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Increased TIMP-1 Activity Results in Increased Expression of Gelatinases and Altered Cell Motility

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Abstract Matrix metalloproteinases are proteolytic enzymes which play a major role in resorption of collagen and other components of the extracellular matrix. They are controlled by specific inhibitors, so-called tissue inhibitors of metalloproteinases (TIMPs). The balance between matrix metalloproteinases and TIMPs seems to play a major role in controlling extracellular matrix homeostasis and cell migration. The influence of TIMP-1 on migration behaviour was explored in human hepatoma cells transiently and stably transfected with mouse TIMP-1, and incubated with biologically active TIMP-1. Transfection and biosynthesis were verified by Northern blotting, Western blotting, metabolic labeling, and reverse zymography. Overexpression of and incubation with TIMP-1 resulted in suppressed migration and seemed to enhance cell-cell contact. Using gelatin zymography and Western blotting we measured a significant increase of matrix metalloproteinases-2 and matrix metalloproteinases-9 in cells transfected with TIMP-1. This new phenomenon may be of important physiological significance in modulating TIMP and MMP expression. Our results indicate a functional involvement of TIMP-1 in matrix homeostasis and some automatic control in matrix turnover. J. Cell. Biochem. 75:346–355, 1999.

Key words: hepatoma cells; matrix; migration; MMPs; TIMP-1

The integrity of connective tissue is determined by the balance of degradation and synthesis of components of the extracellular matrix (ECM). Degradation is performed by matrix metalloproteinases (MMPs) which are inhibited by their specific inhibitors (TIMPs). The interaction between MMPs and TIMPs modulates the rate of matrix degradation and accumulation.

Tissue inhibitors of metalloproteinases TIMP-1, TIMP-2, TIMP-3, and TIMP-4 belong to the family of collagenase inhibitors. They are extracellular proteins which are able to inhibit all members of the family of metalloproteinases which include collagenases, gelatinases A and B, and stromelysins [Birkedal-Hansen et al., 1993; Denhardt et al., 1993]. TIMPs bind to the active forms of MMPs forming 1:1 complexes thus blocking their activity. TIMP-1 can also bind, albeit rather less tightly, to progelatinase B [Goldberg et al., 1992]. TIMP-2 and TIMP-4 but not TIMP-1 specifically bind to progelatinase A [Bigg et al., 1997]. Although the different forms of TIMPs have overlapping activities, they are located on different chromosomes and their expression is regulated differently [Ray et al., 1994; Roeb et al., 1995]. Differential regulation of the TIMP genes or divergent C-terminal protein sequences may underlie distinct biological functions for each TIMP [Apte et al., 1995]. TIMP-1 is a 28.5 kDa glycoprotein with 12 cysteine residues that have been shown to form disulfide bonds in the enzyme giving a six-loop structure [Williamson et al., 1990]. TIMP-1 is resistant to extremes of temperature and pH. In part this is thought to be due to the presence of six sulfhydryl bridges presumed to maintain the structural integrity of the molecule [Caterina et al., 1997].

The balance between activated MMPs and their free inhibitors determines the net MMP activity and seems to be a crucial determinant of matrix protein turnover associated with a variety of pathological processes, such as tumor cell invasion, metastasis, and fibrosis [Ponton et al., 1991; Kossakowska et al., 1992; Carome et al., 1993; Stetler-Stevenson et al., 1993a].

In the present study we demonstrated that human hepatoma cells transfected with a cDNA coding for murine TIMP-1 synthesize biologi-

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cally active mouse TIMP-1. We studied the influence of TIMP-1 overexpression on MMP activity and motility of cells. An overexpression of TIMP-1 led to enhanced MMP-2 and MMP-9 activity, and there was evidence that TIMP-1 is involved in the regulation of cell migration.

MATERIALS AND METHODS

Materials

The random primed DNA labeling kit, cell proliferation kit (XTT), restriction enzymes, T4 DNA ligase and Klenow enzyme were purchased from Boehringer Mannheim (Mannheim, Germany). Recombinant human TIMP-1, MMP standard, and monoclonal antibodies against MMP-9 (mouse MAb IgGκ) and MMP-2 (mouse MAb $IgG_1\kappa$) were purchased from Dianova (Hamburg, Germany). The antibodies are specific for human MMP-2/-9 as declared by the supplier. [α-^{32}P]dATP (110 TBq/mmol) was obtained from Amersham International (Amersham, UK). A cDNA coding for mTIMP-1 and not cross hybridizing with human TIMP-1 mRNA was kindly provided by D.R. Edwards (Norwich, UK).

Standard cloning procedures were performed as described [Sambrook et al., 1989]. Construction of a bacterial TIMP-1 expression plasmid, preparation of a rabbit polyclonal anti-murine-TIMP-1, and construction of an eucaryotic TIMP-1 expression plasmid were performed as described [Roeb et al., 1994]. Expression and purification of biologically active mTIMP-1 in baculovirus-transfected insect cells has been described [Kurschat et al., 1995]. All polymerase chain reaction-generated sequences were verified by DNA sequencing [Sambrook et al., 1989] using T7 DNA polymerase (Pharmacia, Freiburg, Germany).

Cells and Cell Treatment

Human hepatoma cells (HepG2) were cultured to 50–70% confluency in DMEM containing 10% fetal calf serum, streptomycin (100 mg/l), and penicillin (60 mg/l), at 5% CO_2 in a water-saturated atmosphere. Murine TIMP-1-cDNA was transfected into HepG2 cells using a calcium phosphate precipitation technique according to the methods described [De Clerck et al., 1992]. HepG2 cells for transfection were subcultured for 16 h prior to the addition of the calcium phosphate precipitate; 18 h after transfection the precipitate was removed, and cells were cultured for 48 h in serum-free medium for zymography or migration assay. Control cells received pCDM8 empty vector. Transfection efficiency of transient transfection was 10–50% determined by co-transfection with the β-gal gene.

For selection of stably transfected colonies (for example clone #1) the neomycin resistance gene was co-transfected with the expression vector pCDM8 containing the entire coding region for murine TIMP-1 cloned into the XhoI and filled HindIII site. In order to exclude vector specific modifications and accelerate isolation procedures further clones (#28, #39, #45, #26) were recovered after stable transfection with the expression vector pcDNA3.1-mTIMP-1 already containing the neomycin resistance gene. Colonies resistant to 500 µg/ml neomycin were isolated, clones were established and assessed for the expression of mTIMP-1 by Northern blotting, Western blotting, reverse zymography (see below), and immunoprecipitation.

RNA Isolation and Northern Blot Analysis

Total RNA was isolated from cells with the phenol extraction method as described [Rose-John et al., 1988]. Five micrograms of RNA were heated to 65°C for 10 min in 50% formamide, 20 mmol/L 3-[N-morpholino]propane sulfonic acid (MOPS), 5 mmol/L sodium acetate, 1 mmol/L EDTA, and 2.2 mol/L formaldehyde before gel electrophoresis in 1% agarose containing 2.2 mol/L formaldehyde, 20 mmol/L MOPS, 5 mmol/L sodium acetate, and 1 mmol/L EDTA. Equal loading of the RNA gel was checked by ethidium bromide staining of 18S and 28S ribosomal RNA. The separated RNA was transferred to Gene/Screen Plus membranes (DuPont-De-Nemours, Dreieich, Germany) according to the supplier's instructions. An 825-bp fragment coding for the full-length cDNA of mTIMP-1 was exised from the Bluescript vector pBSTIMP-1 with the restriction endonucleases BamHI and HindIII. The filters were prehybridized at 68°C for 2 h in 10% dextran sulfate, 1 M sodium chloride, and 1% SDS and hybridized in the same solution with cDNA fragments labeled by random priming [Feinberg and Vogelstein, 1983]. After hybridization, unspecifically bound radioactivity was removed by washing in 2× standard saline citrate solution (SSC) at room temperature, followed by two consecutive washes in 2× SSC/1% SDS at 68°C for 30 min each. The filters were then subjected to autoradiography

with intensifying screens at −80°C. Autoradiograms from at least three separate experiments were analyzed.

Western Blot

SDS-polyacrylamide gel electrophoresis utilized separating gels of 10% polyacrylamide and stacking gels of 3% polyacrylamide. Each lane was charged with 2 µg of total protein. Following electrophoresis at 30 V the proteins from serum-free cell supernatants were transferred to a PVDF membrane (Pall Gelman Sciences, Rossdorf, Germany). Protein bands were localized by staining with Ponceau S. Blots were blocked with TBS-N (pH 7.6), containing 10% BSA, 20 mM Tris.HCl, pH 7.5, 137 mM NaCl, 0.1% Nonidet P40, washed and incubated with polyclonal antibodies against TIMP-1, and monoclonal antibodies against MMP-2 or MMP-9 (dilution 1:1,000). Western blotting was followed by development with ECL (Amersham).

Zymography (Assay for Demonstration of MMP Activity) and Reverse Zymography (Assay for Demonstration of Biologically Active TIMPs)

Serum-free media of confluent HepG2 cells were concentrated five-fold, assayed for protein content using Bio-Rad DC protein assay kit, and stored at −20°−C until assay. MMP and TIMP activity in the media was assessed by gelatin zymography and reverse zymography following the methods described previously [De Clerck et al., 1992]. The concentrated media were run on SDS/polyacrylamide gel (12% for reverse zymography, 10% for zymography) containing 1 mg/ml gelatin (and metalloproteinases released by 4β-phorbol 12-myristate 13 acetate (PMA)-stimulated human chondrosarcoma cells in the case of reverse zymography). Each lane was charged with equal amounts of total protein. After electrophoresis in 25 mM Tris base, 250 mM glycine, 1% SDS, the gel was washed at room temperature in 2.5% Triton X-100, 5 mM $CaCl_2$, 50 mM Tris.HCl, pH 7.5, and was incubated again in the same buffer two times for 1 h. After rinsing the gel extensively with six changes of distilled water it was incubated overnight (zymography) or for 20 h (reverse zymography) at 37°C in 5 mM $CaCl_2$, 50 mM Tris.HCl, pH 7.5 followed by Coomassie blue staining (0.5% wt/vol) and destaining in methanol/acetic acid/water (50:10:40). Gelatin zymography depicts MMPs as negatively staining bands of gelatinolytic activity. Dark bands in the reverse zymography indicate TIMP activity.

RESULTS

Figures 1–3 present control experiments performed to demonstrate expression and secretion of active mTIMP-1 after stable transfection. All experiments were repeated three times at least. One representative result was chosen for the figures.

The Generation of HepG2 Cell Lines Secreting Constitutively Large Amounts of mTIMP-1

Two different expression vectors were used to establish cell lines secreting mTIMP-1 constitutively. Firstly human heptoma cells were cotransfected with an expression plasmid containing the entire coding sequence for mTIMP-1 (pCDM8mTIMP-1) and a plasmid containing the resistance to Neomycin (pSV2Neo). In order to exclude vector specific modifications and accelerate isolation procedures the entire coding region for murine TIMP-1 was also cloned into the expression vector pcDNA3.1 already containing the neomycin resistance gene. Cells were selected in a Neomycin-bearing medium and multiple clones were analyzed by Northern blotting (Fig. 1). We isolated several clones expressing mTIMP-1. Mobility of clone #1 mTIMP-1mRNA (expression vector pCDM8mTIMP-1) was different in comparison to mTIMP-1 mRNA of clones #26, #28, #39, and #45 (recovered after stable transfection with the expression vector pcDNA3.1 mTIMP-1). The difference of mobility is due to different additional base pairs in the mammalian expression vectors particularly the polyadenylation signal for enhanced mRNA stability. The clones #1 (pCDM8mTIMP-1), #26, and #45 (both pcDNA3.1mTIMP-1) were chosen for further experiments.

HepG2 cells stably transfected with TIMP-1 do not grow as monolayer. They grow in nests and lie on top of each other. Microscopic analysis revealed that these three independent stably transfected isolates have similar properties but migration patterns were not uniform (Fig. 2).

To demonstrate that mTIMP-1 is really produced by positive clones we carried out immunoprecipitations (data not shown) and Western blots using polyclonal antibodies against mTIMP-1 (Fig. 3a). TIMP-1 was running with the same electrophoretic mobility as recombinant mTIMP-1 used as a positive control (Fig.

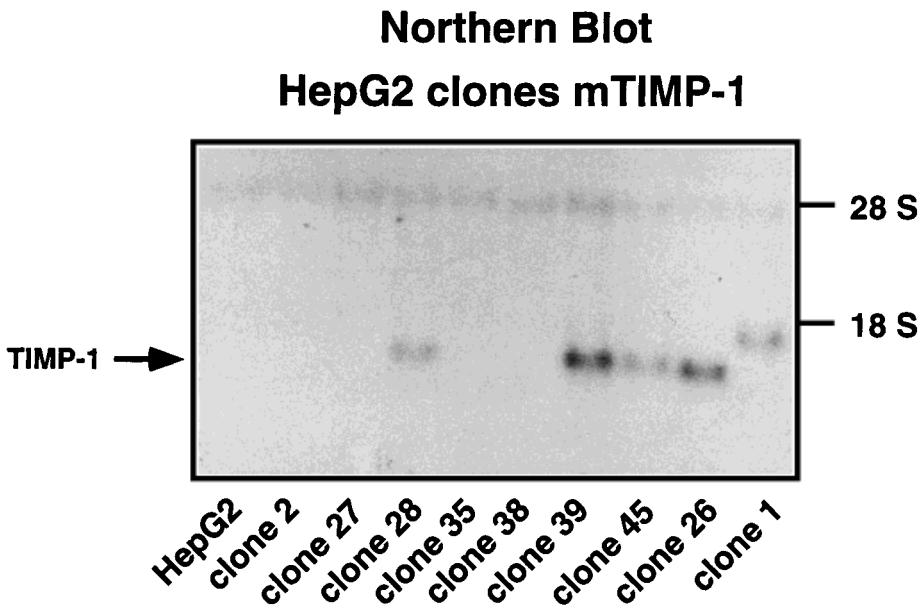

Fig. 1. Northern blot of HepG2 cell clones transfected with mTIMP-1. Northern blot analysis of RNA extracted from different HepG2 cell clones which were stably transfected with an expression vector coding for mTIMP-1. RNA from Wild-type HepG2 cells served as negative control (lane 1, HepG2). Each lane was charged with 5 µg of total RNA. The arrow indicates the position of mTIMP-1 mRNA. Mobility of clone 1 mRNA (expression vector pCDM8) was lower in comparison to mRNA of clones #26, #28, #39, and #45 (expression vector pcDNA3.1-mTIMP-1). Equal loading of the RNA gel was checked by ethidium bromide staining of 18S and 28S ribosomal RNA. Cross-hybridization to 28S ribosomal RNA served as an internal standard. The quality of the mRNA in each lane was also verified with a GAPDH probe (data not shown). Clones #1, #26, and #45 were used for further experiments.

3a, lane 5). Minor amounts of human TIMP-1 could be detected in Wild-type HepG2 cells by Western blotting (Fig. 3a, lane 1).

We performed reverse zymography (Fig. 3b) to determine whether the supernatant of HepG2 cells stably transfected with mTIMP-1 cDNA contained biologically active mTIMP-1 protein. All three mTIMP-1 containing supernatants (clone #1, #26, and #45) exhibited metalloproteinase-inhibitory activity at an electrophoretic mobility corresponding to TIMP-1. Thirty ng of recombinant human TIMP-1 served as positive control. The supernatant of HepG2 cells transfected with the empty expression vector indicates the presence of low endogenous TIMP-1 activity (Fig. 3b, lane 1). As a negative control the supernatant of COS-7 cells transfected with the empty expression vector and known to express no TIMP-1 [Roeb et al., 1994] was used (Fig. 3b, lane 7). Lane 6 indicates molecular weight standards. Coomassie Blue staining indicated that the reverse zymogram bands were not the result of TIMP-1 staining itself. The results demonstrate that mTIMP-1 cDNA transfected into HepG2 cells is functionally active and inhibits gelatinase activity.

Inhibition of Migration of TIMP-1 Transfected HepG2 Cells

To work out the functional role of TIMP-1 we observed the influence of endogenous and exogenous TIMP-1 on migration behaviour of HepG2 cells (Fig. 4). Cells were transiently transfected with an expression vector coding for mTIMP-1 (Fig. 4a) and the empty expression vector pCDM8 (Fig. 4b). The migration behaviour of transfected cells was assessed 48 h after wounding a confluent monolayer on a plastic surface of a petridish by scraping away one half of cells along a straight line. Cells expressing and secreting TIMP-1 showed suppressed migration and kept in contact with each other (Fig. 4a) whereas control cells readily migrated into the empty space as single cells (Fig. 4b).

When non-transfected HepG2 cells were incubated for 48 h with recombinant human TIMP-1 (Fig. 4d) or with the supernatant (DMEM: supernatant = 2:1) of HepG2 cells transiently transfected with mTIMP-1 cDNA (Fig. 4c), the biologically active TIMP-1 containing supernatant resulted in suppressed migration of HepG2 cells, similar to the transfected HepG2 cells

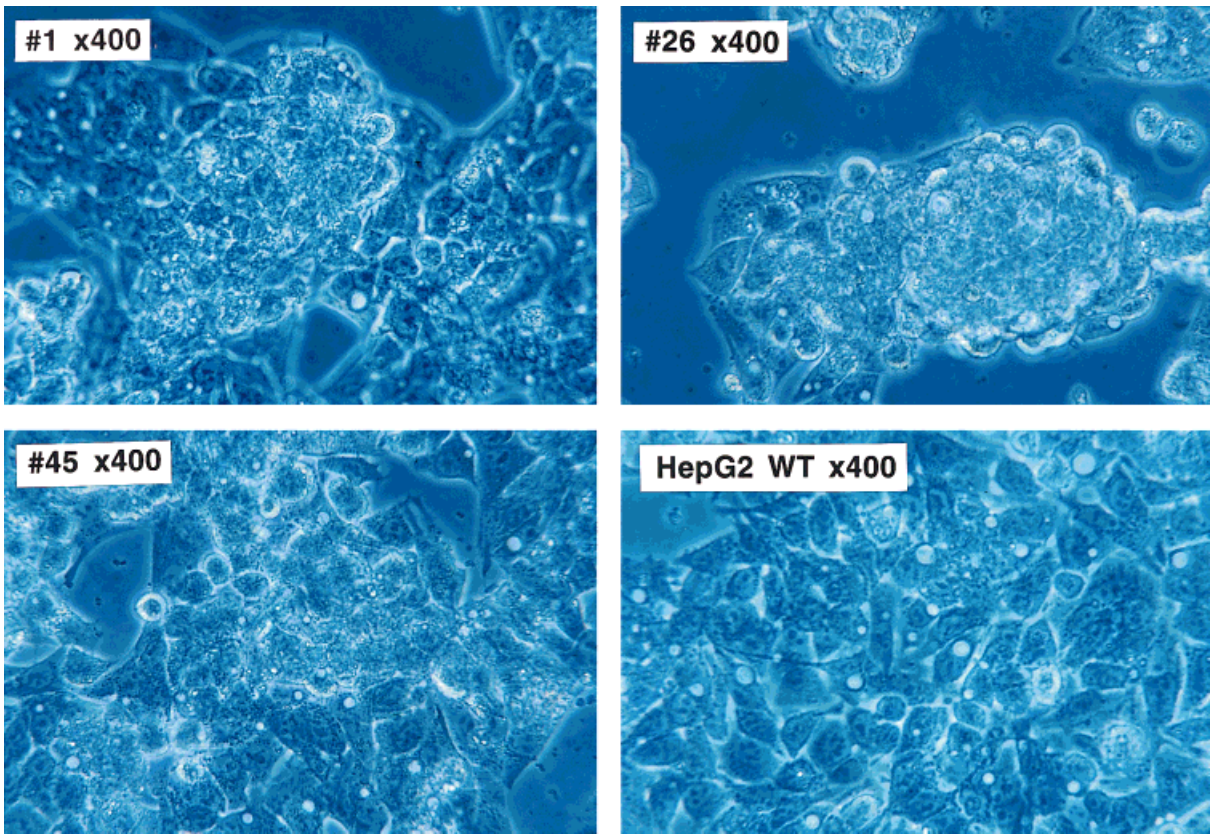

Fig. 2. Morphology of HepG2 cells stably transfected with mTIMP-1. Morphology of Wild-type HepG2 cells and HepG2 cells stably transfected with an expression vector coding for mTIMP-1 (clones #1, #26, #45). Cells expressing and secreting TIMP-1 grow in nests, show suppressed migration and keep in contact with each other. Magnification ×400.

(Fig. 4a). For comparison HepG2 cells incubated with the supernatant from cells transfected with empty expression vector (DMEM: supernatant = 2:1) migrated as single cells (Fig. 4e).

Influence of TIMP-1 on the Expression of MMPs in Transfected HepG2 Cells

In comparison to Wild-type cells, HepG2 cells engineered to express mTIMP-1 at increased levels also expressed higher levels of MMPs 2 and 9. By gelatin zymography, overexpression of MMPs was demonstrated both in HepG2 cells transiently expressing mTIMP-1 and in clone #1 cells (Fig. 5a, lanes 1 and 3). By Western blotting with monoclonal antibodies raised against the hemopexin domain of MMP-2, we could confirm five- to 10-fold higher amounts of 66-kDa MMP-2 in HepG2 cells expressing mTIMP-1 (Fig. 5b). MMP-9 (86 kDa) could also be detected at a significantly higher concentration in the supernatant of clone cells #1, #26, and #45 (Fig. 5c, lanes 2–4) than in the supernatant of HepG2 cells (Fig. 5c, lane 1). For detection, monoclonal antibodies raised against the C-terminal hemopexin domain of MMP-9 were used. Interestingly Wild-type HepG2 as well as TIMP-1 expressing cells appear to express activated forms of MMP-2 (66 kDa) and MMP-9 (86 kDa).

Further analysis showed no difference between Wild-type HepG2 and stably transfected HepG2-mTIMP-1 cells in production of laminin, collagen IV, or acute phase proteins (Haptoglobin, β-Fibrinogen) after stimulation with interleukin-6 (data not shown).

DISCUSSION

In previous reports we have demonstrated that TIMP-1 is synthesized by rat Kupffer cells and rat hepatocytes in primary culture [Roeb et al., 1993; Kordula et al., 1992]. Now we have demonstrated the synthesis and secretion of biologically active mTIMP-1 in transfected hu-

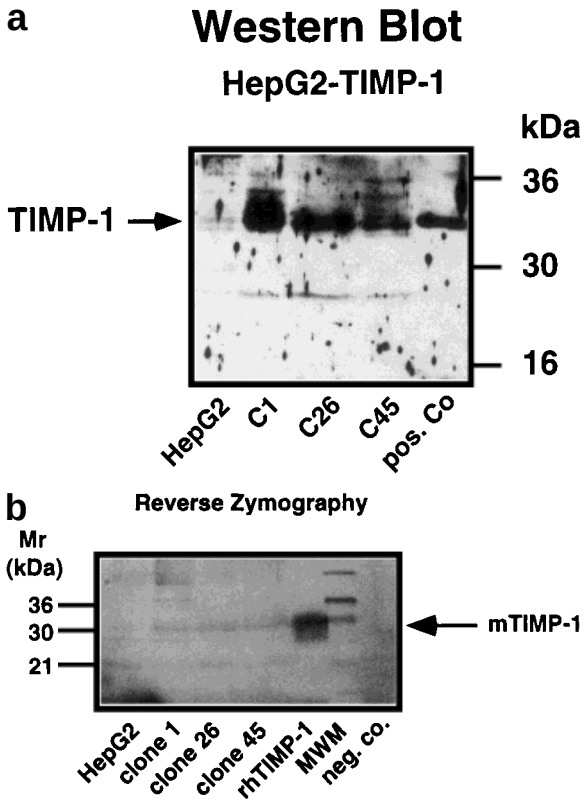

Fig. 3. **a:** Western blot of HepG2 cells stably transfected with mTIMP-1. Western blot of mTIMP-1 from supernatants of HepG2 cell clones stably transfected with an expression vector coding for mTIMP-1 (C1, clone #1; C26, clone #26; C45, clone #45). Lanes 1–4 were charged with 2 μg of total protein each. Recombinant mTIMP-1 (30 ng) was used as positive control (pos. Co). Proteins were separated by SDS-PAGE, blotted and probed with polyclonal antibodies against murine TIMP-1. The arrow indicates the position of TIMP-1 protein. **b:** Inhibitory activity of mTIMP-1 from stably transfected HepG2 cells (reverse zymography). Ten μl of serum-free supernatant from stably transfected HepG2 cells (clones #1, #26, and #45) cultured for 24 h was subjected to reverse zymography. Lane 1 shows the supernatant of HepG2 cells transfected with the empty vector. Thirty ng of recombinant human TIMP-1 served as positive control (lane 5). Lane 6 indicates molecular weight standards. As a negative control (neg. co.) the supernatant of COS-7 cells transfected with the empty expression vector was used (lane 7).

man hepatoma cells (Fig. 1). The human hepatoma cell line HepG2 used for our experiments expresses low endogenous amounts of human TIMP-1 [Kordula et al., 1992]. We have chosen mouse TIMP-1 for transfection into human cell lines in order to distinguish between transfected and endogenous TIMP-1. For TIMP-1 detection we used a polyclonal antibody against mTIMP-1 [Roeb et al., 1994] that does not cross react with human TIMP-1. Successful transfection of HepG2 cells with mTIMP-1 was demonstrated by Northern blotting, Western blotting, and reverse zymography.

Synthesis and secretion of degradative enzymes and the inhibitors controlling them are involved in the turnover of extracellular matrix [Knowlden et al., 1995]. In a model for MMP and TIMP regulation adopted from Overall [1994] growth factors and other mediators differentially modulate MMP, TIMP, and ECM protein expression both spatially and temporally. Normal connective tissue is characterized by a low level of ECM proteins, TIMP expression, and MMP-2 expression. At a remodeling connective tissue site a net resorptive cell phenotype may be characterized by increased MMP and reduced TIMP expression. Normal tissue is likely protected from MMP activity by cells that exhibit an upregulation of TIMP and matrix protein synthesis to both reconstitute local tissue and to buffer adjacent normal tissue from inappropriate MMP activity diffusing from the focus of degradation [Overall, 1994]. The integrity of connective tissue is determined by the balance of resorption and repair of their ECM. The activity of metalloproteinases is rate-limiting for the degradation [Matrisian, 1990]. Metalloproteinases are regulated at many points. Once they are activated the major point of control lies in the activated forms of their specific inhibitors TIMP-1, -2, -3, and TIMP-4 [Murphy et al., 1994]. As a secreted protein TIMP will reduce the activity of MMPs in the extracellular environment. Thus any induction of TIMP expression is likely to lower the level of MMP activity and reduce degradation of macromolecular components in the ECM.

In our experiments TIMP-1 secreting hepatoma cells showed suppressed migration and kept in contact with each other. This phenomenon seems to be independent of the kind of cells because HepG2 and COS-7 cells behaved similarly (data for COS-7 cells were not shown). Previously Walther and Denhardt [1996] demonstrated that for mouse melanoma B16F10 cells endogenously generated TIMP-1 was an effective inhibitor not only of matrix invasion and tumorigenicity but also of cell motility on plastic. Since migration seems to depend on the ability to pass through an extracellular matrix, requiring matrix breakdown by MMPs, overproduction of TIMP inhibits MMPs, thus inducing a possible matrix accumulation and therefore enhancing cell attachment and reducing cell migration. The possibility that different migra-

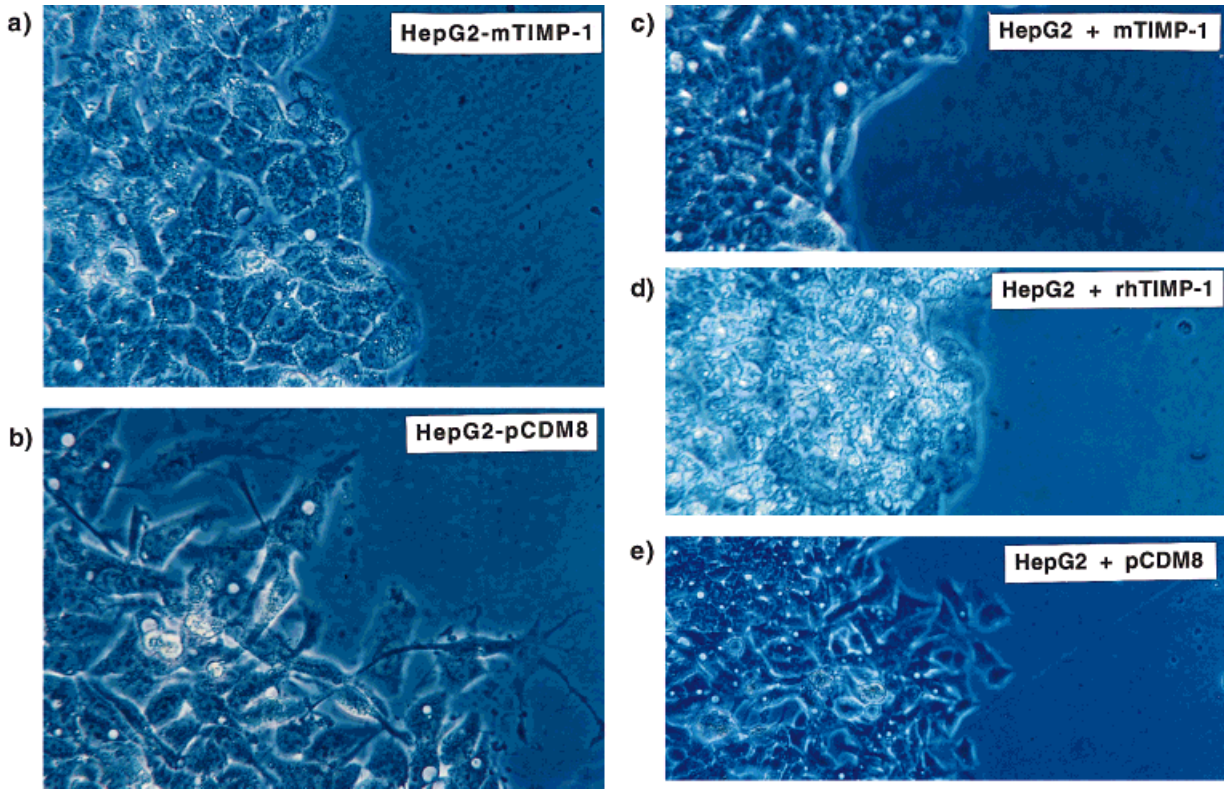

Fig. 4. Influence of endogenous (left side) and exogenous (right side) biologically active TIMP-1 on the migration of HepG2 cells. **a:** Migration of HepG2 cells transfected with the cDNA coding for mTIMP-1 48 h after wounding a confluent monolayer on a plastic surface by scraping away one half of cells along a straight line. One typical migration pattern out of three experiments is shown. Magnification ×400. **b:** Migration of HepG2 cells transfected with the empty expression vector (pCDM8) 48 h after wounding a confluent monolayer on a plastic surface by scraping away one half of cells along a straight line. One typical migration pattern out of three experiments is shown. Magnification ×400. Migration of non-transfected HepG2 cells that were incubated for 48 h with a TIMP-1 bearing supernatant (c,d) resulted in suppressed migration of HepG2 cells, similar as in transfected HepG2 cells (see a). One typical migration pattern out of three experiments is shown. Magnification ×400. **c:** HepG2 cells incubated with the supernatant (DMEM:supernatant = 2:1) from HepG2 cells transiently transfected with mTIMP-1 cDNA. **d:** HepG2 cells incubated with with recombinant human TIMP-1 (1 ng/ml). **e:** HepG2 cells incubated with the supernatant (DMEM:supernatant = 2:1) from cells transfected with empty expression vector pCDM8.

tion and adhesion patterns are due to some unspecific transfection effect could be excluded by addition of recombinant TIMP-1 and TIMP-1 from supernatants of transfected cells to the medium of HepG2 cells. These cells yielded a similar migratory phenotype like transiently TIMP-1 transfected cells. Another observation is also supporting the striking influence of TIMP-1 on cells and their ECM: Close contact and a straight borderline includes all cells on the plate although the efficiency of transient transfection is only 10 to 50%. It has been emphasized that the regulation of MMP and TIMP at the single cell level may not be necessarily reflected at the tissue level [Overall, 1994]. The tissue MMP and TIMP levels are the net result of cell responses at many sites in the tissue, often from mixed cell populations in which not all cells will respond in an identical manner to the same combinations and concentration of growth factors.

We were not able to detect quantitative changes in ECM molecules like fibronectin, laminin, or collagen IV in human hepatoma cells with overexpression of TIMP-1, MMP-2, and MMP-9. So it remains speculative whether reduced migration in our model depends on more connections of cells with their surroundings.

There are a few reports dealing with the enzymatic mechanisms of cell migration. During migration cells have to break down connections and build up new connections with their surroundings. MMPs are important for breaking connections and these MMPs might be inhibited by TIMPs with the effect of reduced migration [Stossel, 1993]. It has been reported that the catalytic activity of MMP-1 is necessary for

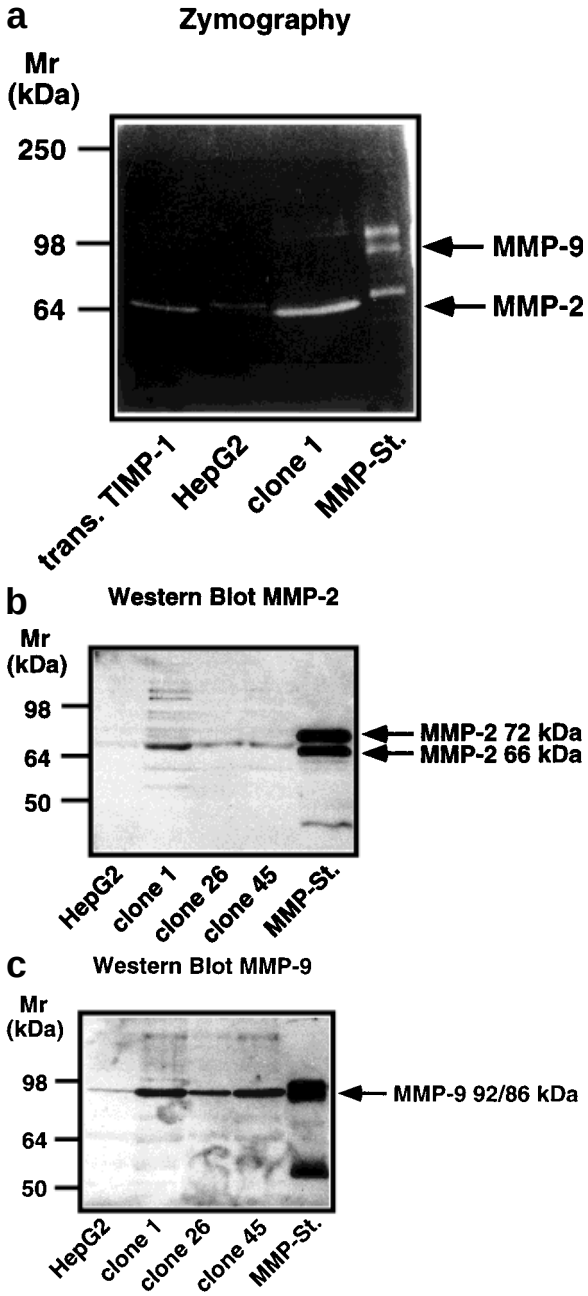

Fig. 5. Influence of endogenous TIMP-1 on the expression of MMPs in stably transfected HepG2 cells. **a:** Zymogram of serum-free supernatants (10 µl) from HepG2 cells (2×10^6). Lane 1 (trans. TIMP-1): supernatant from HepG2 cells transiently transfected with an expression vector coding for mTIMP-1. Lane 2 (HepG2): supernatant from Wild-type HepG2 cells. Lane 3 (clone #1): supernatant from HepG2 cells stably transfected with mTIMP-1. Lanes 1–3 were charged with equal amounts of total protein each. Lane 4 (MMP-St.) MMP standard. Molecular weight markers are indicated in the figure. **b,c:** Western blot analysis of MMP-2 (b) and MMP-9 (c) present in the supernatants of HepG2 cells and clone cells by monoclonal antibodies. Lane 1 (HepG2): serum-free supernatant from Wild-type HepG2 cells. Lanes 2, 3, and 4 (clones #1, #26, and #45): serum-free supernatants from HepG2 cells stably transfected with mTIMP-1. Lanes 1–4 were charged with 2 µg of total protein each. Lane 5 (MMP-St.) MMP standard. Molecular weight markers are indicated in the figure.

keratinocyte migration and that migration was completely blocked by TIMP-1 and by anti-MMP-1 antiserum [Pilcher et. al., 1997]. Glioma cell migration however was modulated by MMP-2 activation [Deryugina et al., 1997]. Inhibition of endothelial cell migration by recombinant TIMP-3 has also been described [Anand-Apte et al., 1996]. Since TIMP-3 is not expressed in total liver or in HepG2 cells [Roeb et al., 1997] TIMP-3 cannot be responsible for depressed migration in our experiments. During human intrahepatic bile duct development MMP might play a critical role in biliary cell migration by degrading ECM proteins [Terada et al., 1995]. The authors also demonstrated that MMP inhibitors (TIMP-1 and TIMP-2) and MMP activators (trypsin, chymotrypsin, and cathepsin B) play an important role in biliary cell migration. Recent studies showed that gelatinase B is a major factor of polymorphnuclear neutrophil migration across basement membrane and that elastase may contribute to this process by activating pro-gelatinase B [Delclaux et al., 1996]. Overall both MMPs and TIMPs have been associated with cell and tumor cell migration.

To our knowledge this is the first report documenting increased expression of gelatinases (MMP-2 and MMP-9) as a result of increased expression of TIMP-1. Moreover, the newly synthesized MMPs appeared to be at least partially activated (Fig. 5). Consequently the net effect of increased TIMP-1 expression in our model might be smaller than had been assumed. This novel phenomenon might be of importance in ECM homeostasis - maybe a kind of self regulation. However, the intracellular mechanisms remain obscure. Further studies will be required to elucidate the molecular background. In vivo, injury to a peripheral nerve is followed by a remodeling process with axonal degeneration and regeneration paralleled by an upregulation of TIMP-1 and gelatinase B mRNAs [La Fleur et al., 1996]. In that case TIMP-1 may protect basement membrane from uncontrolled degradation after injury. In our case of TIMP-1 overexpression its the other way round. MMPs may protect cells from excessive deposition of extracellular matrix.

We have documented a role for TIMP-1 in liver cell adhesion and liver cell contact. There is increasing evidence that matrix accumulation in liver fibrosis is a dynamic pathologic process in which altered matrix degradation plays a significant role (Arthur, 1994). In kid-

ney, increased TIMP-1 mRNA levels altering matrix degradation were already associated with inflammation and fibrosis [Eddy and Giachelli, 1995]. In scleroderma (systemic sclerosis: SSc) excessive extracellular matrix deposited in skin and internal organs has also been connected with high TIMP-1 synthesis. Cells from these patients produce less stromelysin suggesting that alterations in the regulation of matrix enzymes and their inhibitors may play an important role in the molecular pathology of SSc [Bou-Gharios et al., 1991]. High TIMP activities should inhibit matrix metalloproteinases—demonstrable by reverse zymography (Iredale et al., 1992; Kurschat et al., 1995; Yoneda et al., 1997]—resulting in matrix accumulation by impairment of matrix degradation. However, it remains speculative whether TIMP-1 is the functional promotor of changes in matrix homeostasis like fibrosis. HepG2 cells stably expressing mTIMP-1 might provide an interesting model for the study of TIMP-1 in liver cells.

There are reports that TIMP-1 can inhibit in vitro invasion and in vivo metastasis, for example in melanoma cells [Stetler-Stevenson et al., 1993b]. In metastasis an excess of protease activity results in uncontrolled matrix degradation and disruption of the cell-matrix interaction required for migration or invasion. Alteration of the MMPs/TIMPs balance in tumor cells results in increased matrix deposition that limits local invasion [De Clerck, 1996]. Since increased MMP activity could facilitate tumor metastasis by breaking down ECM barriers, TIMP might inhibit tumor development [Khokha et al., 1989; Alexander and Werb, 1992]. Recent studies showed that the recombinant TIMP-1 and TIMP-2 inhibited bone resorption and osteolytic bone metastasis of breast cancer [Yoneda et al., 1997]. Tumor invasion and metastasis are major obstacles for successful cancer therapy. A well-known model system for tumor invasion [Stetler-Stevenson et al., 1993a] consists of three steps: tumor cell adhesion, ECM proteolysis, and tumor cell migration. In conclusion our results have provided new insights in the process of migration by the effects of TIMP-1 overexpression. These results underline a functional involvement of TIMP-1 in matrix homeostasis and indicate a kind of automatic control in matrix turnover.

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