genes Dev 2000; 14: 163–176
5. Ratzinger G, Stoitznzer P, Ebnner S, Lutz MB, Layton GT, Rainer C, Senior RM, Shipley JM, Fritsch P, Schuler G, Romani G, Mani N. Matrix metalloproteinases 9 and 2 are necessary for the development of cancer. J Clin Invest 1996; 98: 11069–11076
6. Coussens LM, Werb Z. Matrix metalloproteinases and the development of cancer. Annu Rev Pathol 2010; 5: 201–205

References: 1. Stetler-Stevenson WG, Azzavoorian S, Liotta LA. Tumor cell interactions with the extracellular matrix during invasion and metastasis. Annu Rev Cell Biol 1993; 9: 541–573
2. Coussens LM, Werb Z. Matrix metalloproteinases and the development of cancer. Cancer Chem 1996; 8: 895–904
3. Shapiro SD. Matrix metalloproteinase degradation of extracellular matrix: biological consequences. Curr Opin Cell Biol 1998; 10: 602–608
4. Yu Q, Stamenovic I. Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. Genes Dev 2000; 14: 163–176
5. Ratzinger G, Stoitznzer P, Ebnner S, Lutz MB, Layton GT, Rainer C, Senior RM, Shipley JM, Fritsch P, Schuler G, Romani G, Mani N. Matrix metalloproteinases 9 and 2 are necessary for the development of cancer. J Clin Invest 1996; 98: 11069–11076
6. Coussens LM, Werb Z. Matrix metalloproteinases and the development of cancer. Annu Rev Pathol 2010; 5: 201–205
Campbell SL, Khosravi-Far R, Rossman KL, Clark GJ, Der CJ. Increasing complexity of Ras signaling. *Oncogene* 1998; 17: 1395–1413.

Nakatsukasa H, Ashida K, Higashi T, Ohguchi S, Tsuboi S, Hino N, Nouso K, Urabe Y, Kinugasa N, Yoshida K, Uematsu S, Ishizaki M, Kobayashi Y, Tsuji T. Cellular distribution of transcripts for tissue inhibitor of metalloproteinases 1 and 2 in human hepatocellular carcinomas. *Hepatology* 1996; 24: 82–88.

Li G, Fridman R, Kim HR. Tissue inhibitor of metalloproteinase-1 inhibits apoptosis of human breast epithelial cells. *Cancer Res* 1999; 59: 6267–6275.

Gomez DE, Alonso DF, Yoshiji H, Thorgeirsson UP. Tissue inhibitors of metalloproteinases: structure, regulation and biological functions. *Eur J Cell Biol* 1997; 74: 111–122.

Murphy G, Willenbrock F. Tissue inhibitors of matrix metalloendopeptidases. *Methods Enzymol* 1995; 248: 496–510.

Khokha R, Zimmer MJ, Graham CH, Lala PK, Waterhouse P. Suppression of invasion by inducible expression of tissue inhibitor of metalloproteinase-1 (TIMP-1) in B16-F10 melanoma cells. *J Natl Cancer Inst* 1992; 84: 1017–1022.

Kato S, Yasukawa H, Fujii T, Yamaguchi M, Miyagi N, Okamoto K, Wada Y, Miyamoto T, Morimatsu M, Fox JC. Coordinate regulation of matrix metalloproteinase-1 and tissue inhibitor of metalloproteinase-1 expression in human vascular smooth muscle cells. *Connect Tissue Res* 2000; 41: 143–153.

Palecek SP, Loftus JC, Ginsberg MH, Lauffenburger DA, Horwitz AF. Integrin-ligand binding properties govern cell migration speed through cell-substratum adhesiveness. *Nature* 1997; 385: 537–540.

Ashida N, Arai H, Yamasaki M, Kita T. Distinct signaling pathways for MCP-1-dependent integrin activation and chemotaxis. *J Biol Chem* 2001; 276: 16555–16560.

Sun Y, Cheng Z, Ma L, Pei G. Beta-arrestin2 is critically involved in CXCR4-mediated chemotaxis, and this is mediated by its enhancement of p38 MAPK activation. *J Biol Chem* 2002; 277: 49212–49219.

Cara DC, Kaur J, Forster M, McCafferty DM, Kubes P. Role of p38 mitogen-activated protein kinase in chemokine-induced emigration and chemotaxis in vivo. *J Immunol* 2001; 167: 6552–6558.

Kerkhoff E, Rapp UR. Cell cycle targets of Ras/Raf signalling. *Oncogene* 1998; 17: 1457–1462.

Liao J, Wolfman JC, Wolfman A. K-ras regulates the steady-state expression of matrix metalloproteinase 2 in fibroblasts. *J Biol Chem* 2003; 278: 31871–31878.

Alhasan SA, Aranha O, Sarkar FH. Genistein elicits pleiotropic molecular effects on head and neck cancer cells. *Clin Cancer Res* 2001; 7: 4174–4181.

Holvoet S, Vincent C, Schmitt D, Serres M. The inhibition of MAPK pathway is correlated with down-regulation of MMP-9 secretion induced by TNF-alpha in human keratinocytes. *Exp Cell Res* 2003; 290: 108–119.

Wang X, Mori T, Jung JC, Fini ME, Lo EH. Secretion of matrix metalloproteinase-2 and -9 after mechanical trauma injury in rat cortical cultures and involvement of MAP kinase. *J Neurotrauma* 2002; 19: 615–625.

Vitale S, Schmid-Alliana A, Breuil V, Pomeranz M, Millet MA, Rossi B, Schmid-Antomarchi H. Soluble fractalkine prevents monocyte chemoattractant protein-1-induced monocyte migration via inhibition of stress-activated protein kinase 2/p38 and matrix metalloproteinase activities. *J Immunol* 2004; 172: 585–592.