

TIMP-1 mediates the inhibitory effect of interleukin-6 on the proliferation of a hepatocarcinoma cell line in a STAT3-dependent manner

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Abstract

The tissue inhibitor of metalloproteinases (TIMP)-1 is a multifunctional protein which is not only an inhibitor of matrix metalloproteinases (MMPs) but also to have a possible "cytokine-like" action. Here, we first compared mRNA expression of TIMP-1 and MMP-9 in BEL-7402 (a hepatocellular carcinoma cell line), L-02 (a normal liver cell line) and QSG-7701 (a cell line derived from peripheral tissue of liver carcinoma) using real-time quantitative RT-PCR. By evaluating the variation of the MMP-9/TIMP-1 ratio as an index of reciprocal changes of the expression of the two genes, we observed that the MMP-9/TIMP-1 ratio was about 13- and 5-fold higher in BEL-7402 than in L-02 and QSG-7701, respectively. Significantly, overexpression of TIMP-1 decreased the MMP-9/TIMP-1 ratio in BEL-7402 and then inhibited the cell growth to 60% and reduced the migration to about 30%. Meanwhile, our data showed that interleukin-6 (IL-6) (100 ng/mL) could also inhibited the cell growth of BEL-7402. Further studies indicated that TIMP-1 mediated the inhibitory effect of IL-6 on BEL-7402 cell proliferation in a STAT3-dependent manner, which could further accelerate the expression of the cyclin-dependent kinase inhibitor p21. A dominant negative STAT3 mutant totally abolished IL-6-induced TIMP-1 expression and its biological functions. The present results demonstrate that TIMP-1 may be one of the mediators that regulate the inhibitory effect of IL-6 on BEL-7402 proliferation in which STAT3 signal transduction and p21 up-regulation also play important roles.

Key words

- Tissue inhibitor of metalloproteinases-1
- Hepatocellular carcinoma
- Interleukin-6
- STAT3
- p21

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Research supported by the Natural
Science Fund of Jiangsu Province
(No. 2000SWX000B501).

Received April 24, 2006
Accepted January 19, 2007

Introduction

Four tissue inhibitors of metalloproteinases (TIMP) have been identified; TIMP-1 has been shown to be particularly interesting. In addition to its classical role as a broad specific inhibitor of matrix metalloprotein-

ases (MMP) (1), TIMP-1 has also been reported to possess growth factor-like (2) and anti-apoptotic properties (3-5). TIMP-1 expression can be stimulated by a wide variety of agents including serum, growth factors, phorbol esters, cytokines, and viruses (6-8). It has been reported that the activation of

hepatic stellate cells (HSCs), a key event in the pathophysiology of liver fibrosis (9), is also accompanied by induction of TIMP-1 activity and the expression of its mRNA (10, 11). It was shown that transgenic TIMP-1 inhibits simian virus 40 T antigen-induced hepatocarcinogenesis by impairing hepatocellular proliferation and tumor angiogenesis (12). All of these studies suggested that TIMP-1 might be involved in the process of liver pathogenesis.

The pleiotropic cytokine interleukin-6 (IL-6) elicits a wide variety of biological activities in different cell types, including hematopoietic and neuronal cells, keratinocytes, osteoclasts, and vascular endothelial cells. IL-6 also modulates the hepatic expression of acute-phase response genes during inflammation (13). When HSCs are activated, they secrete and respond to a wide range of cytokines and growth factors including IL-6. Furthermore, activated HSCs also secrete and deposit most of the excess extracellular matrix (ECM) in the fibrotic liver through increased secretion of ECM proteins, including TIMP-1 and TIMP-2 (14). IL-6 has also been identified as a key protective factor from stress. During hyperoxic lung injury, IL-6 can protect lung cells from apoptosis by inducing the expression of Bcl-2 and TIMP-1 (15).

In the present study, we examined the functional involvement of IL-6 and TIMP-1 during liver carcinogenesis. Using a liver carcinoma cell line, we found that IL-6 was able to increase TIMP-1, that IL-6 and TIMP-1 were able to inhibit cell proliferation, and that IL-6 induction of TIMP-1 and p21 expression was STAT3 dependent.

Material and Methods

Antibodies and cytokine

The recombinant human IL-6 was obtained from Merck Inc. (Whitehouse Station, NJ, USA). Rabbit polyclonal antibodies for TIMP-1 and β -tubulin were from Santa

Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). All other chemicals were from Amersco Inc. (Kaysville, UT, USA).

Plasmids and adenovirus

Dr. David Young (Department of Cell Biology, Harvard Medical School, Boston, MA, USA) kindly provided the reporter plasmid pGL3-TIMP-1, in which the promoter region of human TIMP-1, spanning a fragment from -1714 to +17 bp was inserted before a luciferase reporter gene. The plasmids pcDNA3-TIMP-1 and pEGFP-TIMP-1 were constructed in our laboratory. The plasmid p21(Waf1) luciferase and the control reporter plasmid pSV- β -galactosidase were gifts of Dr. Zhi-Ming Yin (Life Sciences College, Nanjing Normal University, Nanjing, China). The cDNA encoding STAT3 and dominant negative STAT3 were gifts from Dr. Xin-Yuan Fu (Department of Pathology, Yale University School of Medicine, New Haven, CT, USA) and were subcloned into expression vector pcDNA3.0 (pcDNA-STAT3wt and pcDNA-STAT3cyf). STAT3cyf is a mutation of Stat3 tyrosine 705 to phenylalanine (Y705F) which causes a reduction of the tyrosine phosphorylation of wild-type STAT3 and inhibits the action of endogenous STAT3 in transfected cells (16). Dr. Xin-Yuan Fu also provided the M67 reporter plasmid in which STAT3 DNA-binding sites were inserted before a luciferase reporter gene.

Adenoviral vectors expressing human TIMP-1 were generated in our laboratory using the method described by He et al. (17). Briefly, the cDNA encoding full-length human TIMP-1 was cloned into the shuttle vector, pAdTrack-CMV, which contains a GFP expression cassette driven by a separate cytomegalovirus (CMV) promoter. pAdTrack CMV-TIMP-1 was recombined with backbone pAdEasy-1 in BJ5183 bacteria. Linearized recombinant plasmid DNA was then transfected into 293 cells, an adenoviral packaging cell line, to generate the recombinant adenovi-

rus expressing TIMP-1 and GFP (Ad1-TIMP-1). We used a GFP-expressing recombinant adenovirus (Ad1) as a parallel empty control.

The adenoviral backbone vector and shuttle vector were kindly provided by Dr. Tong-Chuan He (The Howard Hughes Medical Institute, Baltimore, MD, USA) (17). The simplified system described by He et al. (17) was used for adenovirus generation and amplification and titer determination. Viral particles were purified by cesium chloride density gradient centrifugation.

Recombinant adenovirus infection

Adenoviruses were incubated with cells in a small volume of serum-free medium at 37°C. After adsorption for 2 h, fresh complete growth medium was added and the cells were further incubated for the following experiments.

Cell culture and transfection

The BEL-7402, QSG-7701 and L-02 cell lines were purchased from the Shanghai Institute of Cell Biology, China, and were grown in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) supplemented with 10% NCS (HyClone, Hampton, NH, USA), 2 mM L-glutamine, 20 U/mL penicillin, and 20 mg/mL streptomycin. The cultures were kept in a 5% CO₂ and 95% air humidified incubator at 37°C.

Transfections were performed using the calcium phosphate method. All promoter-luciferase fusions were co-transfected with the pSV- β -galactosidase plasmid to estimate transfection efficiency.

Cell growth assay

Subconfluent monolayer cells were trypsinized and plated onto a 6-well plate at a density of 10⁴ cells per well. After overnight post-plating, the cells were either treated with IL-6 (100 ng/mL), or infected with

Ad1-TIMP-1. The cell numbers were counted with a hemocytometer after trypsinization at different time points.

Real-time quantitative RT-PCR

Total RNA was isolated from cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the protocol of the manufacturer. Total RNA (2 μ g) was used for a reverse transcriptional reaction (Amersham Biosciences, Piscataway, NJ, USA). The primers were synthesized to amplify specific segments of the cDNA sequences of human TIMP-1, MMP-9, and β -actin; the β -actin was used as an internal control. The primer sequences for each gene were as follows: TIMP-1 forward primer: 5' TTC CGA CCT CGT CAT CAG GG 3'; TIMP-1 reward primer: 5' ATT CAG GCT ATC TGG GAC CGC 3', MMP-9 forward primer: 5' CCT GGA GAC CTG AGA ACC AAT C 3'; MMP-9 reward primer: 5' GAT TTC GAC TCT CCA CGC ATC 3', actin forward primer: 5' TCC TGT GGC ATC CAC GAA ACT 3'; actin reward primer: 5' GAA GCA TTT GCG GTG GAC GAT 3'.

The qPCR assays were performed with the MyiQ SyBr Green Supermix system (BioRad, Hercules, CA, USA) and 300 nM sense and antisense primers. The cycling conditions were as follows: initial denaturation at 95°C for 2 min, followed by 40 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The expression level of each mRNA was determined relative to the standard curve using the LightCycler computer software (Roche Diagnostics, Castle Hill, NSW, Australia). The specificity of the SyBr Green assays was confirmed by melting point analysis and gel electrophoresis. The gene expression of β -actin was used for normalization.

Western blotting analysis

Cells were washed and lysed in lysis buffer (20 mM Tris-HCl, pH 8.0, 75 mM NaCl, 1.5

mM MgCl₂, 1 mM EGTA, 1.0% NP-40, 1 mM PMSF, 10 µg/mL leupeptin, and 10 mg/mL aprotinin). Protein concentrations were determined by Bradford assays. Samples of the extract containing equal amounts of total protein were submitted to 10% (w/v) SDS-PAGE and transferred to the PVDF membrane, which was blocked with 3% BSA-blocking buffer and then incubated with polyclonal anti-TIMP-1 antibody diluted 1:500. The membrane was then incubated with the peroxidase-conjugated secondary antibody for 40 min. The blotting patterns were developed using the ECL system (Roche, Mannheim, Germany). β-tubulin was blotted as a control of protein loading.

ELISA

The secreted TIMP-1 protein in culture medium was examined by indirect ELISA. Briefly, the wells of polystyrene microtiter plates (Greiner Bio-One, Maybachstrasse, Frickenhausen, Deutschland) were coated with 150-µL culture medium after 72 h of infection with adenovirus. After overnight incubation at 4°C, the wells were washed three times with 0.1 M PBS containing 0.05% Tween 20, pH 7.4. The coated wells were blocked with 200 µL 2% BSA for 1 h at 37°C and then incubated with 150 µL polyclonal antibody anti-TIMP-1 diluted 1:1000. After incubation for 2 h at 37°C, the wells were washed as before and then incubated with 150 µL of horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5000 dilution, Sigma, St. Louis, MO, USA) for 1 h at 37°C. After washing, *o*-phenylenediamine (Shanghai Chemicals, Shanghai, China) was used as the substrate. The reaction was stopped after 30 min with 50 µL 2 M H₂SO₄ and absorbance was measured at 490 nm using an ELx800 Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VE, USA).

Luciferase assay

BEL-7402 cells were transfected with the luciferase reporter construct (0.2 µg/each

well) when cells reached about 60% density on 6-well plates. At 24 h after transfection, cells were further stimulated with exogenous IL-6 (100 ng/mL) or infected with Ad1-TIMP-1. Luciferase activities in cell lysates were determined with a luminometer (Lumat LB 9507, Berthold, Germany) 48 h after transfection, using a luciferase assay kit (Promega, WI, USA). Luciferase activity was normalized to β-galactosidase activity to correct transfection efficiency. Each experiment was performed in triplicate and was repeated at least three times. P values ≤5% were considered to be significant.

Single-cell migration assay

After pEGFP-TIMP-1 transfection, cells were plated onto a grid glass coverslip at low-cell density. In order to trace the movement of the cells, randomly selected fields were photographed every hour with a Leica digital camera. pEGFP-TIMP-1-expressing cells were distinguished under a fluorescence microscope and photographed with a Spot Cool CCD (Diagnostic Instruments, Sterling Heights, MI, USA). All pictures were merged together using the Photoshop software. The migration distance was compared between the “Green” cells (TIMP-1-expressing cells) and normal cells.

Motility assay

The wounding assay was carried out according to the method described by Sato and Rifkin (18). Briefly, BEL-7402 cells were grown to confluence on 24-well plates in the presence of Ad1-TIMP-1 adenovirus, Ad1 control adenovirus, or mock control. Wounds were made in the monolayer by scraping it with a disposable pipette tip to remove a channel of cells. The cells were washed immediately with PBS and then cultured for 24 h. The distance from the wound margin to the leading edge of the migrating BEL-7402 cells was determined. For each treatment,

eight measurements were made at identical marked regions.

Statistical analysis

All experiments were repeated at least three times. Data are reported as means \pm standard deviation. Statistical differences between the two groups were evaluated using the paired Student *t*-test (Statistica, Statsoft Inc., Tulsa, OK, USA). *P* values less than 0.05 were considered to be significant.

Results

Overexpression of TIMP-1-inhibited cell proliferation of BEL-7402

BEL-7402 is an hepatocellular carcinoma (HCC) cell line extensively used in the

field of hepatoma research in China, while L-02 is a normal liver cell line used as a control and QSG-7701 is a cell line derived from peripheral tissue of liver carcinoma. Their genetic background, in particular regarding TIMP-1 expression, remains largely unknown. The result of real-time quantitative PCR showed that the expression of TIMP-1 tended to be decreased in BEL-7402 cells compared to normal cell lines, whereas the amount of mRNA of MMP-9 presented an inverse trend. In addition, we evaluated the variation of the MMP-9/TIMP-1 ratio as an index of reciprocal changes of the expression of the two genes and observed that the MMP-9/TIMP-1 ratio was significantly higher (about 13- and 5-fold, respectively, $P < 0.001$) in BEL-7402 than in L-02 and QSG-7701, respectively (Figure 1A).

When cells overexpressed TIMP-1 by in-

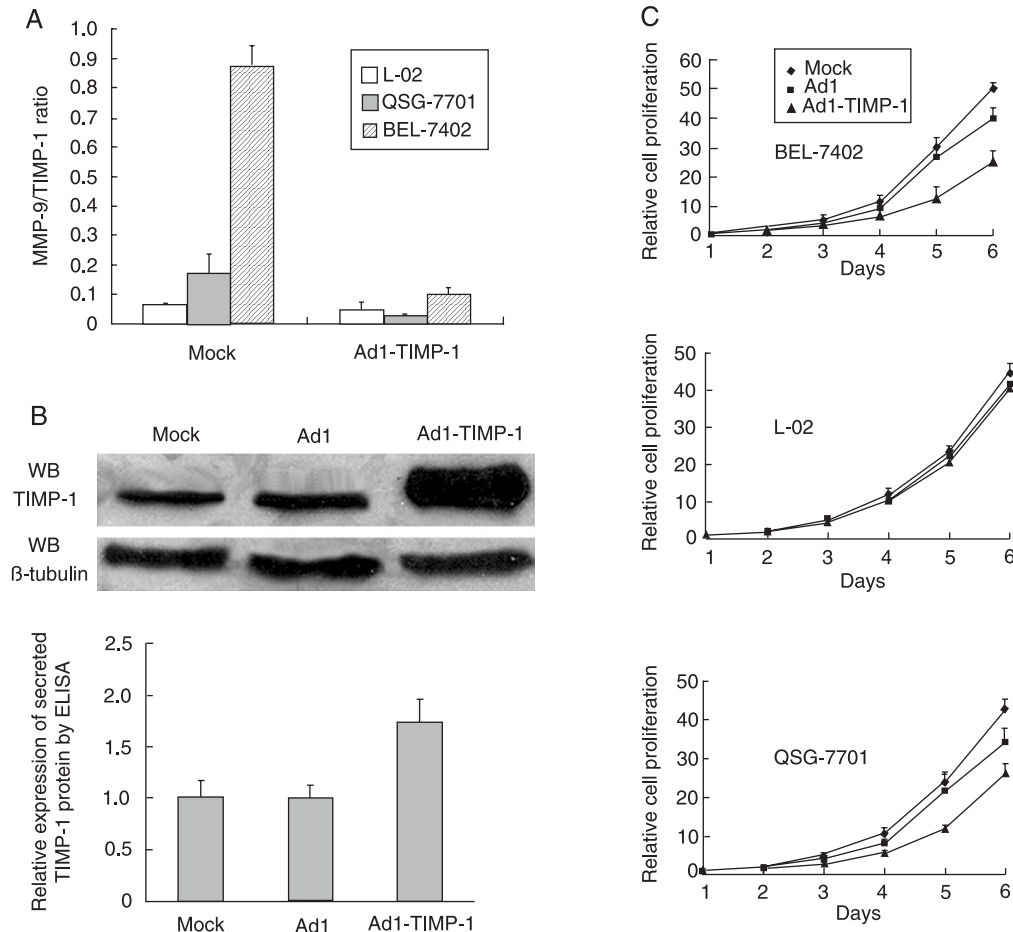


Figure 1. Overexpression of tissue inhibitor of metalloproteinases (TIMP-1) could inhibit the proliferation of the hepatocellular carcinoma BEL-7402 cell line. A, Comparison of the MMP-9/TIMP-1 ratio in three different cell lines with recombinant adenovirus carrying TIMP-1 (Ad1-TIMP-1) and without (mock) TIMP-1 overexpression. B, Detection of TIMP-1 overexpression in BEL-7402. Western blotting (WB) analysis was processed with β -tubulin as internal control. The active protein of TIMP-1 secreted in the culture medium was detected by ELISA. The mock here indicated the cells without any treatment which was an internal control. C, Inhibition of BEL-7402, L-02 and QSG-7701 proliferation by TIMP-1. The cells were infected with Ad1-TIMP-1, Ad1 control and mock control. The number of cells was counted daily with a hemocytometer after trypsinization and the growth curves were calculated.

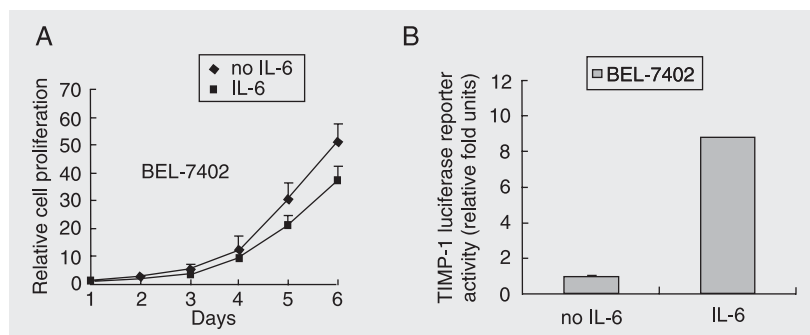


Figure 2. Inhibition by interleukin-6 (IL-6) of the proliferation of the hepatocarcinoma cell line BEL-7402 was accompanied by induced tissue inhibitor of metalloproteinases (TIMP-1) expression. **A**, BEL-7402 cells were collected by trypsinization and resuspended in culture medium after being treated or not with IL-6. The number of cells was counted with a hemocytometer. **B**, Cells were transfected with the TIMP-1 reporter plasmid and treated or not with IL-6. TIMP-1 expression induced by IL-6 was analyzed according to the luciferase activity of the TIMP-1 reporter construct. The β -gal reporter plasmid was co-transfected to normalize the transfection efficiency.

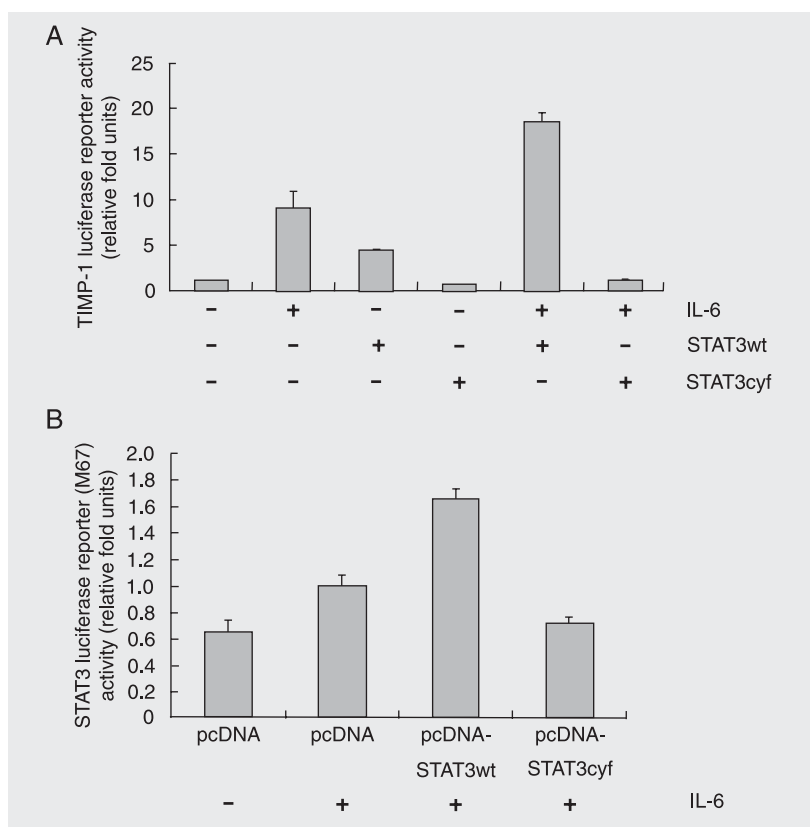


Figure 3. **A**, The induction of tissue inhibitor of metalloproteinases (TIMP-1) by interleukin-6 (IL-6) was STAT3 dependent, as demonstrated by a TIMP-1 luciferase reporter activity assay. Cells were transfected with TIMP-1 reporter plasmids, co-transfected together with the STAT3wt or STAT3cyf constructs. The cells were then treated or not with IL-6. **B**, Functional identification of STAT3wt/cyf protein using a STAT3 reporter (M67) activity assay. Cells were transfected with M67 reporter plasmids, co-transfected together with the pcDNA, pcDNA-STAT3wt or pcDNA-STAT3cyf constructs. The cells were then treated or not with IL-6. Each group was co-transfected with the β -gal reporter plasmid to normalize the transfection efficiency.

fection of recombinant adenovirus carrying the TIMP-1 gene, the MMP-9/TIMP-1 ratio was significantly decreased to almost the same level in BEL-7402, QSG-7701 and L-02 (Figure 1A). Cell growth was inhibited in BEL-7402 and QSG-7701 cell lines (Figure 1C). The number of BEL-7402 cells declined to only about 60% of the number of untreated cells ($P < 0.01$) after 6-day culture by induction of TIMP-1, but L-02 was not observably affected by TIMP-1 overexpression. Interestingly, an obvious growth-inhibitory effect on QSG-7701 ($P < 0.01$) was also observed. The methyl thiazolyl tetrazolium (MTT) assay displayed similar results (data not shown).

IL-6 inhibited the proliferation of the hepatocarcinoma cell line BEL-7402 and induced TIMP-1 expression

Increasing evidence has demonstrated that IL-6 plays an important role in regulating cell proliferation. To investigate the role of IL-6 in the HCC cell line, we cultured BEL-7402 cells in a medium containing IL-6 (100 ng/mL) and found that cell proliferation was inhibited. After 6 days of culture, cell numbers decreased to only about 75% of untreated cells ($P < 0.05$; Figure 2A). This was also confirmed by the MTT assay (data not shown). The results also showed that IL-6 only began to have visible effects after 4 days of treatment, which suggests that other factors induced by IL-6 inhibited the proliferation of these cells.

To address whether the inhibitory effect of IL-6 on BEL-7402 was accompanied by induction of TIMP-1, we checked the activity of luciferase that was under the control of the TIMP-1 promoter. The data suggested that IL-6 was able to induce TIMP-1 expression. The luciferase activity increased about 9-fold after IL-6 treatment (Figure 2B).

IL-6 induction of TIMP-1 was STAT3 dependent

Two functional binding sites for activa-

tor protein-1 and STAT which responded to IL-6/oncostatin have been identified in the TIMP-1 promoter M (19). Here we examined whether STAT3 signaling was involved in TIMP-1 production induced by IL-6 in BEL-7402. As shown in Figure 3, the TIMP-1 reporter luciferase activity of BEL-7402 cells transfected with wild-type STAT3 expression vector was up-regulated about 4.4-fold compared to control cells, and IL-6 treatment elevated TIMP-1 expression up to 18-fold ($P < 0.01$) in these cells. The dominant negative form of STAT3 totally inhibited the expression of TIMP-1 both in IL-6-treated and -untreated cells. Thus, we found that the activation of TIMP-1 gene expression by IL-6 in BEL-7402 cells was dependent on STAT3 signaling.

p21 was up-regulated by TIMP-1 and IL-6

To elucidate the mechanism of IL-6-induced inhibition of cell proliferation in BEL-7402, we checked the p21(Waf1) expression level using a luciferase reporter assay. IL-6 enhanced p21 expression about 3.3-fold compared to control cells, in which the transient overexpression of STAT3 augmented the luciferase reporter activity. When the cells

were transfected with STAT3wt and treated with IL-6, they displayed up to 6.7 times more p21 expression than control cells. Moreover, dominant negative STAT3 blocked the stimulation of IL-6 to control levels (Figure 4A).

TIMP-1 itself was also able to accelerate p21 expression. The transfection of TIMP-1 resulted in a 5-fold higher expression of p21 than the vehicle vector transfection control and blank control (Figure 4B).

TIMP-1 inhibited the migration of BEL-7402

Both the single-cell migration detection assay and wounding assay showed that cells overexpressing TIMP-1 migrated much slower than control cells did. In Figure 5A, a, b, c, d indicate the cell center at successive time points. The migration route of each cell can be considered to be $a \rightarrow b \rightarrow c \rightarrow d$ approximately, the average motion distance of the transfected cells (green cells) was only about 33% compared to the normal cells (Figure 5B).

The mobility of cell groups was detected by the wounding assay. Confluent monolayers of three groups of BEL-7402 cells were wounded and then cultured for 24 h. The

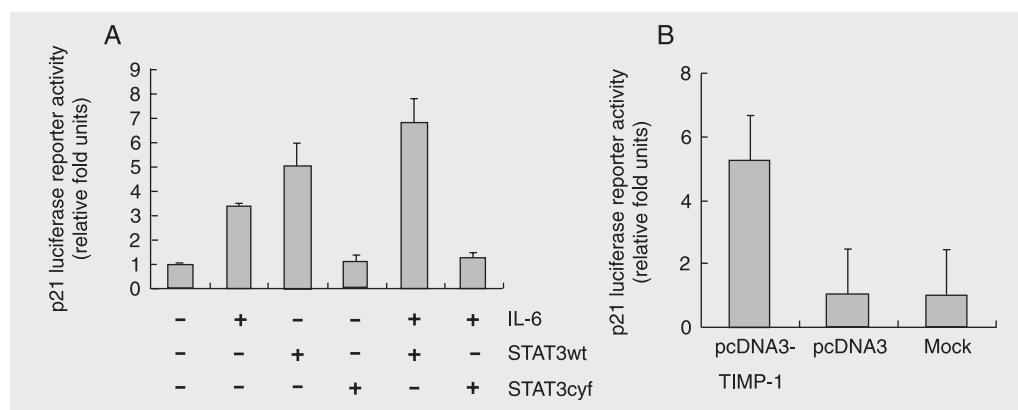


Figure 4. Up-regulation of p21 induced by tissue inhibitor of metalloproteinases (TIMP-1) and interleukin-6 (IL-6) in BEL-7402 cells analyzed by the luciferase assay. *A*, Cells were transfected with p21(Waf1) reporter, co-transfected together with pcDNA3-STAT3wt or pcDNA3-STAT3cyf constructs. The cells were then treated or not with IL-6. *B*, Cells were transfected with p21(Waf1) reporter, co-transfected together with pcDNA3-TIMP-1 or pcDNA3 as an empty parental vector control. Mock was a blank control representing the cells transfected by nothing. Each group was co-transfected with the β -gal reporter plasmid to normalize the transfection efficiency.

cells were photographed and the migration distance was determined (Figure 5C,D). The data showed that the migration rates of the Ad1-TIMP-1 group and the Ad1 group were 28 and 98%, respectively, of the migration of the mock group. Both experiments indicated that TIMP-1 inhibited the migration of BEL-7402 cells.

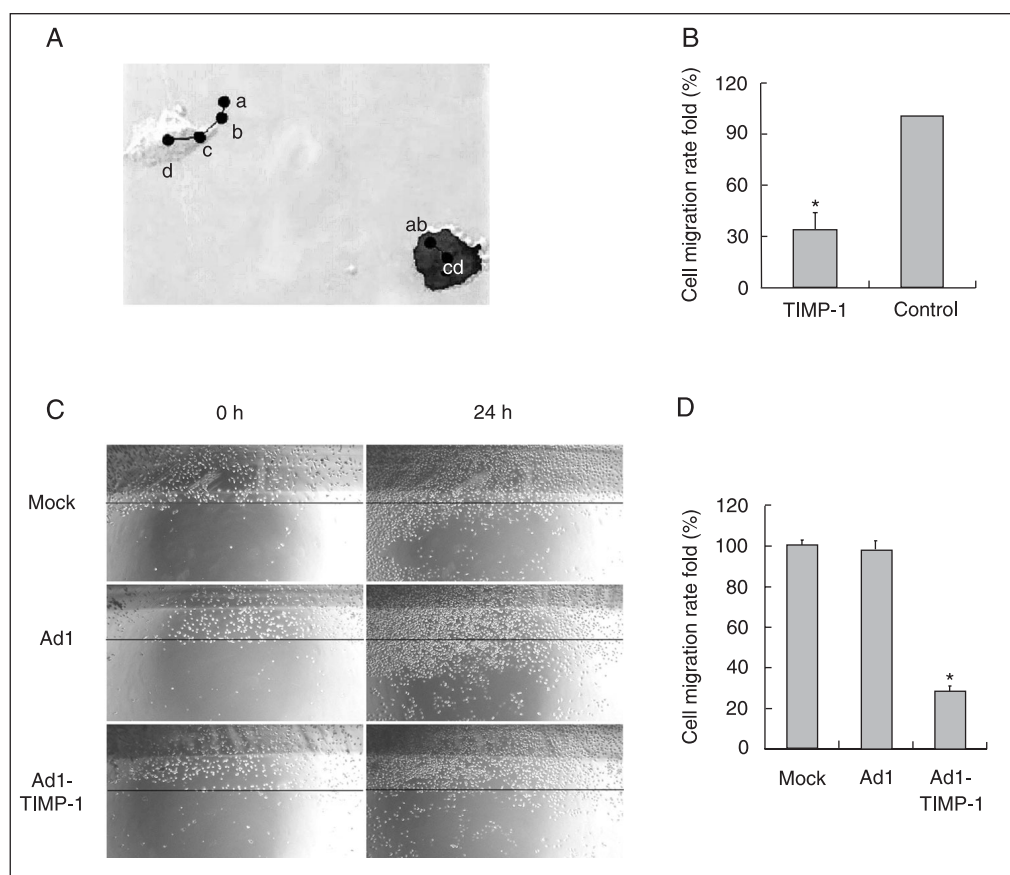
Discussion

The ECM forms a microenvironment that can modulate cellular behavior by affecting the contact of the cell with the outside world (20,21). Degradation or activation of ECM proteins by proteolysis can change the cellular microenvironment rapidly and irreversibly. The turnover of ECM is delicately regulated by a pair of contrary functional molecules: MMP and its tissue inhibitor (TIMP).

MMP produces subtle changes of matrix structure by cleaving and also releasing matrix-bound growth factors and thereby controlling differentiation.

MMP-9 has been reported to play an important role in the invasion and metastasis of cancers (22). A previous report showed that the MMP-9/TIMP-1 mRNA ratio differed between the stage II-III group and the stage IV group of lung cancer (23). Imbalance of the ratio between MMP-9 and TIMP-1 may cause breakdown of the basement membrane and ECM and is an essential step in tumor invasion and metastasis (24). In the present study, BEL-7402 originated from a Chinese patient with HCC which exhibited a high metastasis rate. Real-time PCR data showed that the MMP-9/TIMP-1 ratio was noticeably higher in BEL-7402 than in the normal liver cell line L-02 and QSG-7701,

Figure 5. Inhibitory effect of tissue inhibitor of metalloproteinases (TIMP-1) on the migration of BEL-7402 cells. **A**, The centers of cells monitored every hour are shown by a, b, c, d. The cell on the right overexpressed TIMP-1. **B**, The relative average velocity of single cell migration was calculated for TIMP-1-transfected and control cells. **C**, Ability to migrate affected by TIMP-1 using a wounding assay. Confluent monolayers of three groups of BEL-7402 cells were scraped using a tip and then cultured for 24 h. The cells were photographed and the migration distance was determined. The bee-line indicated the same location. **D**, The migration distance of three groups was determined. * $P < 0.01$ for 7402/Ad1-TIMP-1 compared with the 7402/Ad1 group (Student *t*-test).



which may be one explanation for the high invasive ability of BEL-7402 compared to the other two lines.

TIMP-1 is a multifunctional protein with MMP-dependent and -independent actions for the regulation of cell death, cell proliferation, and angiogenesis (25). After TIMP-1 was initially characterized as a homologue of erythroid potentiating activity factor, it was found to display growth- and survival-promoting activity for a wide range of normal or transformed cells (26,27). Increasing evidence demonstrates a much more complex role for TIMP-1 during tumor progression and angiogenesis, in addition to its regulation of MMP-mediated ECM degradation. It has been well established that TIMP-1 has anti-apoptotic activity on many different cell types through both MMP-dependent (28,29) and -independent pathways (30,31).

Regarding the role of TIMP-1 in cell proliferation, conflicting data have been reported suggesting that TIMP-1 is mitogenic in certain cancer cells and lymphoid cells (2,32) while it inhibits the growth of normal mammary epithelial cells (33). In the present study, TIMP-1 reduced the growth rate of the HCC cell line BEL-7402 by up-regulating the cyclin-dependent kinase inhibitor p21(Waf1) and reducing the MMP-9/TIMP-1 ratio, suggesting that both MMP-9-TIMP-1 balance and TIMP-1 expression as a "cytokine-like" protein are required for the regulation of tumor progression.

Previous studies have shown that IL-6 has a pivotal role in stimulating hepatocytes to produce acute phase proteins cytotoxic to the liver; moreover, IL-6 has been shown to be a cell cycle progression factor for liver cells (34). After 70% partial hepatectomy, liver regeneration is dramatically impaired in IL-6^{-/-} livers. Treatment with IL-6 clearly accelerated the proliferative response in both IL-6^{+/+} and IL-6^{-/-} livers (35). Intense research over the past decade has shown that IL-6 could inhibit the proliferation of some cancer cells (36-38). Zeng and Fan (39)

suggested that IL-6 could inhibit the growth of the hepatocarcinoma cell line BEL-7402 by increasing the influx of Ca²⁺ and decreasing the expression of Bcl-2 in the cell.

Acting via gp130, IL-6 activates multiple signaling pathways, including the JAK-STAT, the Src family of protein tyrosine kinases, and phosphatidylinositol 3-kinase. It is also known that IL-6 can induce TIMP-1 expression (40). As indicated in a previous paper, IL-6 inhibits the proliferation of BEL-7402 by promoting apoptosis (39). We found that IL-6 treatment did not inhibit BEL-7402 proliferation until the fourth day of treatment, suggesting that there might be another mediator induced by IL-6 which actually exerts the inhibitory effect. Our data showed that TIMP-1 may be the mediator of the inhibitory effects of IL-6, inducing the remodeling of the ECM. Actually cell growth and proliferation result from a balance between the number of dividing cells and the number of dying cells. And since TIMP-1 has been shown to inhibit apoptosis, our results suggested the presence of a potential downstream pathway regulated by TIMP-1 distinct from the anti-apoptosis pathway.

The present results showed that IL-6 induced cell growth inhibition through TIMP-1 production, which was STAT3 dependent. The downstream inhibitory molecule of IL-6 and TIMP-1 was p21. IL-6 could accelerate p21 expression through the STAT3 signaling pathway which may act by decreasing MMP activity.

Acknowledgments

We are grateful to Dr. David Young (Department of Cell Biology, Harvard Medical School, Boston, MA, USA) and Dr. Tong-Chuan He (The Howard Hughes Medical Institute, Baltimore, MD, USA) for their generous gift of the construct of the pGL3-TIMP-1 reporter plasmid or adenoviral backbone and shuttle vectors. We would like to thank Dr. Xin-Yuan Fu (Department of Pa-

thology, Yale University School of Medicine, New Haven, CT, USA) for providing STAT3 wild-type, dominant negative form constructs and M67 reporter plasmid, and thank Dr. Zhi-Ming Yin (Life Sciences Col-

lege, Nanjing Normal University, Nanjing, China) for providing the p21(Waf1) luciferase plasmid and the control reporter plasmid pSV- β -galactosidase.

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