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Induction of hepatoma cells migration by phosphoglucose isomerase/autocrine motility factor through the upregulation of matrix metalloproteinase-3

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Abstract

Phosphoglucose isomerase/autocrine motility factor (PGI/AMF) catalyzes the isomerization between glucose-6-phosphate and fructose-6-phosphate, and is involved in cytokine activity, mitogenesis, differentiation, oncogenesis, and tumor metastasis. Presently, we demonstrate that exogenous PGI/AMF stimulates the migration of Huh7 and HepG2 hepatoma cells, but not Hep3B cells. Inhibition of PGI/AMF by PGI/AMF specific inhibitor 5-phospho-D-arabinonate markedly repressed the cellular migration. RT-PCR was used to examine the expression profile of matrix metalloproteinases (MMPs). MMP-3 transcripts, protein level, and secreted form were significantly upregulated in PGI/AMF-treated Huh7 and HepG2 cells, but not in Hep3B cells. MMP-3 inhibition abolished the PGI/AMF-induced cell motility. The observations are consistent with a downstream mediation role of MMP-3 in PGI/AMF-stimulated tumor cell metastasis.
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Phosphoglucose isomerase/autocrine motility factor (PGI/AMF) is a ubiquitous cytosolic enzyme, which catalyzes the isomerization between glucose-6-phosphate (G-6-P) and fructose-6-phosphate (F-6-P) in the glycolysis and gluconeogenesis pathways [1,2]. In humans, an autosomal recessive PGI/AMF deficiency leads to non-spherocytic hemolytic anemia that has variable clinical manifestations [3–5]. Serum activity of PGI/AMF is also a hallmark of tumor marker in gastrointestinal and renal cancer patients [6,7]. Recently, it has been shown that neuroleukin (NLK) [8–10], maturation factor [11,12], and autocrine motility factor (AMF) [11,13], are closely related to PGI and they are single gene products with pleiotropic functions. Cell culture-based studies have demonstrated that exogenous PGI/AMF stimulates various migrating cells, including human umbilical vein endothelial cells (HUVECs) [14], A2058 melanoma cells [15], human HT1080 fibrosarcoma cells [16], and hepatoma cells [17].

Receptor interaction mediates the activity of a cell surface-localized seven transmembrane glycoprotein (gp78) [18]. This activity precedes pertussis toxin-sensitive G-protein activation, inositol triphosphate production, protein kinase C activation, and the enhanced production of an arachidonic acid metabolite [19]. The small GTPase Rho is required for gp78 activation [20]. The forced expression of PGI/AMF can result in oncogenic and antiapoptotic activities, apparently as an accelerated G1 to S cell cycle transition [21], and the upregulation of the phosphatidylinositol 3-kinase (PI 3-K)/Akt signaling pathway [22].

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors in the tropics and Far East countries [23], including Taiwan. A frequent hallmark of HCC is an early invasion into blood vessels as well as intrahepatic metastasis; extrahepatic metastasis occurs subsequently [24]. Invasion and metastasis are the major

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cause of treatment failure in patients with HCC. Invasion of malignant tumor cells requires destruction of basement membranes (BM) and proteolysis of extracellular matrix (ECM) [25]. The ECM degrading proteases produced by various tumor calls play a key role during this process [26]. It is now clear that the matrix metalloproteinases (MMPs) appear to be particularly involved in the metastatic cascades, due to their broad ECM and BM degrading activities [27]. MMPs secreted as inactive zymogens must be activated extracellularly by the removal of an amino terminal propeptide. Once secreted and activated, MMPs can be inhibited by the formation of high affinity 1:1 non-covalent complexes with the tissue inhibitors of metalloproteinases (TIMPs) [28,29]. The balance between the levels of activated MMPs and free TIMPs determines the overall MMP activity.

Hepatoma cells produce PGI/AMF, and the PGI/AMF receptor is strongly detected in hepatoma cells invading into stroma and in tumor thrombi in the portal vein [30]. Moreover, PGI/AMF enhances hepatoma cell invasion through activation of β1 integrins [17]. Despite these known behaviors, the precise role and mechanism of the PGI/AMF in HCC metastasis remains unclear. In the present work, we examined the effect of PGI/AMF on hepatoma cell lines by focusing on cell motility. Our results clearly show that PGI/AMF induces hepatoma cells locomotion through the upregulation of MMP-3.

Materials and methods

Cell culture and reagents. Human hepatoma cell lines were cultured in defined minimal essential medium containing 10% heat inactivated fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in an atmosphere of 5% CO_2. Purified human PGI/AMF and PGI/AMF inhibitor 5PA were kindly provided by Dr. Menghsiao Meng. Rabbit muscle PGI/AMF was purchased from Sigma Chemical (St. Louis, MO). MMP-3 inhibitor was purchased from Calbiochem (San Diego, CA, Cat. No. 44218). Rabbit anti-human MMP-3 antibody was purchased from Chemicon (Temecula, CA). Human MMP-3 enzyme-linked immunosorbent assay (ELISA) system reagents and horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody were purchased from Roche (Nutley, NJ).

Cell motility assay. The cell motility was assayed using transwell cell culture inserts (Becton–Dickinson, Franklin Lakes, NJ). As many as 2×10^4 cells were placed in the top part of the chamber. The bottom part of the chamber was filled with DMEM or with medium supplemented with PGI/AMF. For the 5PA and MMP-3 inhibitor assays, the PGI/AMF and either inhibitor were pre-incubated for 2 h before filling the bottom chamber. Incubation was carried out at 37 °C for 16 h. The filters were removed and fixed with 4% paraformaldehyde for 5 min at room temperature. Cells on the upper filter surface were removed with a cotton swab. The filters were stained with crystal violet, washed with phosphate-buffered saline (pH 7.4), and observed under a light

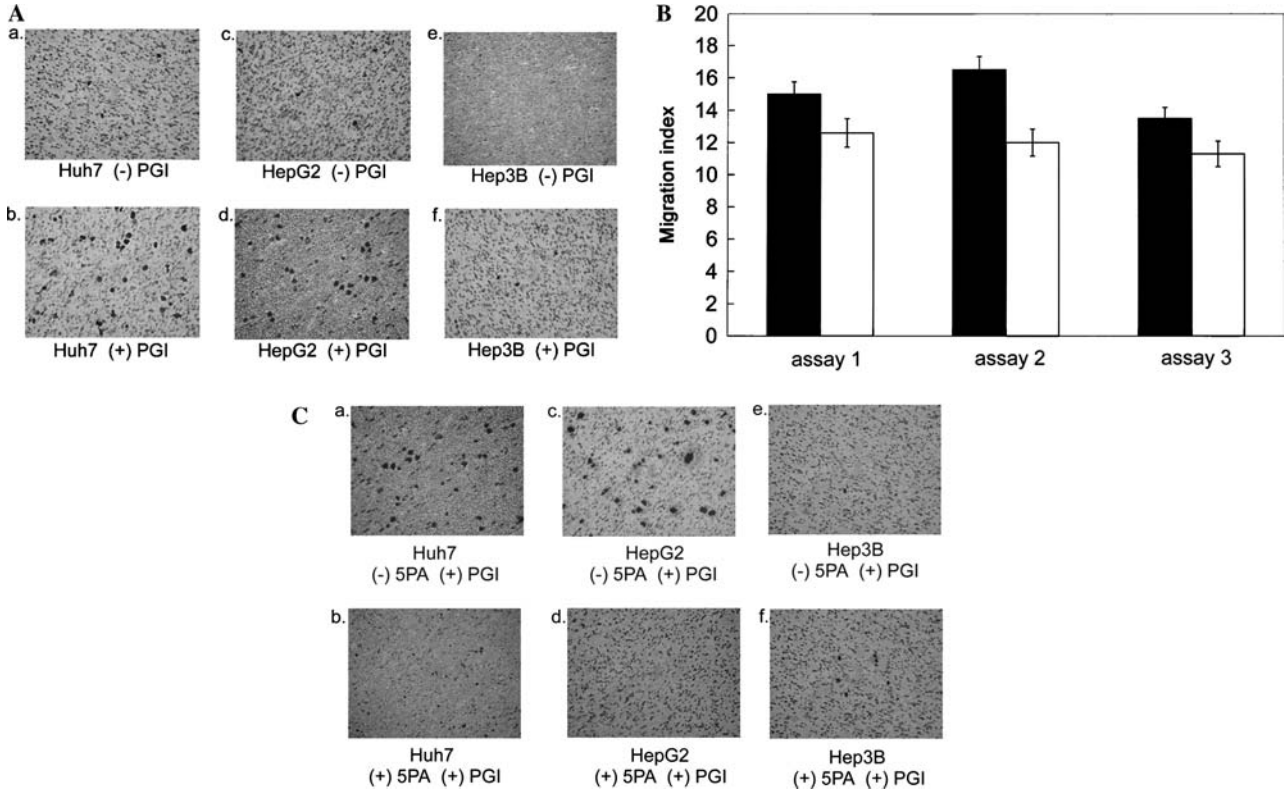

Fig. 1. PGI/AMF stimulates Huh7 and HepG2 cell migration and PG/AMF specific inhibitor 5PA suppressed this effect. (A) As many as 2×10^4 cells were placed in the top section of the transwell chamber and 10 μg/ml purified human PGI was added to the lower section. After 16 h of incubation, cells migrated across the filter were stained, counted, and pictured under light microscope. Migrating cells manifested as dark color. (B) Migration index quantitation of stained cells as determined by visual enumeration. Data were obtained from three independent experiments. (C) The presence of 500 μM of a PGI inhibitor 5PA almost completely suppressed the PGI-induced cell motility. The magnification of images was 200×.

microscope operating at 200× magnification. The migration index was defined as the ratio of the percent migration obtained with migratory cells (Huh7 and HepG2) to the percent migratory obtained with non-migratory cells (Hep3B).

Semi-quantitative RT-PCR. Total cellular RNA was extracted from cell lines using TRIzol reagent (InVitrogen, Carlsbad, CA) according to the manufacturer's instructions. Total RNA was reacted with RNase-free DNase (Promega, Madison, WI) to remove genomic DNA. The PCR primer sequences, reaction locations, and reaction condition were reports by Kim et al. [31]. cDNA was synthesized using 5 μg of total RNA as the template and oligo$(dT)_{20}$ as primers as described in the manufacturer's protocol of AMV reverse transcriptase (Promega). The cDNA mixtures were subjected to PCR using *Taq* polymerase (Promega) and oligonucleotide primer pairs specific to the cDNA for various MMPs, TIMP-1, TIMP-2, and actin [31]. So that amount of specific PCR product semiquantitatively reflected the level of mRNA, each primer pair was located near the polyadenylation signal of the mRNA. The number of PCR cycles was restricted to 28–30, representing the late exponential range for PCR product [31]. The PCR products were analyzed by agarose gel electrophoresis.

SDS–PAGE and immunoblotting. Cells were lysed with a lysis buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, and 2 mM phenylmethylsulfonyl fluoride. Thirty micrograms total protein from the supernatant was subjected to 10% SDS–PAGE and blotted onto polyvinylidene fluoride membrane. The blots were blocked with 5% non-fat dried milk in Tris-buffered saline and incubated (4 °C overnight shaking) with anti-MMP-3 polyclonal antibody (1:5000). The blots were then incubated with HRP-conjugated anti-rabbit antibody (1:2000) at room temperature for 2 h. The labeled bands were detected by chemiluminescence using ECL Western blotting detection reagents and exposure to X-ray film.

Enzyme-linked immunosorbent assay. The level of total secreted MMP-3 was assayed by ELISA using a commercially available kit (Amersham Biosciences, Buckinghamshire, UK). Briefly, a sample was allowed to react with anti-MMP-3 on the surface of microplate wells. After incubation and washing, the bound immunocomplexes were treated with peroxidase labeled Fab' anti-MMP-3 antibody to MMP-3. The amount of bound peroxidase-label antibody was measured by the addition of TMB substrate, with the resultant color qualified at 450 nm in a microplate spectrophotometer.

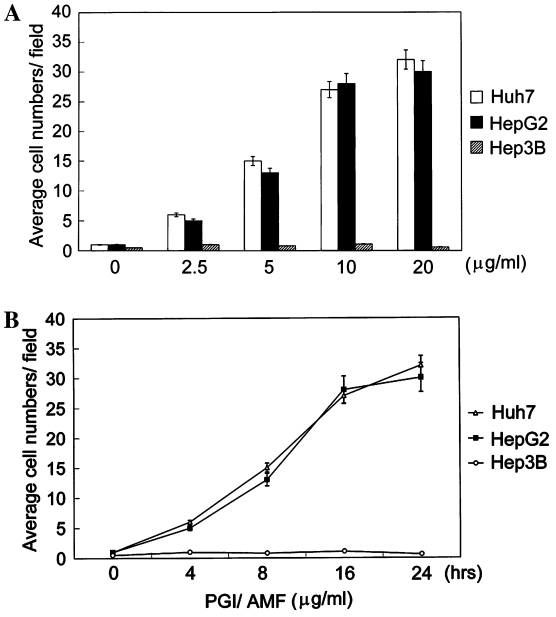

Fig. 2. PGI/AMF induced cell motility in a concentration- and time-dependent manner. Migrated cells were enumerated by counting 10 individual fields under 200× magnification. Data obtained from two independent experiments are presented as means and standard deviations. (A) Three cell lines were treated with various concentrations of purified human PGI as indicated and incubated for 16 h at 37 °C. (B) Cells were treated with 10 μg/ml purified human PGI for different incubation times as indicated. Data from three independent experiments are presented as means and standard deviations.

Results

PGI/AMF influenced on cell motility

PGI/AMF was originally identified as a cytokine that enhances tumor cell migration. Therefore, we questioned whether PGI/AMF influences hepatoma cell migration. Purified human PGI/AMF was added in cell culture medium in the lower part of the transwell culture and incubation in cell culture condition. Huh7 and HepG2 cells migrated across the cell culture insert, while Hep3B cells did not (Fig. 1A). Fig. 1B represented the result as migratory index. The migratory index which indicates the ability to distinguish between migratory and non-migratory cells, calculated as the number of cells migrate (Huh7 and HepG2) divided by the number of cells non-migrated (Hep3B). PGI/AMF-stimulated cell motility, was markedly suppressed by addition of 5PA, a potent and specific inhibitor of PGI (Fig. 1C). Earlier studies reported that in the presence of 0.1 mM 5PA, the PGI/AMF enzyme activity is markedly decreased by 86% and efficiently inhibited CT-26 mouse colon cancer cell migration [32]. The same results were obtained by addition of PGI/AMF from rabbit muscle (data not shown). To ascertain whether the 10 μg/ml PGI/AMF represented the optimum concentration for cell motility induction, cells were treated with PGI/AMF at various concentrations and incubated for varying times. As shown in Fig. 2, the PGI/AMF-induced cell motility displayed a concentration- and time-dependent response.

Upregulation of MMP-3 by PGI/AMF

It is now well known that MMP family and their antagonistic TIMPs are critical modulators of ECM composition and thus crucial in neoplastic cell invasion and metastasis. Presently, various MMP and TIMP primers were synthesized to evaluate the relative mRNA levels for MMPs and TIMPs affected by PGI/AMF. The MMP expression profile is showed in Fig. 3A. We found out that MMP-3 transcripts were significantly elevated in Huh7 and HepG2 cells following PGI/AMF treatment (Fig. 3B). MMP-3 transcripts were not detected in Hep3B cells (Fig. 3B). Furthermore, the levels of TIMP-1 transcripts were reduced in PGI/AMF- treated Huh7 and HepG2 cells (Fig. 3C). The increased detection of

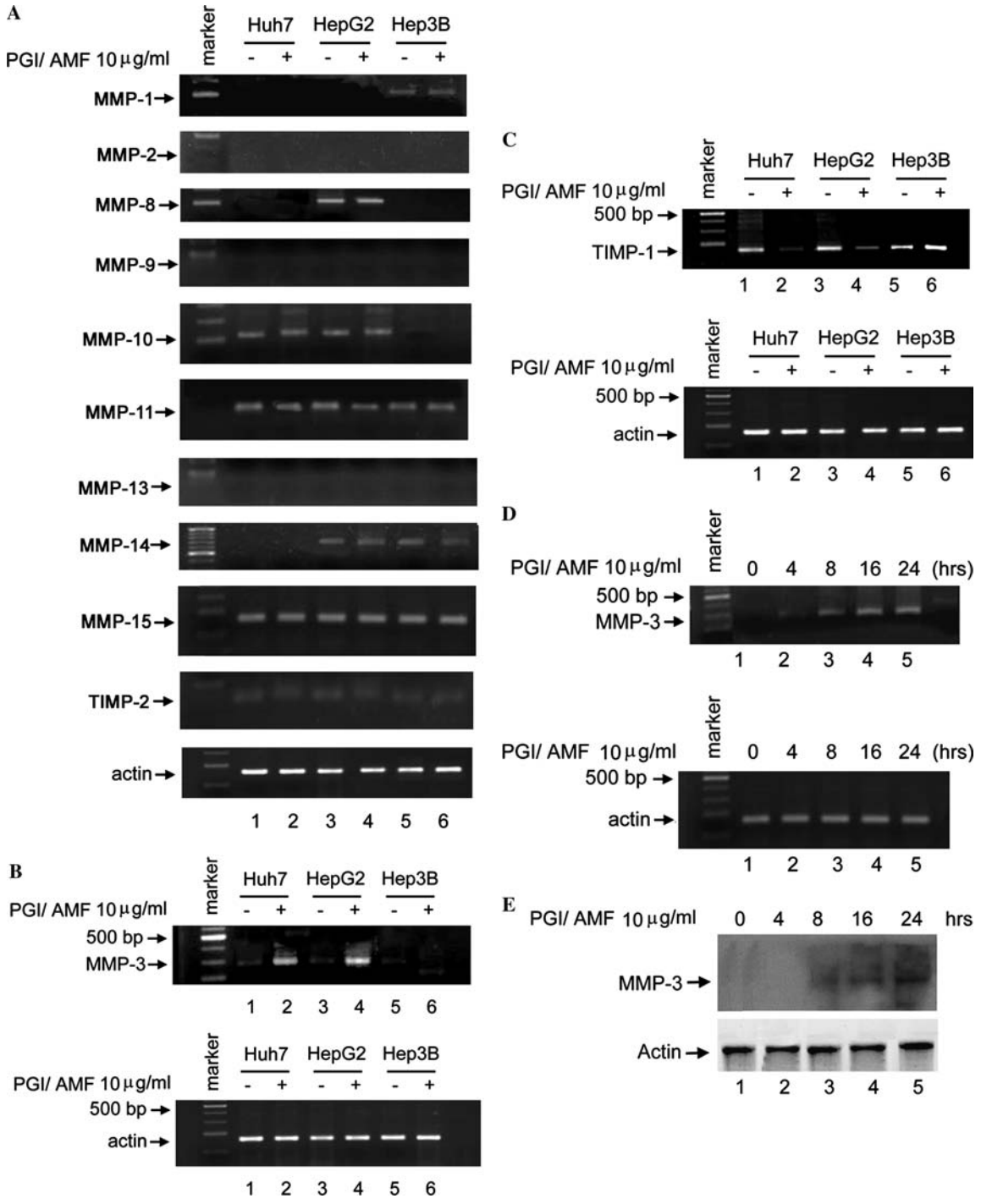

Fig. 3. The MMP-3 expression was upregulated in Huh7 and HepG2 cells by PGI/AMF treatment. (A) In cells treated with 10 μg/ml purified human PGI for 16 h, various MMPs, TIMPs, and actin were shown. (B) Two hundred and ninety base pairs of MMP-3 transcripts were shown as indicated (upper panel). Actin was included in RT-PCR as internal control (lower panel). (C) TIMP-1 transcripts were downregulated in Huh7 and HepG2 cells treated with purified human PGI (upper panel). Actin was included in RT-PCR as internal control (lower panel). (D) PGI/AMF induced MMP-3 transcript elevation in a time-dependent manner. Huh7 cells treated with 10 μg/ml purified human PGI for various time points as shown. The level of MMP-3 transcripts and position were indicated (upper panel). Actin was included in RT-PCR as internal control. (lower panel). (E) Western blot analysis by using anti-MMP-3 antibody to detect MMP-3 expression level. Equal amount of total proteins are loaded on SDS–PAGE and transferred to PVDF membrane. Followed by PGI/AMF treatment, the protein level of MMP-3 was increased in a time-dependent manner, clearly in 16 and 24 h (upper panel). The same blot was reacted with anti-actin antibody to verify the equal amount of proteins (lower panel).

the MMP-3 transcripts was obvious by 16 h following PGI/AMF exposure and occurred in a time-dependent pattern (Fig. 3D). MMP-3 protein expression showed a similar increase pattern in Huh7 (Fig. 3E). Experiments were conducted in HepG2 cells and the same results were obtained (data not shown).

PGI/AMF influences on MMP-3 secretion

Although PGI/AMF induced an upregulation of MMP-3 transcripts and protein expression. As we know, indeed, a functional MMPs have to be secreted outside the cells. The conditioned medium collected from the three PGI/AMF-treated cell lines was analyzed by ELISA assay. As illustrated in Fig. 4, the optical density of the medium increased in a time-dependent manner following PGI/AMF treatment in Huh7 and HepG2 cells, consistent with the accumulated MMP-3 secretion in the presence of PGI/AMF.

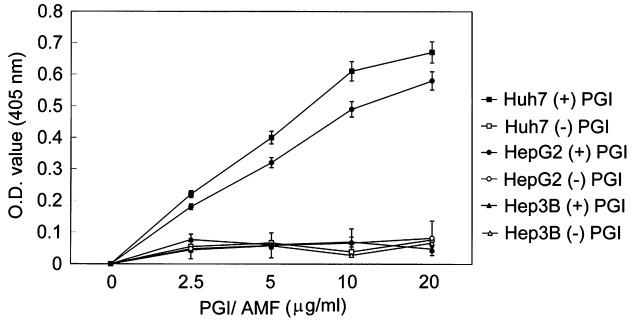

Fig. 4. The secreted form of MMP-3 was elevated followed by PGI/AMF treated Huh7 and HepG2 cells. Three hepatoma cell lines were incubated with various concentrations of PGI/AMF for 16 h. One hundred microliters of conditioned medium was applied onto microplates to perform a MMP-3 ELISA assay as described in Materials and methods. The *y*-axis represented the OD value at 450 nm. Data are presented as means and standard deviations of three independent experiments, each performed in duplicate.

MMP-3 specific inhibitor abolished the PGI/AMF-induced cell migration

To determine the relevancy between MMP-3 elevation and PGI/AMF-stimulation cell locomotion, a specific MMP-3 peptide inhibitor Ac-Arg–Cys–Gly–Val–Pro–Asp-NH_2 was employed to block MMP-3 action. The hexapeptide sequence specific inhibited MMP-3 with low IC_{50} values (5 μM) [34,35]. The inhibitor was not deleterious to the cells (data not shown) and did not alter the other MMP2 expression (Fig. 5A). Upon specific inhibition of the MMP-3 inhibitor, PGI/AMF-induced migration was almost fully suppressed (Fig. 5B). The result indicated that the MMP-3 was a downstream mediator involved in PGI/AMF-stimulated cell migration. The related mechanism about the ability of PGI/AMF to induce the upregulation of MMP-3 needed further investigations.

Discussion

The present study has convincingly demonstrated that the exogenous PGI/AMF elicits Huh7 and HepG2 hepatoma cell migration, with MMP-3 being the mediator of this effect. But PGI/AMF treatment does not affect the motility of another hepatoma cell line Hep3B. Our current research identified a new mediator involved in PGI/AMF-induced cell migration process. Previous report by Torimura et al. [17], indicated PGI/AMF stimulates hepatoma cells invasion through the activation of β1 integrins, with Rho activity and phosphorylation of MEK1 and MEK2 being required in PGI/

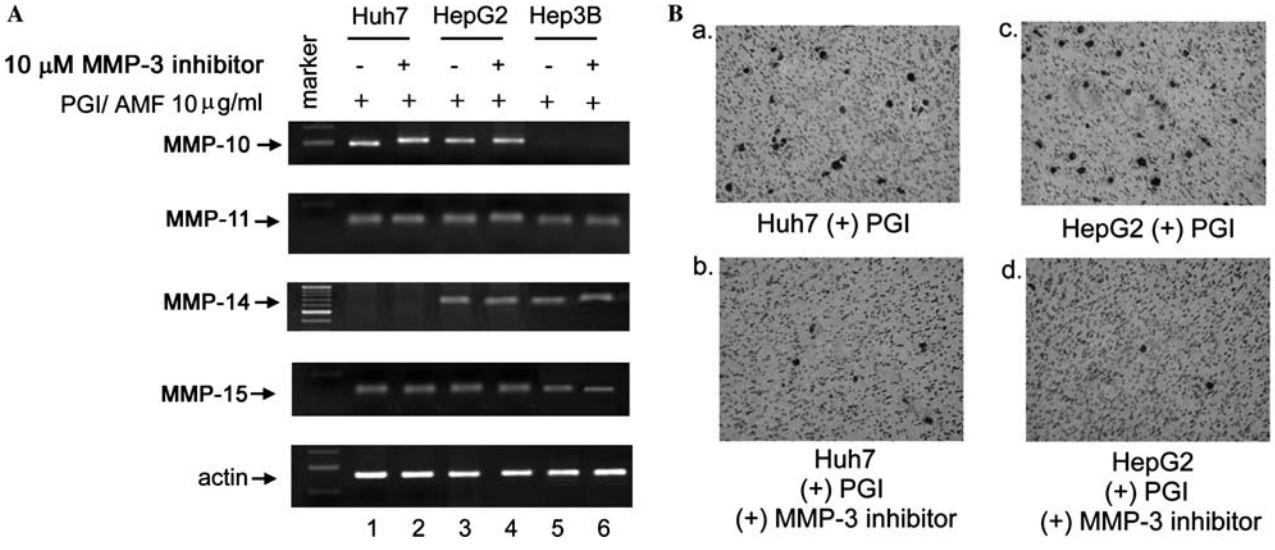

Fig. 5. MMP-3 specific inhibitor abolished PGI/AMF-induced cell motility. (A) RT-PCR illustrated that 10 μM MMP-3 inhibitor did not alter MMP-10, MMP-11, MMP-14, and MMP-15 expression. (B) Cells seeded in transwell insert upper part and incubated with 10 μg/ml PGI/AMF combined with or without MMP-3 inhibitor for 16 h. The migrated cells were visualized by crystal violet staining under microscope observation. The magnification of images was 200×.

AMF signal transmission [20]. The role of cell adhesion molecule, including integrins, cadherins has yet to be tested in the cell lines utilized in the present study.

The strong overall immunochemically evident expression of MMP-3 and MMP-10 in HCC tissue [33] is consistent with a significant role for, especially MMP-3, in the mediation of hepatoma cell migration. However, the failure to detect MMP-3 transcripts in Hep3B cells presently and in another study [31] is counter to this speculation. The involvement of other MMPs in mediating cell motility cannot be as yet be fully excluded.

As the PGI/AMF required its cognate receptor for signal transduction, the expression status and functional role of PGI/AMF receptor will be addressed in our model. Preliminary data that PGI/AMF receptor transcripts are not altered in the presence or absence of PGI/AMF (data not shown) are consistent with the suggestion that the different responses to PGI/AMF in the Huh7, HepG2, and Hep3B cell lines was due to downstream transducing components such as MMP-3. But the interference of other molecules by PGI/AMF cannot be excluded. However, in the presence of PGI/AMF, the MMP-3 transcripts were induced only in migrating Huh7 and HepG2 cells, a result that is expected if PGI/AMF is capable of signal transmission to certain trans-acting factors, which in turn activates MMP-3 transcription. We are pursuing this line of reasoning through MMP-3 promoter analysis to identify *cis*- and/or *trans*-acting elements involved in PGI/AMF mediated MMP-3 activation.

In conclusion, on the basis of these observations, we propose that by developing specific MMP or PGI/AMF inhibitors may ameliorate the severity of HCC-associated metastasis. Blockage of MMPs, in combination with other chemotherapeutic agents, may prevent the progress of HCC.

Acknowledgments

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