Jpn. J. Infect. Dis., 68, 221–229, 2015

Original Article

Different Responses in MMP/TIMP Expression of U937 and HepG2 Cells to Dengue Virus Infection

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SUMMARY: Disease severities following dengue virus (DV) infection are the result of increased vascular permeability leading to hypovolemic shock. Matrix metalloproteinases (MMPs) are believed to play a key role in promoting such severities. A previous study reported that supernatants of DV-infected dendritic cells (DCs), which contained high levels of MMP-2 and MMP-9, induced vascular leakage in a mouse model. In the present study, we investigated whether hepatocytes (HepG2) and monocytes (U937) could be additional sources of MMPs during DV infection. HepG2 and U937 cells were exposed to DV serotype 2 strain 16681. The secretion of MMP-2 and MMP-9 was detected using gelatin zymography. We found that DV infection in the HepG2 cells promoted MMP-2 production while that in the U937 cells promoted MMP-9 production. Semi-quantitative RT-PCR results also confirmed that DV infection in the HepG2 cells up-regulated the expression of MMP-2 mRNA, whereas that in the U937 cells enhanced the expression of MMP-9 mRNA. We monitored the expression of endogenous TIMP-1 and TIMP-2. DV infection induced TIMP-1 expression in the U937 cells. However, lower expression of TIMP-2 was observed in the infected HepG2 cells. We believed that following DV infection, monocytes and hepatocytes can act as MMP-9 and MMP-2 producers, respectively. Their responses could be attributed to the disturbance of TIMP expression by DV in different cell types.

INTRODUCTION

Infection with dengue virus (DV), a mosquito-borne flavivirus, causes dengue fever, which is a major public health problem in several tropical and subtropical countries. Rapid transmission of 4 serotypes of DV (DV serotypes 1–4) is typically facilitated via *Aedes* mosquito vectors abundant in densely populated urban areas. It has been estimated that 50 million infected cases are reported each year (1). Clinical manifestations following DV infection may develop as an acute, indistinguishably febrile (DF) illness with limited bleeding (2). In severe patients, leucopenia develops together with an abrupt reduction in platelets and plasma leakage, leading to dengue hemorrhagic fever (DHF) (3,4). Lethal dengue shock syndrome (DSS) occurs if there is increased capillary permeability resulting in extensive bleeding (3). Although the clinical parameters of the progression of dengue fever to greater severity forms (DHF and DSS) have been documented, physiological mechanisms underlining the development of DHF and DSS remain largely elusive.

Matrix metalloproteinases (MMPs), a family of more than 24 proteolytic enzymes, exhibit a number of physiological functions during both normal health conditions and several pathological circumstances (5). The role of MMPs in promoting disease severity has increasingly been documented in certain flaviviral infections. Recent studies showed that the cerebrospinal fluid and sera of Japanese encephalitis virus (JEV) patients contained higher levels of MMP-2 and MMP-9 than those of healthy individuals (6). MMP-9 expression was found to be induced in macrophages (7), which greatly contributed to the neuro-inflammatory process and severe encephalitic conditions. Elevated levels of MMP-1, MMP-3, and MMP-9 have been previously reported in West Nile virus (WNV)-infected mice (8).

For DV infection, MMPs, together with inflammatory cytokines such as IL-1, IL-6, and TNFα, are believed to markedly contribute to the progression of a severe pathology (9,10). Clinical investigation of DV infection revealed a substantial increase in MMP-9 in the plasma of DV patients with severe DF conditions (11). In addition, peripheral blood cells from patients who developed DHF showed greater expression of MMP-9 than those with milder, DF stage (12). Tissue damage leading to malfunction of major organs, particularly the liver and endothelium, has been evident in cases of DHF and DSS (1,9,13,14). With regard to the endothelium, it has been previously found that after DV infection, human microvascular endothelial cells stimulate the overproduction of MMP-2 and MMP-9, causing the disruption of vascular endothelium (VE)-cadherin cell-cell adhesion while further enhancing endothelial permeability (15). In addition, a number of immune competent cells, including dendritic cells (DCs), monocytes, macrophages, and B cells have been reported to be targeted by DV (9). DV induced the overproduction of MMP-9 and MMP-2 by immature DCs. Administration of supernatants from DV-infected DCs disrupted the expression

Received January 13, 2014. Accepted September 10, 2014.
J-STAGE Advance Publication January 20, 2015.
DOI: 10.7883/yoken.JJID.2013.481

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of platelet endothelial cell adhesion molecule-1 (PECAM-1) and VE-cadherin cell adhesion molecules, leading to increased vascular permeability in a mouse model (16). However, little is known about how other cellular constituents of the major visceral organs (especially the liver), and other antigen-presenting cells (monocytes) could contribute as MMP sources during DV infection.

In the present study, we used HepG2 and U937 cells representing hepatocytes and monocytes, respectively, to investigate their role as additional MMP producers in response to DV infection. We also examined the expression of endogenous inhibitors of MMPs, known as TIMPs, particularly TIMP-1 and TIMP-2, to clarify whether DV interferes with the balance of MMPs/TIMPs, which could potentially be the cause of vascular leakage and progression of severe DHF.

MATERIALS AND METHODS

Virus and cell lines: The DV serotype 2 strain 16681, the prototype Southeast Asian strain (17), was propagated in LLC-MK2 cells. Only up to the 4th passage of DV was used in the experiments. Viral titer was determined by a plaque-forming assay, as described previously (16). Human monocytic cells (U937) and hepatic cells (HepG2) were purchased from CLS Cell Lines Service (Eppelheim, Germany). The U937 cells were cultured in RPMI1640 (Hyclone Laboratories, Logan, UT, USA) supplemented with 10% heat-inactivated FBS (GE Healthcare, Fairfield, CA, USA), 200 U/ml penicillin and 100 μg/ml streptomycin (Life Technologies Corp, Grand Island, NY, USA). The HepG2 cells were grown in MEM (Hyclone Laboratories) with similar supplements. Cell lines were maintained at 37°C in a 5% CO_2 incubator.

In vitro infection: The U937 cells were placed in 24-well culture plates at 2×10^6 cells/well. Following 24-h incubation, they were exposed to DV suspended in a serum-free medium (at MOI = 0.05) at 37°C for 90 min, with a shaking interval every 30 min. The infected U937 cells were washed twice with PBS. RPMI containing 5% FBS was then added to each well. An aliquot of the HepG2 cells (2×10^6 cells) were seeded and cultured in MEM for 48 h at 37°C prior to being infected with DV (at MOI = 0.05) at 37°C for 90 min in a manner similar to that for the U937 cells. The uninfected cells (mock infection) were exposed to DV-free media only and were used as controls. For determination of MMP activities and expression of TIMPs, culture supernatants were harvested at 0 and 72 h following infection.

Western blot analysis: The U937 and HepG2 cells were washed twice with PBS, homogenized in cold lysis buffer [Tris–HCl, pH 7.5 containing 150 mM NaCl, 1 mM EDTA, 2 mM DTT, 2 mM PMSF (Sigma-Aldrich, St. Louis, MD, USA) and 1% Triton X-100 (Bio-Rad, Hercules, CA, USA)] and centrifuged at 10,000 *g* for 15 min. Protein concentrations were determined using the Bradford protein assay (Bio-Rad). Equal amounts of protein were loaded onto a 10% SDS-PAGE gel and were electrophoretically separated (Mini-Protean Tetra cell™, Bio-Rad). Proteins were electroblotted onto nitrocellulose membranes (Hybond-ECL™ [GE Healthcare]) using Mini Trans-Blot™ cell equipment (Bio-Rad) and subsequently blocked with 5% skimmed milk. Membranes were then incubated with mouse anti-human TIMP-1 monoclonal antibody (clone 102D1; Chemicon International, Billerica, MA, USA) or mouse anti-TIMP-2 (clone 3A4; Abcam, Cambridge, UK) antibody at 4°C overnight. Following washing, blots were incubated with horseradish peroxidase-conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) at a dilution of 1:1,000 for 1 h at room temperature. After washing, bands were visualized by adding TMB membrane peroxidase substrate (KPL Laboratories, Gaithersburg, MA, USA).

Gelatin zymography: Gelatinolytic activities of MMP-2 and MMP-9 in cell culture supernatants were assessed by gelatin zymography. In brief, 30 μg protein was mixed with nonreducing Laemmli sample buffer before being electrophoresed in 10% acrylamide gel containing 1 mg/ml gelatin in the presence of SDS. After electrophoresis, the gels were washed three times with 50 mM Tris-HCl (pH 7.5) containing 2.5% Triton X-100, 5 mM $CaCl_2$, and 1 μM $ZnCl_2$ and were subsequently incubated overnight in the same buffer containing 1% Triton X-100. Gelatinase activity was visualized by staining the gels with 0.5% Coomassie blue prepared in 30% methanol/10% acetic acid and de-staining with 2% acetic acid.

Semi-quantitative RT-PCR: Total RNA from the U937 and HepG2 cells was extracted using TRIzol® reagent (Invitrogen Corp, Carlsbad, CA, USA). First-strand cDNA were synthesized from 1 μg total RNA using RevertAid™ M-MuLV reverse transcriptase (Thermo Scientific, Pittsburg, PA, USA). One-sixth of a portion of cDNA was amplified using TaqDNA polymerase (Thermo Scientific) according to the manufacturer's instruction, and GAPDH gene expression was examined as an internal control. PCR was performed using a Mastercycler® personal thermocycle (Eppendorf, Hamburg, Germany) under the following conditions: initial pre-denaturation at 94°C for 1 min; 40 cycles of denaturation at 94°C for 1 min, annealing at 64°C (DV, TIMP-1), 56°C (MMP-9, GAPDH), and 55°C (MMP-2, TIMP-2) for 2 min; and extension at 72°C for 1 min. The PCR products were separated on 1% agarose gel and visualized by ethidium bromide staining. Band intensities on the gels were determined by Scion Image analysis (Scion Corporation, MD, USA). The primers used in RT-PCR are listed in Table 1.

Statistical analysis: Comparison of MMPs and expression of their inhibitors between the mock infection and DV infection groups were determined statistically by independent Student's *t*-test (SPSS Statistics, IL, USA). The difference was considered significant when $P < 0.05$. Arithmetic means of three replicates ± SD are shown. Data shown are representative of at least three independent experiments.

RESULTS

DV infection induced MMP-9 expression in U937 cells and MMP-2 expression in HepG2 cells: Semi-quantitative RT-PCR was used to determine mRNA expression of MMP-2 and MMP-9 in U937 and HepG2 cells. We found that both U937 (Fig. 1A) and HepG2 cells

Table 1. Nucleotide sequences of the primers used for RT-PCR

mRNA	Sense primer	Antisense primer
DV envelope	5′-AAGGTGAGATGAAGCTGTAGTCTC-3′	5′-CATTCCATTTTCTGGCGTTCT-3′
MMP-2	5′-GTGTTCTTTGCAGGGAATGAAT-3′	5′-ACGACGGCATCCAGGTTATC-3′
MMP-9	5′-GAAGATGCTGCTGTTCAGCG-3′	5′-ACTTGGTCCACCTGGTTCAA-3′
TIMP-1	5′-GGTACCATGGCCCCCTTTGAGCCCCT-3′	5′-AAGTTTCACAAGCAATGAGTGCCACTCTG-3′
TIMP-2	5′-CGACATTTATGGCAACCCTATCA-3′	5′-GCCGTGTAGATAAACTCTATATCC-3′

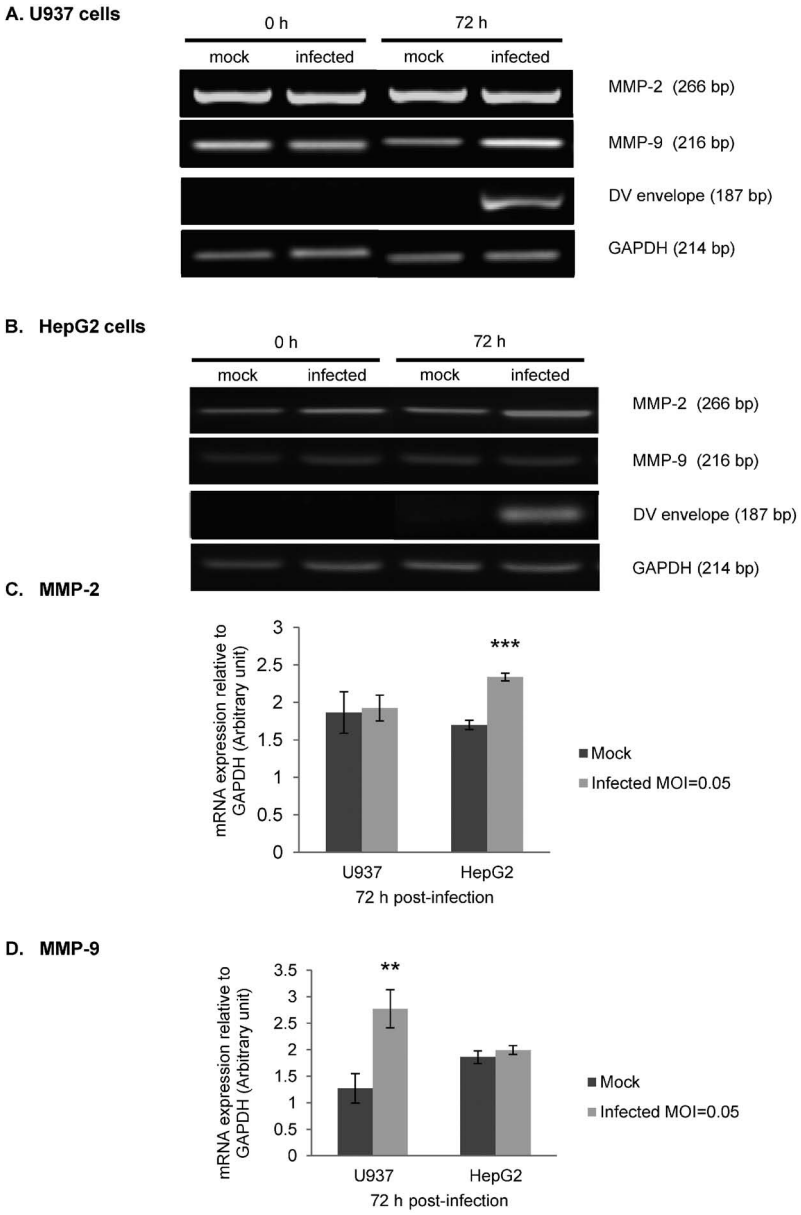

Fig. 1. Expression of MMP-9 and MMP-2 in U937 and HepG2 cells. RT-PCR analysis of MMP-2, MMP-9 and DV envelope gene transcription in U937 (A) and HepG2 (B) cells, in mock and infected cells at 0 and 72 h post-infection. Relative expression of MMP-2 mRNA (C) and MMP-9 mRNA (D) to GAPDH mRNA in U937 and HepG2 cells at 72 h post-infection in mock (dark grey) and DV-infected cells (light grey). Bars show means ± SD of at least three replicates, representatives for three experiments. Statistically significant differences compared with the mock. **, $P < 0.01$; ***, $P < 0.001$.

(Fig. 1B) constitutively expressed MMP-2 and MMP-9. However, by 72 h following exposure to DV, HepG2 cells markedly up-regulated the expression of MMP-2 (Fig. 1C) but not that of MMP-9 (Fig. 1D). Expression of MMP-9 was found to be induced in U937 cells following infection of DV at 72 h (Fig. 1D), compared with that in mock control cells. However, DV infection did not alter the expression of MMP-2 in U937 cells (Fig. 1C). We also confirmed the presence of DV by monitoring the expression of an envelope gene in the in-

fected U937 and HepG2 cells (Fig. 1). By 24 h, we were able to detect a DV gene within both cell lines. Apparently, increased expression of an envelope gene in the U937 cells, which might indirectly reflect DV replicative capacity, was recorded as infection time passed to 72 h. However, in the infected HepG2 cells, those levels were comparable between 24 and 72 h (Fig. 2).

Following DV infection, U937 and HepG2 cells enhanced production of secreted MMP-9 and MMP-2, respectively: Gelatin zymography was performed to examine the gelatinolytic activities of MMP-2 and MMP-9 in cell culture supernatants. We were able to detect soluble MMP-2 (72 kDa) and MMP-9 (92 kDa) in the uninfected U937 and HepG2 cells (Fig. 3A, B). We identified increase in the levels of MMPs in supernatants as the culture time increased. However, greater amounts of soluble MMP-9 from the infected U937 cells were observed as brighter bands during zymography, compared with uninfected cells (Fig. 3A, D). However, the production of MMP-2 was comparable between the DV-infected and uninfected U937 cells (Fig. 3A, C). In addition, DV infection substantially induced MMP-2 secretion in the HepG2 cells (Fig. 3B, C). However, soluble MMP-9 levels were not different between the infected and control HepG2 cells, which was similar to what we observed with mRNA levels (Fig. 3B, D).

Differential expression of TIMP-1 and TIMP-2 in U937 cells and HepG2 cells in response to DV infection: The expression and function of MMPs are regulated by different TIMPs. Therefore, we investigated whether overexpression of MMP-9 and MMP-2 in DV-infected U937 and HepG2 cells might result from aberrant expression of endogenous TIMPs. We studied the expression of TIMP-1 and TIMP-2, which have extensively been reported to preferentially control MMP-9 and MMP-2, respectively. In the U937 cells, DV infection

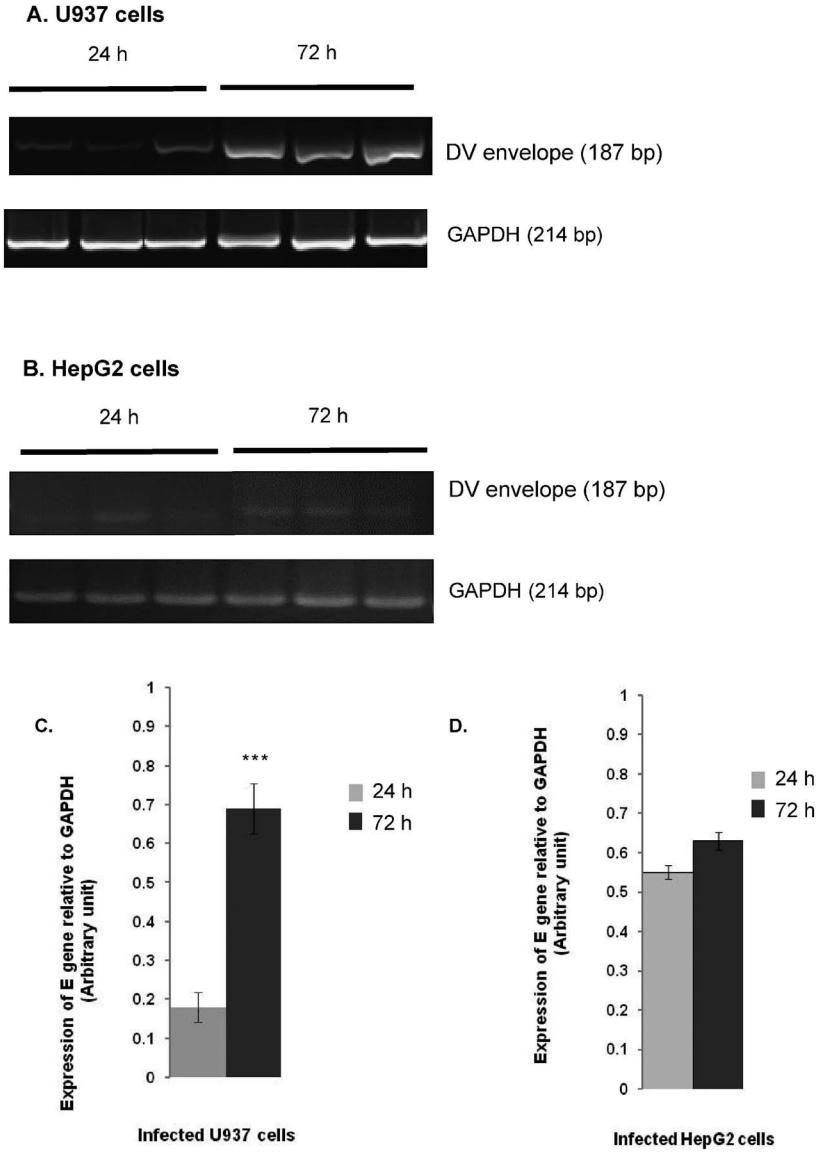

Fig. 2. Semi-quantitative RT-PCR analysis of DV envelope gene in the infected U937 (A) and HepG2 (B) cells at 24 and 72 h post-infection. Relative expression of DV envelope to GAPDH mRNA in U937 (C) and HepG2 (D) cells at 24 h (light grey) and 72 h (dark grey) post-infection. Bars show means ± SD of at least three replicates, representatives for two experiments. Statistically significant differences compared between two time points. ***, $P < 0.001$.

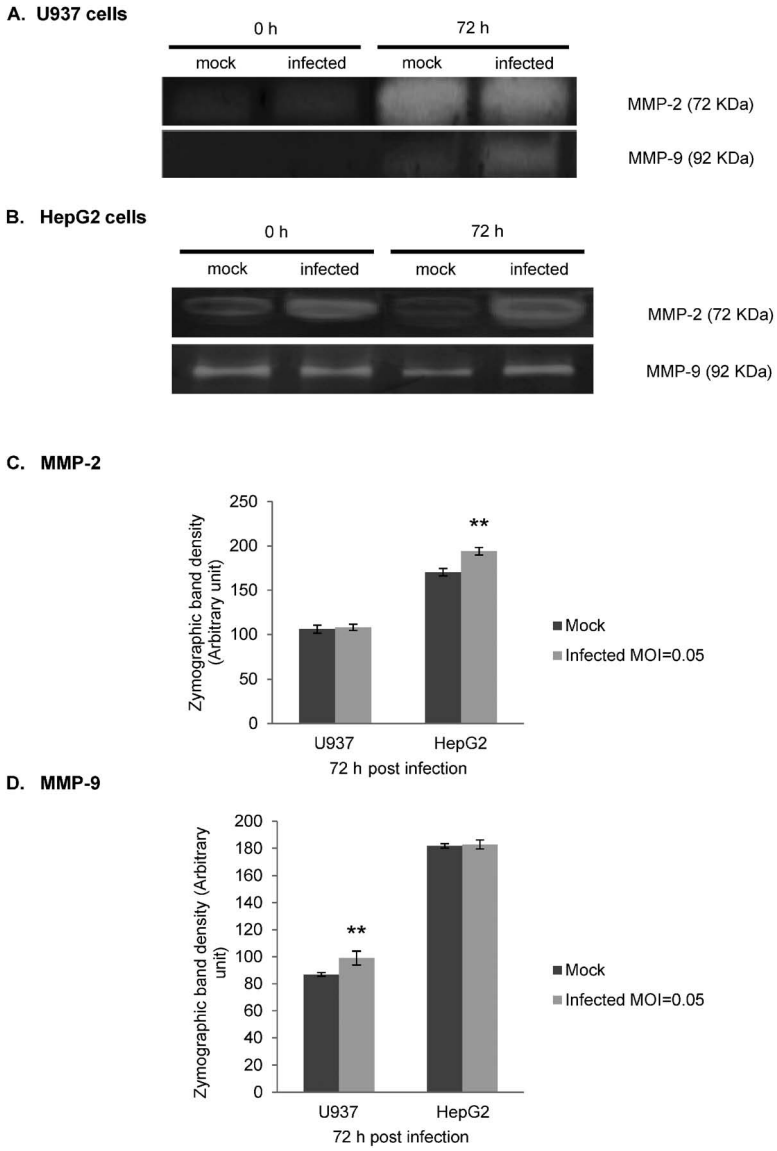

Fig. 3. Gelatinolytic activities of MMP-9 (gelatinase B, 92 kDa) and MMP-2 (gelatinase A, 72 kDa) in culture supernatants of U937 and HepG2 cells. Gelatin zymographic analysis of secreted MMP-9 and MMP-2 in U937 (A) and HepG2 (B) cells in mock and infected cells at 0 and 72 h post-infection. Band intensity of MMP-9 (C) and MMP-2 (D) from the infected cells and mock controls at 72 h post-infection. Bars are means ± SD of at least three replicates, representatives for three independent experiments. Statistically significant differences compared with the mock. **, $P < 0.01$.

induced the expression of TIMP-1 mRNA by 72 h, while the expression of TIMP-2 was unaffected (Fig. 4A). Soluble amounts of TIMP-1 in supernatants at 0 h were below detectable levels, but enhanced levels of TIMP-1 protein were observed only in the infected U937 cells (Fig. 5A). TIMP-2 expression was recorded as early as 0 h, but we could not detect the apparent change in TIMP-2 expression following DV infection in the U937 cells (Fig. 5A). The HepG2 cells constitutively expressed both TIMP-1 and TIMP-2 at transcriptional levels (Fig. 4B). In the presence of DV, the HepG2 cells retained their expression of TIMP-1 but down-regulated that of TIMP-2. Similar to the results from RT-PCR, using Western blot analysis, we found decreased levels of TIMP-2 expression in the infected HepG2 cells, but no obvious difference in soluble TIMP-1 levels between the infected and control cells (Fig. 5B).

DISCUSSION

In the present study, we showed that hepatocytes can act as a cellular source of MMP-2 following the exposure of DV. As a cancerous cell line, HepG2 cells, originating from hepatocellular carcinoma cells, were reported to constitutively release active MMP-2 and MMP-9 (18). However, this cell line has been widely used to investigate a number of DV interactions with host cells and to assess the intracellular signaling caused by DV infection such as cell entry, viral replication and apoptosis induction (19).

The liver has well been identified as a DV target organ because DV antigens have been discovered in hepatocytes of lethal cases (20). Hepatic encephalopathy with massive bleeding and increased liver enzyme levels have often been reported in severe DHF and DSS (1,9,21). However, the detailed mechanisms underlining the

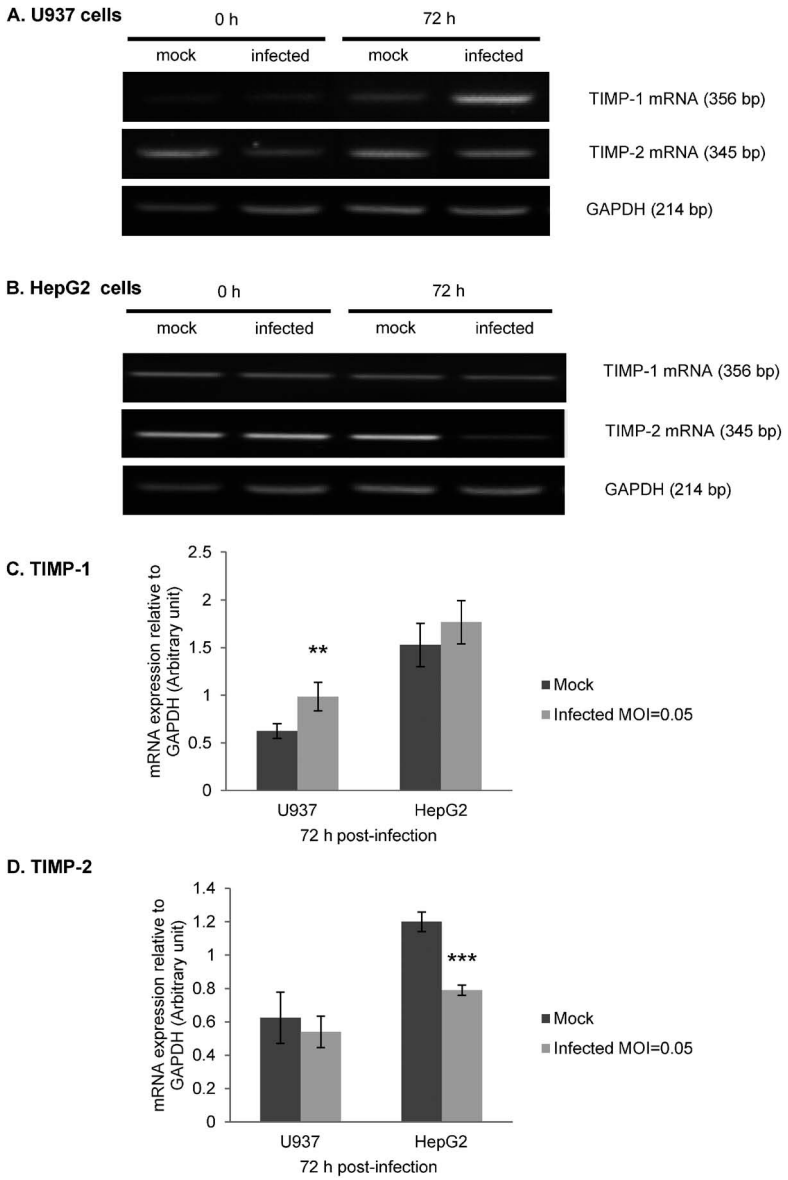

Fig. 4. Expression of TIMP-1 and TIMP-2 in U937 and HepG2 cells. RT-PCR analysis of TIMP-1 and TIMP-2 in U937 (A) and HepG2 (B) cells. Relative expression of TIMP-1 mRNA (C) and TIMP-2 mRNA (D) to GAPDH mRNA in U937 and HepG2 cells at 72 h post infection in mock (dark grey) and DV-infected cells (light grey). Bars show means ± SD at least three replicates representatives for three independent experiments. Statistically significant differences compared with the mock. **, $P < 0.01$; ***, $P < 0.001$.

hepatic injury observed in several cases leading to liver failure have never been clarified. Immunohistochemical analysis of hepatic lesions from DHF patients revealed the enhanced production of pro-inflammatory mediators, including IL-6, IL-12, IL-18 and iNOS, in the portal tract and hepatic acinus (22). Such a microenvironment is believed to promote apoptosis in DV-infected hepatocytes, which have been reported through endoplasmic reticulum stress, and caspase activation as well as alteration of the mitochondrial transmembrane potential (23). In addition, signaling via the extracellular signal-regulated kinase 1/2 (ERK1/2) cascade has been recently reported as a key mechanism stimulating hepatic apoptosis in a mouse model. Use of an ERK inhibitor, revealed marked reduction in caspase genes leading to abrogated DV-induced liver injury (24). The overproduction of MMP-2 in the HepG2 cells observed in this study might result from the activation of ERK signaling, similar to that shown in hepatitis C infection (25). However, interaction of specific DV proteins and cellular signaling pathways triggering MMP expression is currently under investigation to clarify this point.

In the infected HepG2 cells, we found considerable up-regulation of MMP-2 expression at the transcription level, as evidenced by RT-PCR, which correlated well with greater activities of secreted MMP-2 analyzed by gelatin zymography. In addition, TIMP-2 mRNA expression and soluble TIMP-2 levels were down-regulated in the HepG2 cells following DV infection. The transformation mechanism of proMMP-2 to active MMP-2 requires an optimal concentration of soluble TIMP-2 and membrane type 1(MT1)-MMP. It has been previously described that to generate active MMP-2, proMMP-2 molecules must bind a receptor complex

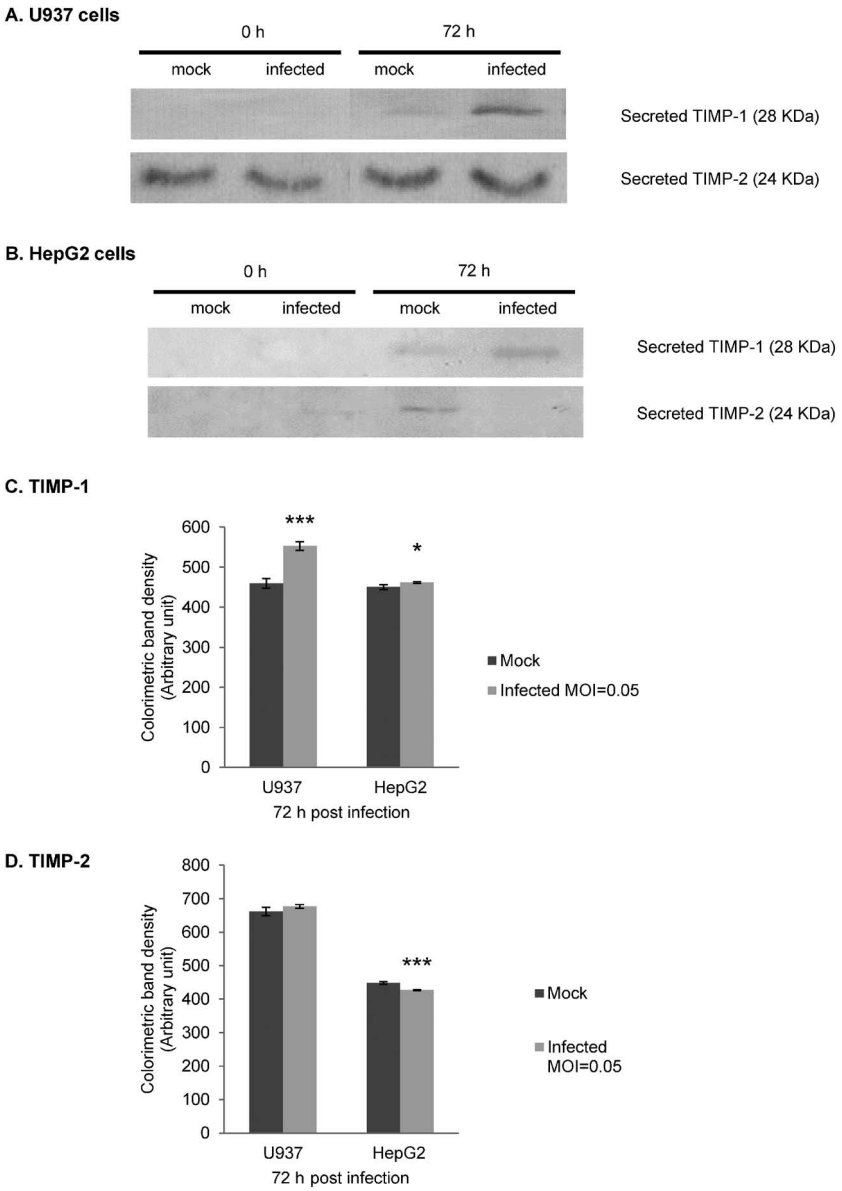

Fig. 5. Production of TIMP-1 (28 kDa) and TIMP-2 (24 kDa) in culture supernatants of U937 and HepG2 cells. Western blot analysis of soluble TIMP-1 and TIMP-2 in U937 (A) and HepG2 (B) cells in mock and infected cells at 0 and 72 h post-infection. Band intensity of TIMP-1 (C) and TIMP-2 (D) from the infected cells and mock control at 72 h post-infection. Bars are means ± SD of three replicates, representatives for three independent experiments. Statistically significant differences compared with the mock. *, $P < 0.05$; ***, $P < 0.001$.

formed by TIMP-2 and MT1-MMP on the cell surface (26). Only a low concentration of TIMP-2 that interacts with MT1-MMP is allowed for appropriate cleavage of proMMP-2 by adjacent free MT1-MMP, releasing fully active MMP-2 (27,28). Alternatively, another mechanism is that homodimerization of MT1-MMP results in the activation of proMMP-2 to MMP-2, in which no TIMP-2 is needed (29). In our study, overexpression of MMP-2 and down-regulation of TIMP-2 in the HepG2 cells might have resulted from an altered TIMP-2/MT1-MMP regulation in MMP-2 activation triggered by DV infection. The persistence of reduced TIMP-2 levels may facilitate increased MMP-2 synthesis in hepatocyte responses to DV.

Monocytes are antigen-presenting cells that play a crucial role in mediating inflammatory responses. They can travel through lymphatic and vascular systems and differentiate to macrophages in peripheral tissues. Monocytes are a prime target of DV infection (30). They allow DV to not only get entry but also facilitate its replication (31). The human monocytic U937 cell line has been extensively employed to examine DV infectivity (30,32). This in vitro study revealed that DV-infected monocytic cells are capable of producing MMP-9. Following infection with DV, the monocytes substantially elevated the expression of MMP-9 but not that of MMP-2. Similar to what we observed in DV infection, monocytes act as a cellular source of MMP-9 during responses to a number of infectious pathogens including *Mycobacterium tuberculosis* (33), human cytomegalovirus (34) and *Porphyromonas gingivalis* (35). Increased MMP-9 expression is required by inflammatory monocytes to translocate themselves to peripheral tissues (35,36).

TIMP-1 is known to be an inhibitor of MMP-9 (37). The stoichiometric balance of 1:1 between MMP-9: TIMP-1 is crucially maintained under normal physiologic conditions (38). Enhanced TIMP-1 production during pathologic circumstances has been hypothesized as causing counterbalance in the overproduction of functional MMP-9 (37). Although significant increase in MMP-9 and TIMP-1 production have been previously recorded in malarial (39) and bacterial infections (40), a strict balance of MMP-9/TIMP-1 is easily disturbed leading to the MMP-9 overexpression. The alteration in MMP-9 and TIMP-1 levels could possibly represent our DV-infected U937 cells since we could detect a remarkable increase in TIMP-1 and MMP-9. Imbalance of MMP-9/TIMP-1 could therefore have a plausible role in monocytes against DV infection.

To our knowledge, this study provides new evidence that in response to DV infection, hepatocytes and monocytes can act as cellular MMP producers, particularly MMP-2 and MMP-9, respectively. Their function could result in the aberrant expression of TIMP-2 and TIMP-1, which might interfere with the delicate balance between MMPs/TIMPs in a number of different DV-susceptible cells. Our findings also suggest that there is a feasible role for hepatocytes and monocytes in facilitating the progression of disease toward more severe hemorrhagic fever.

Acknowledgments We thanked Rujira Trakulyingcharoen, Siriwat Sukphokkit, and Kittiya Islam for technical assistance. This work was supported by the Office of the Higher Education Commission, Thailand Research Fund (Grant no. MRG5080143 to SK) and Mahidol University under National Research Universities Initiatives Grant to VL. PS was a recipient of Science Achievement Scholarship of Thailand.

Conflict of interest None to declare.

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