Biochimica et Biophysica Acta 1842 (2014) 1720–1732

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

Involvement of the nuclear high mobility group B1 peptides released from injured hepatocytes in murine hepatic fibrogenesis

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ARTICLE INFO

Article history:
Received 5 January 2014
Received in revised form 24 May 2014
Accepted 16 June 2014
Available online 23 June 2014

Keywords:
Hepatic stellate cells
Oxidative stress
TGF-β1 signaling
Smad2 phosphorylation
RAGE
TLR4

ABSTRACT

This study investigated the pro-fibrogenic role of high mobility group box 1 (HMGB1) peptides in liver fibrogenesis. An animal model of carbon tetrachloride (CCl_4)-induced liver fibrosis was used to examine the serum HMGB1 levels and its intrahepatic distribution. The increased serum HMGB1 levels were positively correlated with elevation of transforming growth factor-β1 (TGF-β1) and collagen deposition during fibrogenesis. The cytoplasmic distribution of HMGB1 was noted in the parenchymal hepatocytes of fibrotic livers. In vitro studies confirmed that exposure to hydrogen peroxide and CCl_4 induced an intracellular mobilization and extracellular release of nuclear HMGB1 peptides in clone-9 and primary hepatocytes, respectively. An uptake of exogenous HMGB1 by hepatic stellate cells (HSCs) T6 cells indicated a possible paracrine action of hepatocytes on HSCs. Moreover, HMGB1 dose-dependently stimulated HSC proliferation, up-regulated de novo synthesis of collagen type I and α-smooth muscle actin (α-SMA), and triggered Smad2 phosphorylation and its nuclear translocation through a TGF-β1-independent mechanism. Blockade with neutralizing antibodies and gene silencing demonstrated the involvement of the receptor for advanced glycation end-products (RAGE), but not toll-like receptor 4, in cellular uptake of HMGB1 and the HMGB1-mediated Smad2 and ERK1/2 phosphorylation as well as α-SMA up-regulation in HSC-T6 cells. Furthermore, anti-RAGE treatment significantly ameliorated CCl_4-induced liver fibrosis. In conclusion, the nuclear HMGB1 peptides released from parenchymal hepatocytes during liver injuries may directly activate HSCs through stimulating HSC proliferation and transformation, eventually leading to the fibrotic changes of livers. Blockade of HMGB1/RAGE signaling cascade may constitute a therapeutic strategy for treatment of liver fibrosis.

1. Introduction

The progression of liver fibrosis has been considered as a complex process of wound healing in response to a variety of chronic stimuli [1,2] and is characterized by an excessive deposition of extracellular matrix (ECM) proteins, primarily the fibrillar collagens including type I collagen [2,3]. Being the primary hepatic cellular component responsible for excessive collagen synthesis during hepatic fibrosis [4], hepatic stellate cells (HSCs) undergo a complex transformation or activation process following liver injury and thereby change from a quiescent to an activated, myofibroblast-like phenotype [2,5]. The changes accompanying HSC activation encompass the increases in DNA synthesis, cellular proliferation, ECM production as well as morphological transformation, including the appearance of cytoskeletal protein α-smooth muscle actin (α-SMA). Among the reported HSC activators, transforming growth factor-β1 (TGF-β1) is the key cytokine mediator markedly up-regulated during hepatic fibrogenesis. Through activation of type I TGF-β receptor, the post-receptor signal transducers, Smad2 and Smad3, are phosphorylated. The phosphorylation of Smad2 and Smad3 results in the formation of a stable complex with Smad4, which translocates into the nuclei to act as transcriptional regulators. In addition to the canonical Smad pathway, TGF-β1 also activates different members of the mitogen-activated protein kinase (MAPK) family in HSCs, including

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http://dx.doi.org/10.1016/j.bbadis.2014.06.017
0925-4439/

p38 MAPK, extracellular signal-regulated kinase (ERK), and c-Jun N-terminal kinase (JNK) [6–9].

High mobility group box 1 (HMGB1) is originally identified as a nuclear non-histone DNA-binding protein that functions as a structural co-factor critical for proper transcriptional regulation in somatic cells. With moderate affinity but without sequence specificity, HMGB1 binds to the minor groove of linear DNA and bends it into a helical structure in the nucleus, hereby interacting with and recruiting various transcription factors [10]. The pathogenetic role and regulatory function of extracellular HMGB1 have long been discussed in the development of many organ diseases involving immune dysfunctions. Nuclear HMGB1 actively secreted from innate immune cells could act as an indicator for the extent of tissue injury and as an immunostimulatory signal to enhance other cytokine expression, promote the recruitment of mononuclear cells to clear cellular debris, and protect against possible infection that often follows trauma [11,12]. The active and passive release of HMGB1 from hepatocytes has been previously noted in the hepatocytes under ischemic and reperfusion stimuli [13,14]. Although HMGB1 induces a distinct pattern of gene expression compared to bacterial endotoxin [15], the high levels of circulating HMGB1 in patients with infectious and inflammatory disorders are significantly correlated with poor disease prognosis including infectious and sterile inflammation and, hence, also regarded as a therapeutic target [16–20].

Despite the extracellular release of HMGB1 peptides has been implicated in the pathogenesis of hepatic and retinal ischemia/reperfusion injury [13,14,21] and pulmonary fibrosis [22], the role of HMGB1 in hepatic fibrogenesis has not been clearly defined. This study, therefore, aimed to determine the role of HMGB1 during the development of liver fibrosis by examining its serum profile and intrahepatic distribution pattern in a murine model of carbon tetrachloride (CCl_4)-induced liver fibrosis. Meanwhile, primary hepatocytes and HSC-T6 cells were used to elucidate the interactive mechanism in determining whether the HMGB1 released from injured hepatocytes might exhibit fibroproliferative and transforming effects on HSCs via the canonical TGF-β receptor-Smad and relevant non-canonical signaling pathways. Additionally, an immunological blockade of HMGB1 signaling axis was performed to determine the prophylactic efficacy in CCl_4-induced murine liver fibrosis.

2. Materials and methods

2.1. Reagents

Recombinant HMGB1 peptides and monoclonal antibodies against HMGB1, α-SMA and type I collagen α1 (COL1A1) were purchased from Sigma-Aldrich Chemicals (St. Louis, MA). Endotoxin level in working solution with recombinant HMGB1 protein was less than 0.25 EU determined by an LAL-based endotoxin test kit (Pierce Thermo, Rockford, IL). Antibodies against phosphor-Smad2 (Ser465/467), Akt, phosphor-Akt (Ser473), ERK1/2, phosphor-ERK1/2 (Thr185/Tyr187), JNK, phosphor-JNK (Thy183/Tyr185), p38 MAPK, phosphor-p38 MAPK (Thy180/Tyr182), and acetylated lysine were from Cell Signaling (Beverly, MA), while those against 4-hydroxynonenal (4-HNE) and 8-hydroxydeoxyguanosine (8-OHdG) were from Millipore (Temecula, CA). Those antibodies against Smad2 and Actin were from Santa Cruz (Santa Cruz, CA), while secondary HRP-conjugated ones were from Jackson ImmunoResearch Laboratories (West Grove, PA). Neutralizing antibodies for blocking ligand interaction of toll-like receptor 4 (TLR4) and the receptor for advanced glycation end-products (RAGE) were from eBioscience (San Diego, CA) and R&D Systems (Minneapolis, MN), respectively.

2.2. Animal model of liver fibrosis

Six to eight week-old male ICR mice were raised at 20–22 °C with 12 h light–dark cycle. All animal experimental procedures were approved by the Institute of Animal Care and Use Committees at Kaohsiung Chang Gung Memorial Hospital and EDA Hospital. Liver fibrosis was induced by subcutaneous CCl_4 administration at a dose of 1 mL/kg (1:1 mixture with mineral oil) twice a week for 5 consecutive weeks [23]. For a time-course observation, serum samples and liver tissues were collected at 18 and 35 days after initiation of CCl_4 injection. For a prophylactic anti-RAGE therapy, an isotype-matched control IgG (Jackson ImmunoResearch Lab, West Grove, PA) and an anti-RAGE neutralizing antibody (R&D Systems) were intraperitoneally administrated twice weekly at dose of 10 μg per mouse (average dose 283 μg/kg body weight), starting after 1 week of CCl_4 injection. Sera and liver tissues were collected at 35 days after initiation of CCl_4 injection. All sera were subjected to biochemical measurement as previously described [23].

2.3. ELISA

Mouse sera were subjected to ELISA using the kits for HMGB1 (Shino-Test Corporation, Kanagawa, Japan) and TGF-β1 (Biosource, Camarillo, CA) according to the manufacturers' protocols.

2.4. Histopathology, immunohistochemistry (IHC), Sirius red staining and morphometry

Formalin-fixed paraffin-embedded sections were prepared and processed for H&E, IHC, and Sirius red staining as previously described [23].For morphometrical observation, cytoplasmic location of HMGB1 in liver parenchyma was counted on at least 8 random high power fields per section and expressed as positive percentage per high power field. Collagen deposition in Sirius red-stained sections was measured using automatic thresholding function of ImageJ software (W.S. Rasband, NIH, Bethesda, Maryland, USA) and data are shown as percentage of total scanned area.

2.5. Cell culture

Primary hepatocytes were isolated from SD rats and maintained as previously described [23]. A rat hepatocyte cell line (Clone-9) and a rat HSC cell line (HSC-T6) were grown in F12K and Waymount medium (Sigma), respectively, supplemented with 10% heat-inactivated FBS, penicillin and streptomycin (Invitrogen, Grand Island, NY). Cells were incubated at 37 ºC in a humidified atmosphere of 5% CO_2 in air, and the medium was renewed twice a week. Before experiment, cells were trypsinized and plated on 3.5 cm diameter dishes or 96-well microplates.

2.6. Cell proliferation and BrdU assays

To measure cell proliferation, HSC-T6 cells were seeded onto 96-well plates at the density of 4×10^3 per well 24 h before experiment. After being treated with recombinant HMGB1 at different concentrations for 24 h, a commercially available MTS proliferation assay kit (Promega, Madison, WI) was used as described previously [24]. Alternatively, HMGB1-treated cells were subjected to a cell-based BrdU proliferation assay (Roche Applied Science, Penzberg, Germany) according to manufacturer's instruction. The optical densities in both assays were colorimetrically measured by a microplate reader (MRX II, Dynex technologies, Chantilly, VA) and normalized to control level, which is considered as 100% of viability.

2.7. Immunofluorescent staining

Subcellular localization of HMGB1 and Smad2 proteins was identified by using immunofluorescent staining. Culture cells were seeded onto sterile glass coverslip (2×10^5 cells per slip) and fixed immediately in ice-cold methanol after drug treatment. After washes with PBS, the

cells were permeabilized with 0.5% Triton X-100 in PBS for 10 min, and blocked with blocking reagent (DAKO) for 30 min. Then the slips were incubated for 60 min at room temperature with primary antibodies against either HMGB1, poly-His tag (R&D Systems, Minneapolis, MN), or Smad2. Subsequently, the bound signal was visualized by Alexa Fluor 488- or 594-conjugated anti-rabbit antibody (Molecular Probe, Eugene, OR). Nuclei were counterstained with 2 μg/mL of Hoechst 33342 (Molecular Probe).

2.8. Quantitative PCR (qPCR)

Total RNA was extracted from cultured HSCs using the TriZol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. After spectrophotometrical quantification, 2 μg of total RNA was used for reverse transcription reaction, followed by conventional and qPCR analyses using gene-specific primers for *β-actin*, *TGF-β1*, *COL1A1*, and *α-SMA* (shown in Supplemental Table S1). After qPCR amplification, a final melting curve protocol was performed to confirm the PCR specificity. Gene expression levels were normalized to *β-actin* reference gene.

2.9. Western blot and immunoprecipitation (IP) detection

Total proteins were extracted by lysing the cells in ice-cold RIPA buffer in the presence of phosphatase inhibitors (1 mM sodium fluoride and 1 mM sodium orthovanadate) and a cocktail of protease inhibitors (Roche Molecular Biochemicals, Mannheim, Germany). Protein content was quantified using a Coomassie protein assay kit (Pierce Biotechnology, Rockford, IL, USA) and equal amounts of protein were resolved in reducing SDS-PAGE and subsequent immunoblotting detection as previously described [23].

Rabbit antibodies against HMGB1 (Santa Cruz Biotechnology) and acetylated lysine residues (Cell Signaling Biotechnology) were used to specifically immunoprecipitate HMGB1 or acetylated proteins in sera. About 500 μl of pooled mouse sera was first precleared by 30 min of incubation with preclearing matix (Santa Cruz Biotechnology) at 4 °C, and then were incubated overnight at 4 °C with 5 μg of antibody and 50 μl protein A-Sepharose slurry on a rotator for overnight. After incubation and centrifugation, supernatants were aspirated and discarded. The beads were washed five times with PBS, resuspended in 50 μl of reducing electrophoresis buffer, and subjected to immunoblot detection.

2.10. RNA interference of TLR4 and RAGE

To discriminate the role of TLR4 and RAGE in HMGB1-activated signaling and HSC activation, HSC-T6 cells were transfected with either rat-specific TLR4, RAGE small interfering RNA (siRNA), or scramble RNA control (Santa Cruz) at 100 nM with Lipofectamine 2000 (Invitrogen, Grand Island, NY). After verifying the silencing efficiency after 48 h of siRNA transfection by Western blot, the cells received HMGB1 stimulation for indicated duration and were subjected to immunoblot detection as described above.

2.11. Statistical analysis

Spearman's rank correlation analysis was used to determine the dependence between plasma HMGB1 and TGF-β1 levels. In vivo and in vitro data were presented as mean ± standard error of mean (SEM) and mean ± standard deviation (SD), respectively. Comparative in vitro data were presented as normalized values of 1.0 in negative controls. Multiple comparisons among groups were done by one-way ANOVA followed by a Bonferroni post hoc test. Significance is declared when *P* value is less than 0.05.

3. Results

3.1. Elevation of serum HMGB1and its correlation with liver fibrogenesis in mice

Subcutaneous administration of CCl_4 was performed to induce liver fibrosis in ICR mice. A time-course biochemical observation confirmed injured hepatic functions including elevation of serum AST and ALT levels (Fig. 1A). To determine the involvement of HMGB1 in the pathogenesis of liver fibrosis, the mouse sera were collected for measurement of HMGB1 and TGF-β1 by ELISA. Although serum HMGB1 levels in normal mice were not detectable (i.e. <1 ng/mL), the concentrations were remarkably increased to 110.2 ± 10.3 and 128.5 1 ± 6.2 ng/mL after 18 and 35 days of CCl_4 treatment, respectively (Fig. 1B). Immunoblotting detection qualitatively identified the presence of a 29 kD peptide identical to HMGB1 in the sera of these animals with fibrotic livers (Fig. S1). To investigate the relationship between HMGB1 elevation and liver fibrosis, serum TGF-β1 levels were measured using ELISA method. TGF-β1, as a reliable marker for liver fibrosis, was reproducibly increased along with the progression of CCl_4-induced liver fibrosis (Fig. 1C). Simple linear regression and Spearman's rank correlation analyses further indicated a positive correlation between serum levels of HMGB1 and TGF-β1 (Fig. 1D). Moreover, histological observation by H&E staining showed deterioration of hepatic microarchitecture (Fig. 1E). Sirius red stain revealed prominent deposition of collagen fibers (Fig. 1F) and further morphometrical analysis demonstrated significantly aggravated collagen deposition in livers (Fig. 1G). Correlation analysis again indicated a close relationship between the HMGB1 elevation and the severity of liver fibrosis (Fig. 1H).

3.2. Redistribution of nuclear HMGB1 into cytoplasm of hepatocytes in fibrotic livers

To examine the cellular localization of HMGB1 peptides in liver tissues before and after fibrosis induction, IHC staining for HMGB1 was performed to further delineate the possible pathogenetic role of HMGB1. The immunoreactivity of HMGB1 peptides was present in and strictly confined to the nuclei of hepatocytes and those of residing cellular components in the normal livers (Fig. 2A). After 18 days of CCl_4 administration, the nuclear size of parenchymal hepatocytes partially became enlarged possibly due to liver parenchymal damage (Fig. 2B) and the abundance of nuclear HMGB1 peptides obviously increased. The nuclear pattern of signals in smaller size indicated infiltrated inflammatory cells or activated sinusoidal endothelial cells and HSCs. After 35 days of treatment with CCl_4, a diffused pattern of HMGB1 was remarkably observed along with sinusoidal location and a cytoplasmic pattern of HMGB1 was notably manifested in few of parenchymal hepatocytes around the central vein area (Fig. 2C). Quantitative analysis on the cytoplasmic location of HMGB1 under high power field showed an average of 3.5% positivity in fibrotic livers at day 35. Since active extracellular transport of HMGB1 from hepatocytes has been known to be regulated by its posttranslational acetylation [25], IP detection with the sera of CCl_4-treated mice demonstrated a remarkably increased acetylation of MHGB1 (Fig. 2D), indirectly supporting the extracellular release of HMGB1 from parenchymal hepatocytes. These findings suggest that nuclear HMGB1 peptides were relocated from nucleus to cytoplasm and eventually released from the injured parenchymal cells of fibrotic livers. This result implies that the release of nuclear HMGB1 peptides likely contributes to the elevated serum HMGB1 levels and that it may in turn activate HSCs intrahepatically in a paracrine manner, thereby promoting the progression of liver fibrosis.

3.3. Release of HMGB1 from hepatocytes under chemical and oxidative stresses in vitro

Given the importance of oxidative stress in liver fibrogenesis, we next simulated the in vivo oxidative and chemical insults and examined

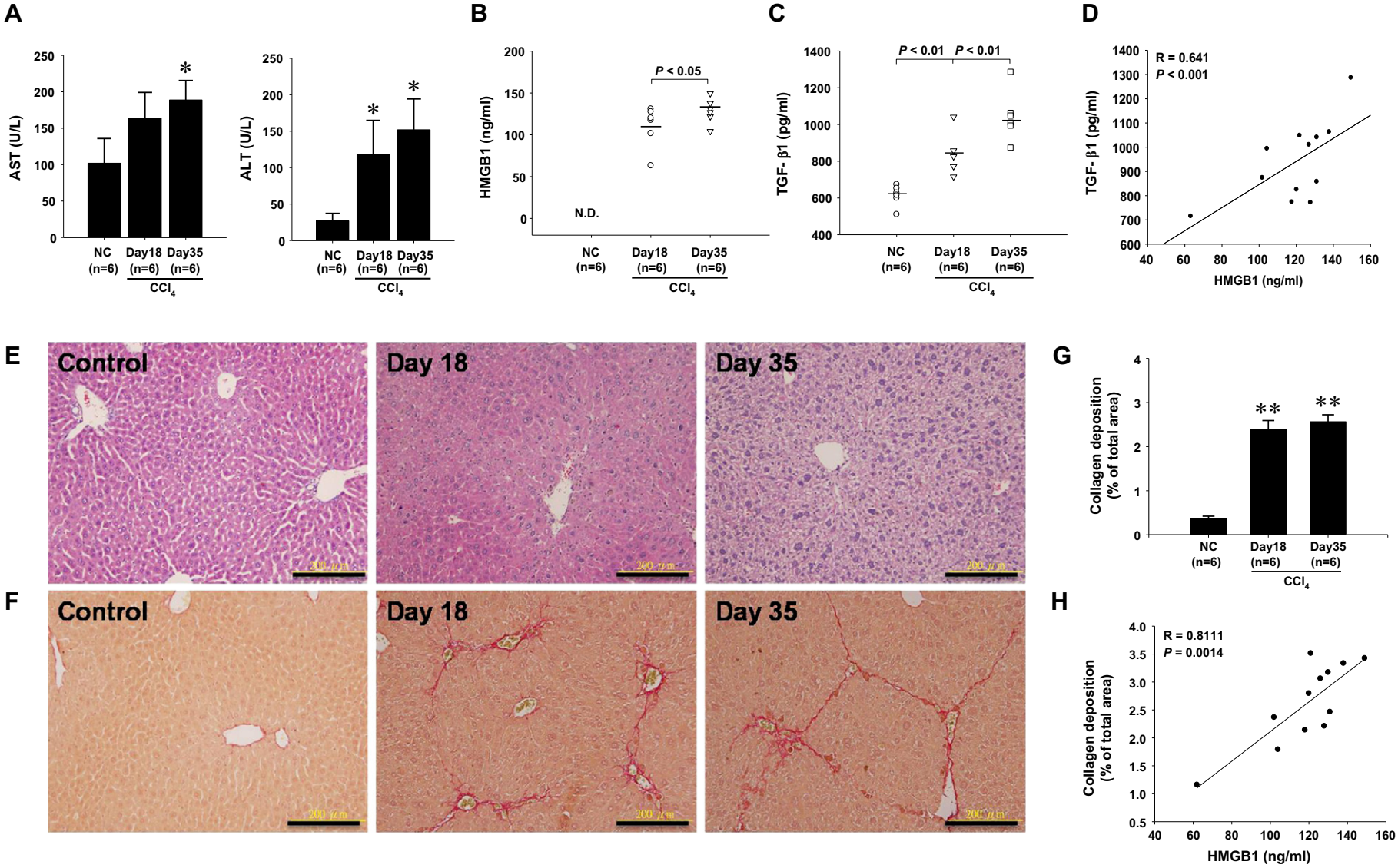

Fig. 1. Correlation of serum HMGB1 elevation with severity of liver fibrosis in the mice with CCl_4 administration. Sera were collected from normal ICR mice and those treated with CCl_4 administration after 18 and 35 days and subjected to biochemical detection for liver function integrity, including AST and ALT (A). Serum HMGB1 (B) and TGF-β1 (C) levels were determined by using ELISA. N.D., not detectable. (D) Spearman's rank correlation analysis showed a positive correlation between serum HMGB1 and TGF-β1 levels in the mice receiving CCl_4 administration. Formalin-fixed and paraffin-embedded liver tissues were sectioned and followed by histopathological examinations, including H&E (E) and Sirius red (F) stains. Morphometrical analysis revealed increased collagen deposition in fibrotic livers (G), which positively correlated with serum HMGB1 levels (H). Data are expressed as mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. negative control using one-way ANOVA.

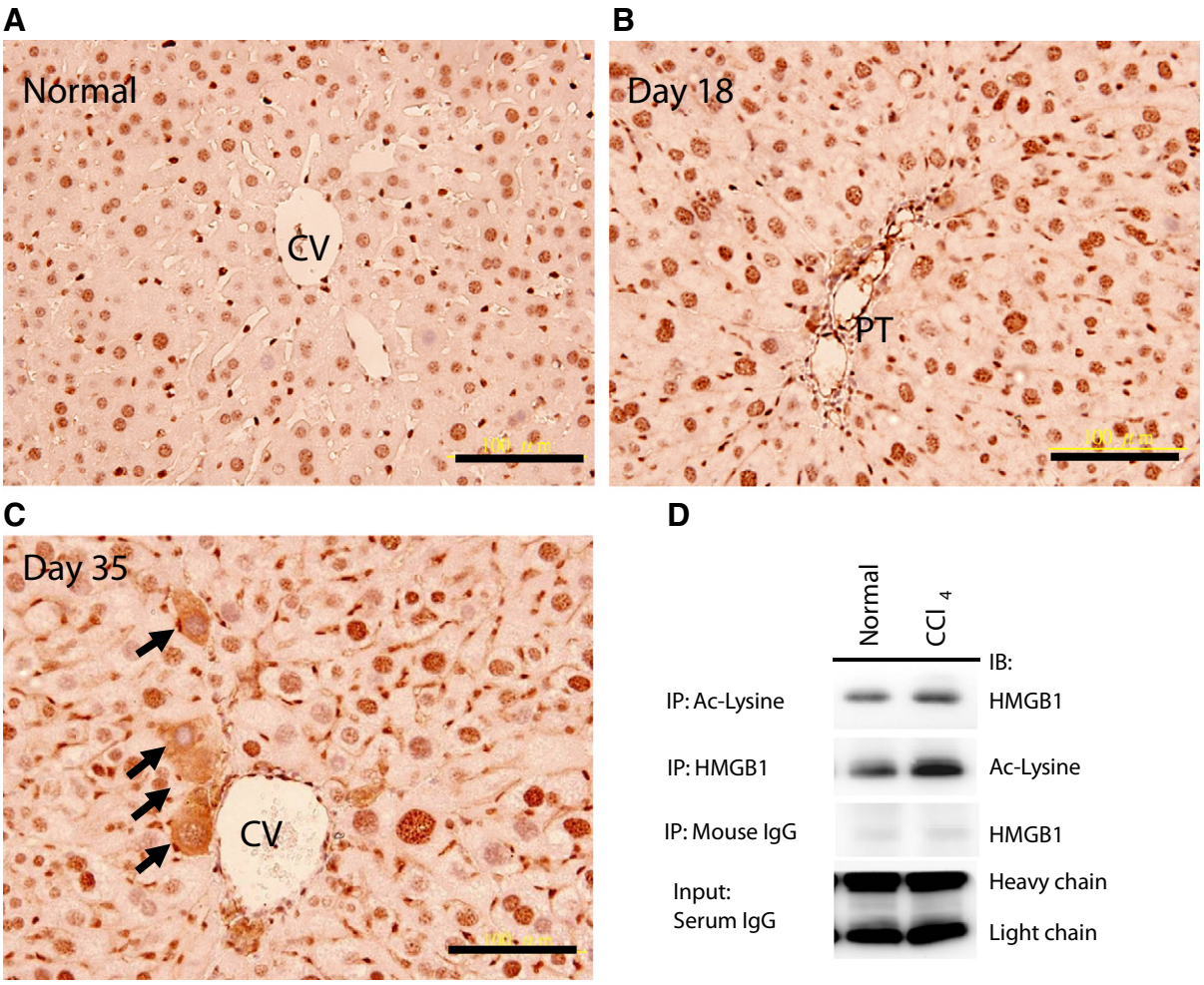

Fig. 2. Abnormal cytoplasmic localization of nuclear HMGB1 peptides in fibrotic livers and increased acetylation of HMGB1 peptides in sera of mice with CCl_4 administration. Immunohistochemistry showing nuclear localization of HMGB1 in normal mice liver (A). The abnormal subcellular distribution of HMGB1 in fibrotic mouse livers after 18 (B) and 35 (C) days of CCl_4 administration. Arrows indicate the cytoplasmic localization of HMGB1. CV, central vein; PT, portal triad. Scale bar, 100 μm. (D) Sera collected from normal mice ($n = 4$) and from the mice with CCl_4 administration for 35 days ($n = 4$) were subjected to immunoprecipitation detection by using antibodies against acetylated lysine residues, HMGB1 peptides, or isotype-matched mouse IgG, followed by immunoblotting visualization. Serum IgG was regarded as input control.

their in vitro outcomes. Cell viability assay characterized the cytotoxicity of hydrogen peroxide (H_2O_2) and CCl_4 in cultured clone-9 hepatocytes. H_2O_2 and CCl_4 at doses higher than 250 μM (Fig. 3A) and 10 mM (Fig. 3B), respectively, exhibited significant cytotoxicity. Annexin V and PI staining followed by flow cytometry assay showed remarkable induction of apoptosis and necrosis by both insults (Fig. 3C). Western blot demonstrated that CCl_4 treatment also generated intracellular oxidative stress as reflected by formation of 4-HNE and 8-OHdG adducts (Fig. 3D). Since H_2O_2 is previously demonstrated to induce active release of HMGB1 from innate immune cells [26] and hepatocytes [13], we sought to determine whether oxidative stress induces extracellular HMGB1 release; clone-9 cells were treated with H_2O_2 at 400 μM. Western blotting showed that nuclear HMGB1 peptides remarkably emerged in conditioned media of clone-9 hepatocytes after 12 h of H_2O_2 treatment, while HMGB1 contents in lysates significantly decreased after 72 h treatment (Fig. 3E). Meanwhile, the mobilization of HMGB1 from nucleus to cytoplasm was also noted in the clone-9 cells treated with H_2O_2 by using immunofluorescent staining of HMGB1 (Fig. S2). The cytoplasmic redistribution of HMGB1 was confirmed again in the primary hepatocytes (Fig. 3F) and clone-9 cells (Fig. S3) with chemical insult of CCl_4 for 24 h.

3.4. Uptake of exogenous HMGB1 peptides by cultured HSCs

To investigate whether exogenous HMGB1 could be taken up by HSCs, the recombinant HMGB1 peptides tagged with poly-histidine were added to the culture medium of HSC-T6 cells. The uptake of HMGB1 by HSC-T6 cells was first evidenced by immunofluorescent staining using anti-His tag antibody (Fig. 4A). To better characterize the time frame of HMGB1 uptake, conditioned media and lysates were collected for HMGB1 detection using Western blot (Fig. 4B). The results showed that, after 24 h of incubation, the immunoreactivities of both HMGB1 and His-tagged proteins in cell lysates encompassing both intracellular and cytoplasmic membrane compartments significantly increased, possibly due to its uptake and adherence onto cell surface, respectively. The cellular uptake of exogenous HMGB1 peaked at 100 ng/mL. Since TLR4 and RAGE are involved in endocytic uptake of HMGB1 by macrophages [27,28], we next to determine which receptor is responsible for the observed HMGB1 uptake. Genetic depletion by siRNA delivery demonstrated that RAGE gene silencing dramatically abolished the cellular uptake of HMGB1 by HSC-T6 cells (Fig. 4C). Taken with earlier findings, it raises the possibility that the release of nuclear HMGB1 protein from the parenchymal hepatocytes under oxidative stress may contribute to activation and/or transformation of HSCs in a paracrine manner during development of liver fibrosis.

3.5. Fibroproliferative effect of exogenous HMGB1 on cultured HSCs

To study whether HMGB1 contributes to HSC population expansion during progression of liver fibrosis, exogenous HMGB1 treatment was used to examine in vitro biological effect. Since posttranslational redox

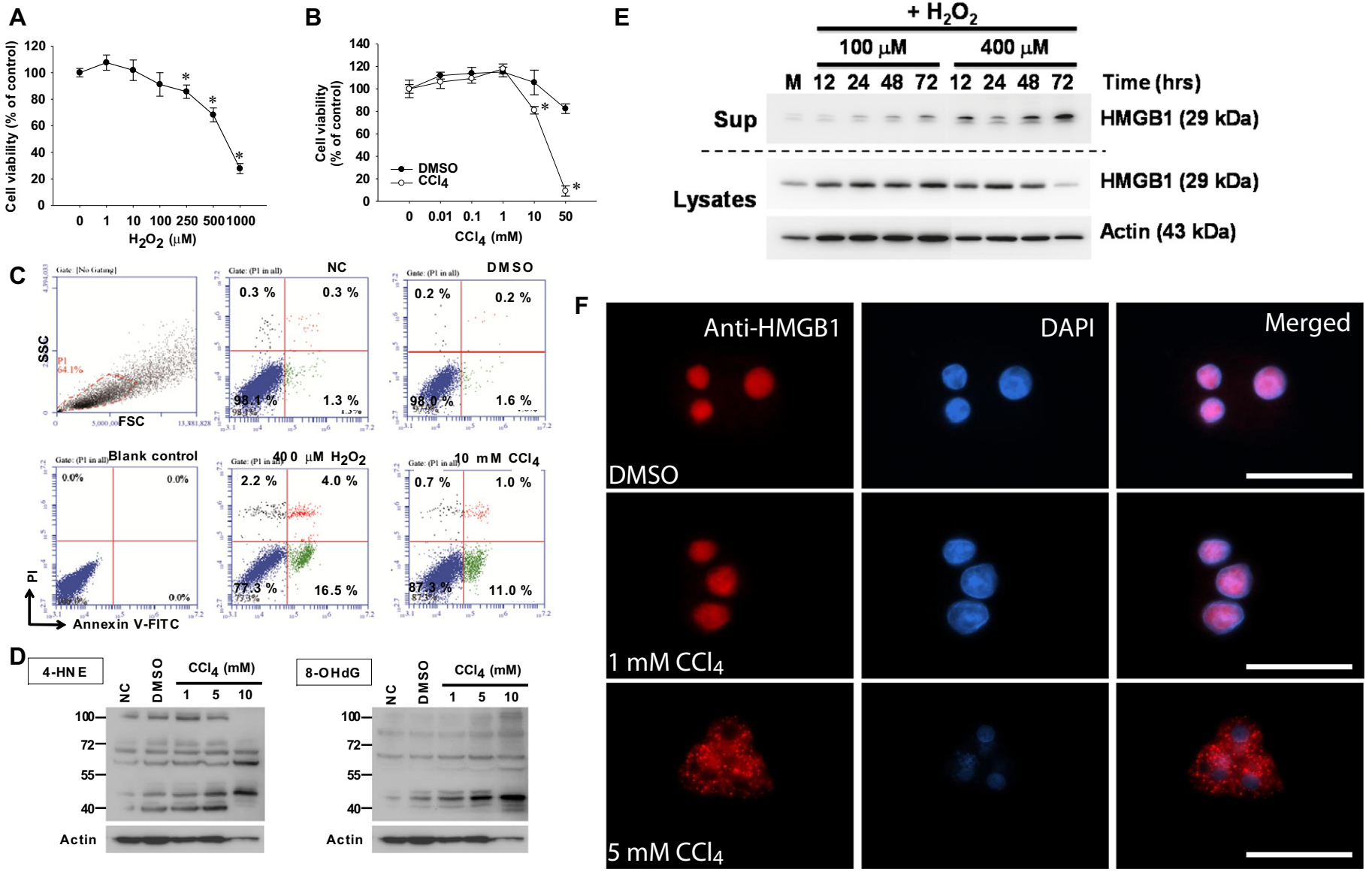

Fig. 3. Mobilization of HMGB1 from nucleus to cytoplasm and its release from cultured hepatocytes with oxidative stimulus. In vitro cytotoxic effect of H_2O_2 (A) and CCl_4 (B) on Clone-9 hepatocytes were characterized by cell viability assay. Data are expressed as mean ± SD. *$P < 0.05$ vs. negative control. (C) Induction of Clone-9 cell death by H_2O_2 and CCl_4 was confirmed by Annexin V–FITC, PI staining, and subsequent flow cytometry. (D) Clone-9 cells were treated with CCl_4 for 24 h and subjected to Western detection for oxidative stress-induced protein modifications, including formation of 4-HNE- and 8-OHdG-modified adducts. (E) Western blotting was used to measure the HMGB1 contents in culture medium (M), in supernatants (Sup) and lysates from Clone-9 hepatocytes under exposure to H_2O_2. (F) Immunofluorescent staining visualized the subcellular mobilization of HMGB1 peptides in primary hepatocytes treated with solvent DMSO or 5 mM CCl_4 for 24 h. Hoechst 33342 was used for nuclear counterstaining. Scale bar, 20 μm.

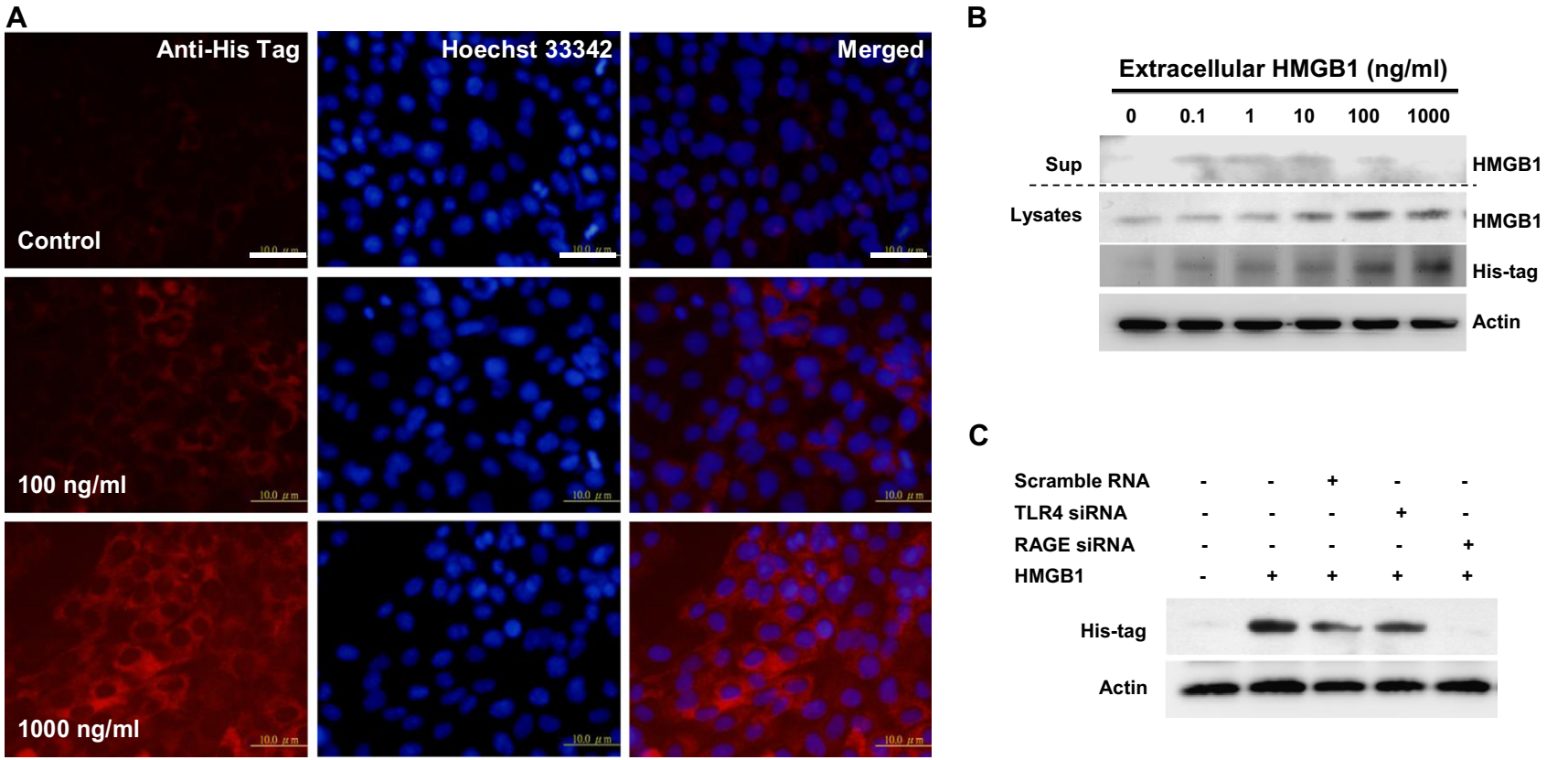

Fig. 4. Uptake of exogenous HMGB1 peptides by cultured HSC-T6 cells. (A) Immunofluorescent staining by anti-His antibody showed cytoplasmic localization of the uptake HMGB1. Scale bar, 10 μm. (B) Recombinant His-tagged HMGB1 peptides were exogenously added into cultured HSC-T6 cells for 24 h. Western blotting detected HMGB1 contents in the supernatants (Sup) and cellular lysates of HMGB1-treated cells as well as the antigenicity of His-tag in lysates. (C) HSC-T6 cells received either scramble RNA, TLR4, or RAGE siRNA transfection for 48 h, followed by exogenous treatment with recombinant His-tagged HMGB1 at 100 ng/mL. Protein lysates after 24 h of uptake were used to measure the antigenicity of His-tag by Western blot.

state of HMGB1 was recently identified to induce different inflammatory activities in immune cells [29,30], redox status of recombinant HMGB1 peptides applied in this study was characterized and all-thiol and dimer forms of HMGB1 peptides were evidenced by reduction treatment (Fig. S4). After being added into cultured HSC-T6 cells under 0.5% FBS for 48 h, the HSC growth rate was measured using MTS- (Fig. 5A) and BrdU-based (Fig. 5B) proliferation assays. Both results consistently indicated that, similar to the mitogenic effect of platelet-derived growth factor (PDGF) [8], HMGB1 also exhibited growth-stimulating effect on HSC-T6 cells. To further determine whether exogenous HMGB1 treatment affects de novo synthesis of α-SMA and COL1A1, cultured HSC-T6 cells were treated with recombinant HMGB1 peptides for 6 h and 24 h, followed by real-time qPCR and Western blotting detection, respectively. The qPCR data revealed that HMGB1 significantly increased the gene transcript contents of both *α-SMA* and *COL1A1* in a biphasic bell-shape pattern (Fig. 5C). Consistently, α-SMA and COL1A1 protein levels were also up-regulated by HMGB1 treatment (Fig. 5D). To examine the exogenous effect of HMGB1, ELISA data indicated that exogenous HMGB1 treatment significantly increased TGF-β1 production in both clone-9 (Fig. 5E) and HSC-T6 cells (Fig. 5F), suggesting the pro-fibrogenic effect of soluble HMGB1 protein through TGF-β1 pathway.

3.6. Phosphorylation and nuclear translocation of Smad2 induced by HMGB1 stimulation

Since TGF-β1 is known to up-regulate both COL1A1 and α-SMA expression in HSCs thereby leading to their activation and transformation [6,8,9], the fibroproliferative effect of HMGB1 (Fig. 5) strongly suggested that HMGB1 may directly activate or enhance the canonical TGF-β1 signaling pathway in cultured HSCs via a cross-talking mechanism. We next sought to answer whether HMGB1 induces Smad2 phosphorylation and its nuclear translocation. HSC-T6 cells were serum-starved for 1 h prior to exposure to HMGB1. Western data clearly showed that treatment with HMGB1 at 1 ng/mL or higher doses remarkably induced Smad2 phosphorylation as normalized to total Smad2 levels (Fig. 6A). A time course observation revealed that the HMGB1-induced Smad2 phosphorylation was significantly manifested at 30 min after treatment

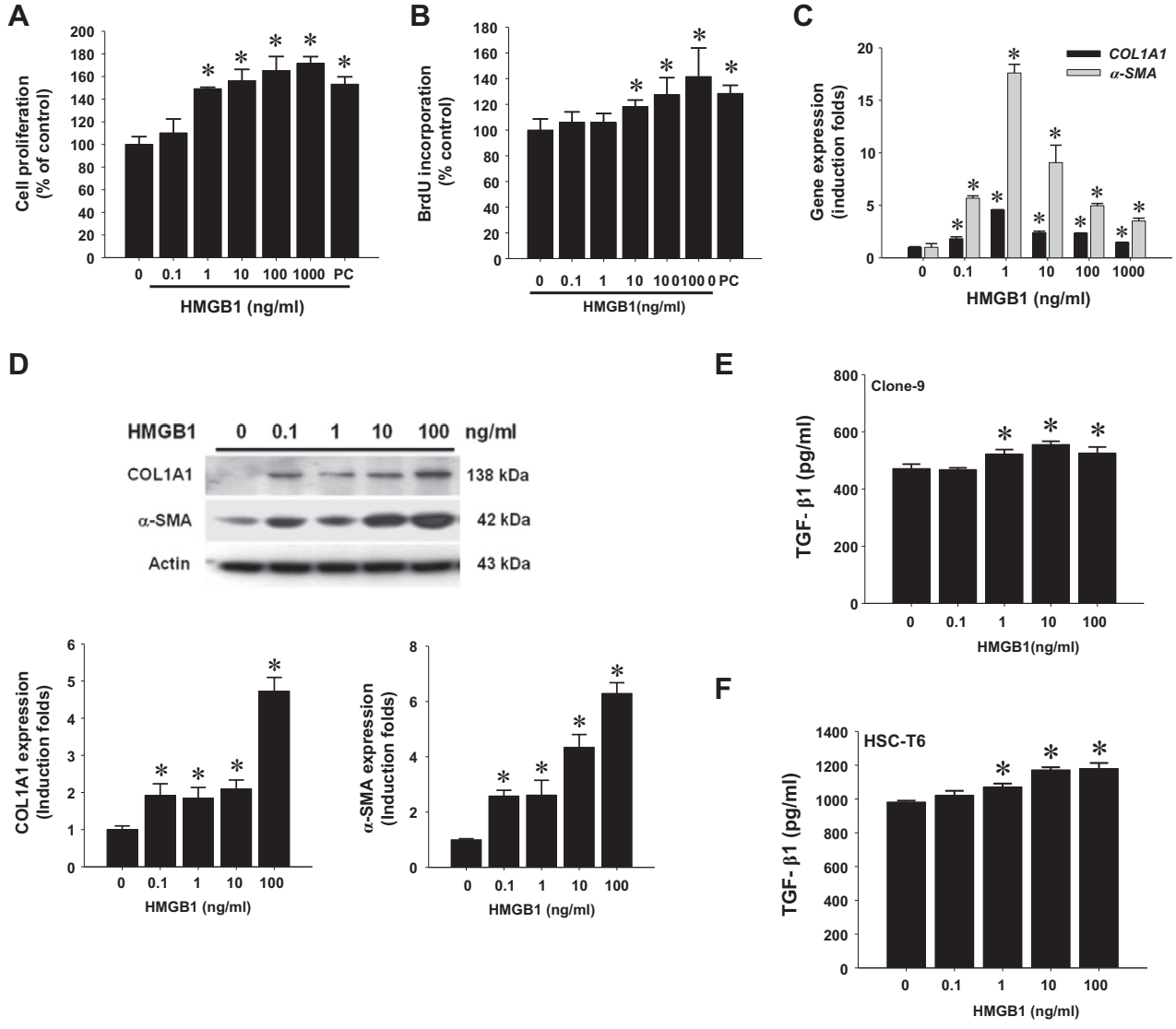

Fig. 5. Fibroproliferative effect of exogenous HMGB1 on cultured HSC-T6 cells. After 16 h of serum starvation, cultured HSC-T6 cells were treated with recombinant HMGB1 for 48 h in the presence of 0.5% FBS and subjected to MTS cell proliferation (A) and BrdU incorporation assays (B). Platelet-derived growth factor at 10 ng/mL was used as the positive control (PC) for stimulating HSC proliferation. (C) Total RNA isolated from the cells with 6 h of HMGB1 treatment was subjected to qPCR detection for *α-SMA* and *COL1A1* gene expression. (D) Representative Western blotting images showing the up-regulation of COL1A1 and α-SMA protein by HMGB1 treatment. Densitometrical analyses for COL1A1 and α-SMA protein expression. Data are expressed as mean ± SD induction folds over control. *$P < 0.05$ vs. negative control using one-way ANOVA. Conditioned media from Clone-9 hepatocytes (E) and HSC-T6 cells (F) with 24 h of HMGB1 treatment were subjected to ELISA detection for TGF-β1 levels. Data are expressed as mean ± SD. *$P < 0.05$ vs. negative control.

and thereafter (Fig. 6B). In comparison, the extent of HMGB1-induced Smad2 phosphorylation was similar to that induced by TGF-β1 (Fig. 6C). The nuclear translocation of phosphorylated Smad2 elicited by HMGB1 was confirmed by the shifting of cytoplasmic Smad2 to nuclear compartment (Fig. 6D). Moreover, the immunofluorescent staining for the Smad2 subcellular location reconfirmed that the exogenous HMGB1 peptides indeed elicited Smad2 nuclear translocation (Fig. 6E), suggesting the involvement of the canonical Smad signaling pathway in the HMGB1-induced HSC activation.

3.7. Involvement of RAGE and ERK1/2 in HMGB1-mediated HSC activation

To further delineate the profiles of HMGB1-activated non-canonical signaling activities, such as PI3K/Akt and MAPKs, HSC-T6 cells were treated with recombinant HMGB1 peptides at 10 ng/mL and subjected to Western blot detection. The results indicated that HMGB1 significantly triggered ERK1/2 phosphorylation (Fig. 7A, B), although it slightly elevated Akt and JNK phosphorylation but did not affect constitutive phosphorylation levels of p38 MAPK (Fig. S5). Since both TLR4 and RAGE, two well-recognized receptors for HMGB1, have been implicated in the pathogenesis of liver fibrosis [12,31–35], we next sought to discriminate the contribution of TLR4 or RAGE in the HMGB1-elicited HSC activation and subsequent cellular transformation. Neutralizing efficiency of antibodies was assured of their blocking effects on lipopolysaccharide-stimulated TNF-α production in murine Raw264.7 monocytes (Fig. S6). The functional blockade of TLR4 receptor using neutralizing antibodies prior to HMGB1 stimulation did not prevent the Smad2 (Fig. 7C, E) and EKR1/2 hyperphosphorylation (Fig. 7D) as well as the α-SMA over-expression (Fig. 7F) induced by HMGB1. By contrast, pretreatment with anti-RAGE antibody completely abolished the HMGB1-elicited Smad2 and ERK1/2 phosphorylation as well as the increased expression of α-SMA (Fig. 7C, D, E, F). To double check the involvement of RAGE-mediated signaling activation, cells received siRNA-mediated gene silencing of TLR4 or RAGE prior to HMGB1 stimulation and subjected to immunoblot detection (Fig. 7G). Although delivery of both TLR4 and RAGE siRNA reduced constitutive ERK1/2 phosphorylation and α-SMA expression, only genetic depletion of RAGE prevented the HMGB1-exhibited phosphorylation of Smad2 (Fig. 7H) and ERK1/2 (Fig. 7I) as well as α-SMA up-regulation (Fig. 7J). Taken together, these findings support that HMGB1 activates HSCs and stimulates their transformation via a RAGE-mediated and TGF-β1-independent Smad signaling pathway.

3.8. Amelioration of anti-RAGE in CCl_4-induced hepatic fibrogenesis

To evaluate the prophylactic effect of RAGE blockade in CCl_4-induced liver fibrosis, mice received intraperitoneal administration of an anti-RAGE neutralizing antibody at 1 week after initiation of CCl_4 injection. Data of biochemistry, qPCR, and ELISA indicated that anti-RAGE treatment significantly attenuated the impairment of liver function

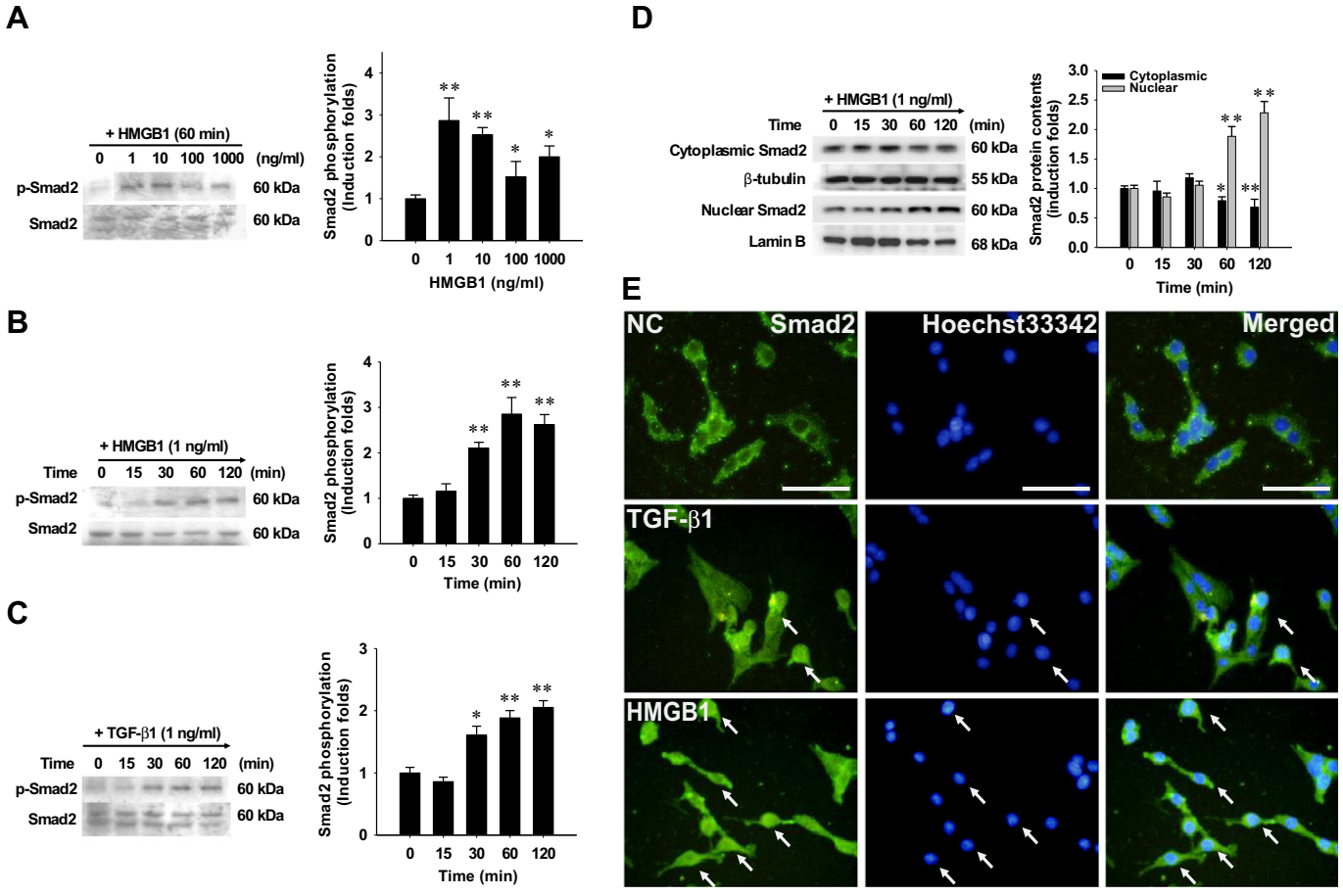

Fig. 6. Induction of Smad2 phosphorylation and its nuclear translocation in cultured HSC-T6 cells by exogenous HMGB1. After 1 h of serum starvation, cultured HSC-T6 cells were treated with recombinant HMGB1 either at indicated dose for 1 h (A) or at 1 ng/mL for indicated duration (B). Total cellular proteins were subjected to Western blotting detection using antibodies against total Smad2 and phospho-Smad2 in parallel, followed by densitometrical analysis. (C) The cells treated with 1 ng/mL of TGF-β1 were used for positive control. (D) Cytoplasmic and nuclear proteins extracted from the cells with HMGB1 at 1 ng/mL for indicated duration, were used to measure Smad2 nuclear translocation. Density data were normalized to control and expressed as mean ± SD of induction folds. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. negative control. (E) After 1 h of serum starvation, cultured HSC-T6 cells either without treatment (normal control, upper panel), with 1 ng/mL TGF-β1 (middle panel), or with 1 ng/mL HMGB1 (lower panel) were used for immunofluorescent visualization of Smad2. Arrows indicate nuclear location. Scale bar, 10 μm.

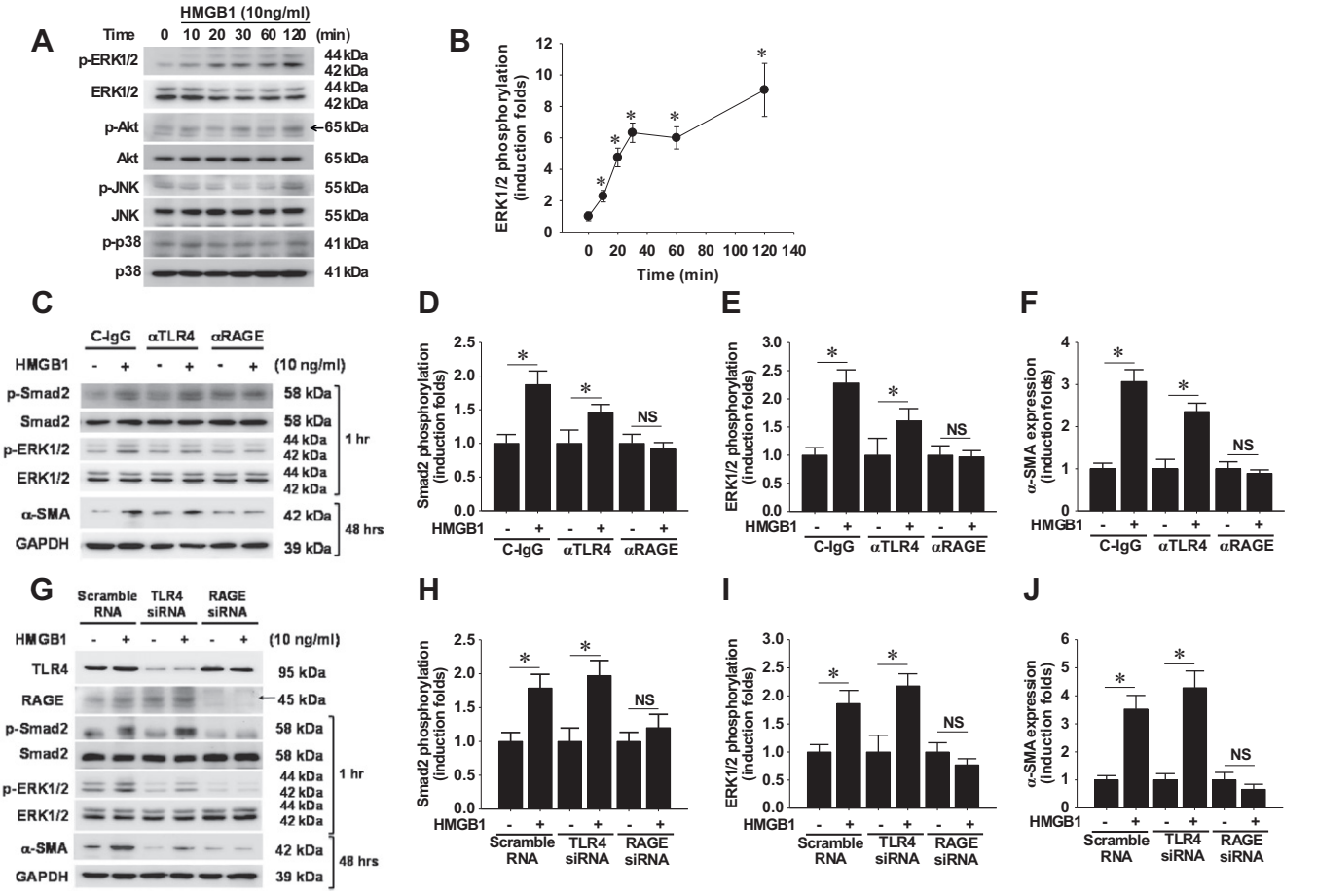

Fig. 7. Involvement of RAGE but not TLR4 in HMGB1-triggered HSC activation. Profiles of HMGB1-induced signaling activities. (A) HSC-T6 cells underwent serum starvation for 1 h before treatment with recombinant HMGB1 peptides at 10 ng/mL. Protein lysates were collected at the indicated time points and subjected to Western blot detection. Subsequent densitometrical analyses indicated that HMGB1 remarkably triggered EKR1/2 phosphorylation (B). (C) Cells were pretreated with either isotypic control IgG (C-IgG), anti-TLR4, or anti-RAGE neurtralizing antibodies at 10 μg/mL for 1 h and simultaneously with serum starvation, followed by HMGB1 stimulation. The protein lysates collected at the indicated time-points were subjected to Western blot detection for Smad2 (D) and ERK1/2 (E) phosphorylation as well as α-SMA expression (F), respectively. (G) The cells received either scramble RNA, TLR4, or RAGE siRNA transfection for 48 h, followed by HMGB1 stimulation. The protein lysates collected at the indicated time-points were subjected to Western blot detection of Smad2 (H) and ERK1/2 (I) phosphorylation as well as α-SMA expression (J), respectively. Density data are representative results from three independent experiments and shown as mean ± SD of induction folds. *$P < 0.05$ as compared between groups. NS: not significant.

(Fig. 8A), the over-expression of gene markers of liver fibrosis (Fig. 8B), as well as the elevation of serum TGF-β1 levels (Fig. 8C) in CCl_4-treated mice as compared to isotype-matched IgG control group. Western blot detection of liver extracts showed that the up-regulated α-SMA and COL1A1 protein expression and Smad2 phosphorylation were prominently reduced by anti-RAGE administration (Fig. 8D). Results of Sirius red staining (Fig. 8E) and subsequent morphometrical analysis (Fig. 8F) clearly demonstrated that functional blockade of RAGE effectively ameliorated hepatic collagen deposition during CCl_4-induced liver fibrosis.

4. Discussion

The present study provides the first evidence showing a marked elevation of serum HMGB1 levels in the mice with liver fibrosis (Fig. 1). This elevation may be attributed to the extracellular release of endogenous HMGB1 from parenchymal hepatocytes, revealed by the abnormal cytoplasmic distribution pattern of HMGB1 protein in injured livers (Fig. 2). In vitro evidence clearly indicated that, under chemical and oxidative insults, the nuclear HMGB1 peptides in both cultured primary hepatocytes and clone-9 cells were mobilized to cytoplasm and eventually released into culture medium (Fig. 3). Besides, HSCs, a pivotal propellant in the development of liver fibrosis, could uptake the exogenous HMGB1 peptides from extracellular micromilieu (Fig. 4). As a consequence, HSCs underwent fibroproliferative alterations, including cell proliferation, COL1A1 overproduction, and α-SMA up-regulation (Fig. 5). Moreover, exogenous HMGB1 mechanistically triggered Smad2 phosphorylation and its nuclear translocation in HSCs (Fig. 6). The HMGB1-elicited signaling involved not only canonical TGF-β receptor-Smad signaling pathway but also RAGE-ERK cascade (Fig. 7). Most importantly, anti-RAGE prophylactic treatment significantly ameliorated CCl_4-induced liver fibrosis (Fig. 8). Therefore, we reasonably proposed that the nuclear HMGB1 peptides released from the parenchyma of injured liver enhance the progression of liver fibrosis through a paracrine RAGE-mediated HSC-activating mechanism (Fig. 9).

The elevated circulating HMGB1 levels have been demonstrated to be a late appearing inflammatory cytokines that gives a wider time frame for clinical intervention against several infectious and inflammatory disorders such as sepsis [16–18]. On the other hand, elevation of circulating antibodies against HMGB1 was reported in patients with autoimmune diseases like rheumatoid arthritis [36]. This elevation implicates the emergence of extracellular HMGB1 in local environments, which may provide a milieu for dendritic cell activation and maturation and has been considered as a marker for disease severity. Similarly, high levels of circulating anti-HMGB1 antibodies in post orthotopic liver transplant patients are closely associated with allograft rejection and post-transplant liver fibrogenesis [37], also suggesting the profibrogenic role of HMGB1 in diseased livers. Moreover, the concurrent elevation of HMGB1 and TGF-β1 in rodent plasma found in this study

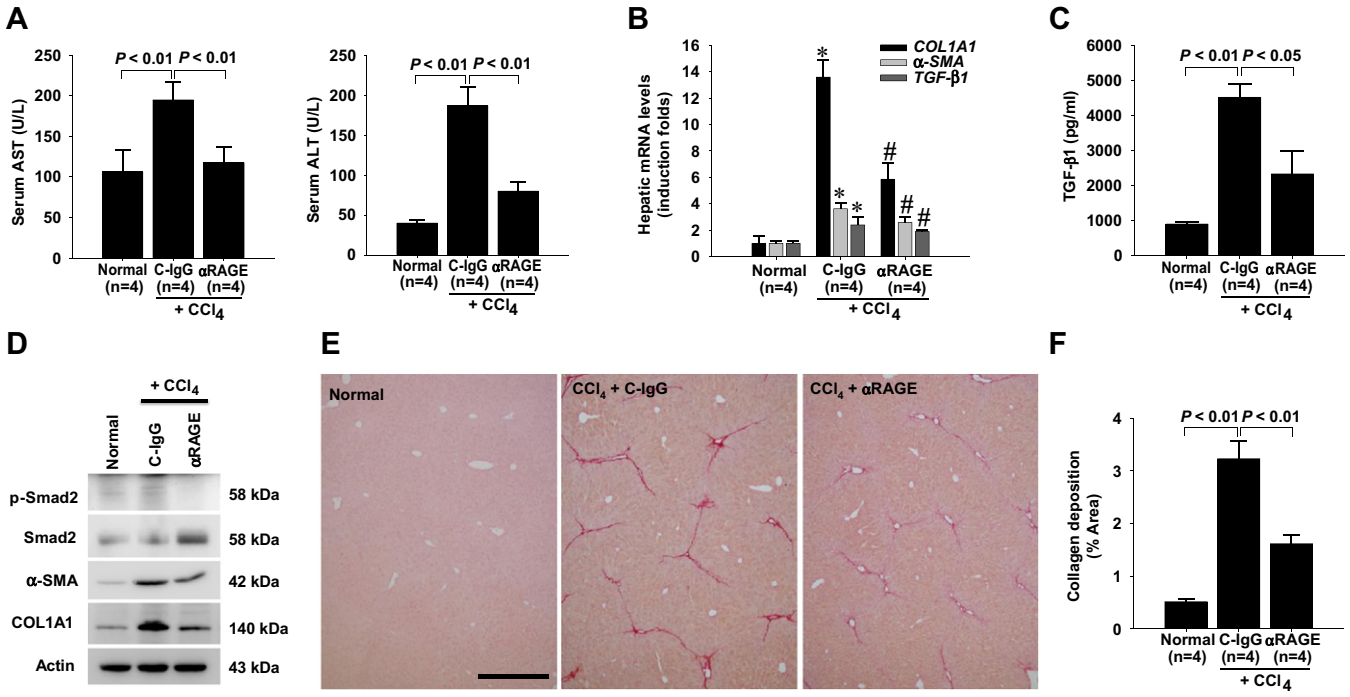

Fig. 8. Amelioration of CCl_4-induced mouse liver fibrosis by anti-RAGE treatment. ICR mice received CCl_4 administration twice weekly for 35 days and simultaneously with treatment of either anti-RAGE neutralizing antibodies or isotype-matched control IgG (C-IgG). (A) Sera were collected from either normal and those treated with and subjected to biochemical detection for liver function integrity, including AST and ALT. (B) qPCR was used to measure gene expression levels of *COL1A1*, *α-SMA*, *and TGF-β1* in normal and fibrotic mouse livers. (C) ELISA detection for serum TGF-β1 levels. (D) Western blotting detection for Smad2 phosphorylaton, α-SMA, and COL1A1 proteins. (E) Representative Sirius red staining images of normal and fibrotic mouse liver sections. (F) Morphometry analysis for the Sirius red-visualized collagen deposition in mouse livers. Data are expressed as mean ± SEM. $^{*}P < 0.05$ vs. normal group; $^{\#}P < 0.05$ vs. C-IgG group.

(Fig. 1) also implies a mutual regulatory mechanism between these two factors. This speculation could be at least in part supported by the fact that exogenous HMGB1 stimulated TGF-β1 production in clone-9 and HSC-T6 cells (Fig. 5E, F). On the other hand, TGF-β1 and other pro-inflammatory cytokines such as tumor necrosis factor-α have been demonstrated to increase HMGB1 expression and secretion by macrophages [12,38,39]. Whether these cytokines are also critical upstream regulators for HMGB1 production and/or release in hepatocytes remains further elucidation.

Although exogenous HMGB1 was recently reported to prevent post-infarction myocardial remodeling and improve myocardial recovery through suppressing TGF-β1/Smad signaling [40,41], the pathogenetic role of HMGB1 has been implicated in a number of organs like pulmonary tissue injury [22] and the ischemia and reperfusion injuries of retina [21] and livers [14]. Indeed, the elevated serum HMGB1 has been significantly noted in the patients with chronic hepatitis B virus infection and low-grade fibrosis [42]. The present study further demonstrated that exogenous HMGB1 stimulated HSC proliferation and up-regulated α-SMA

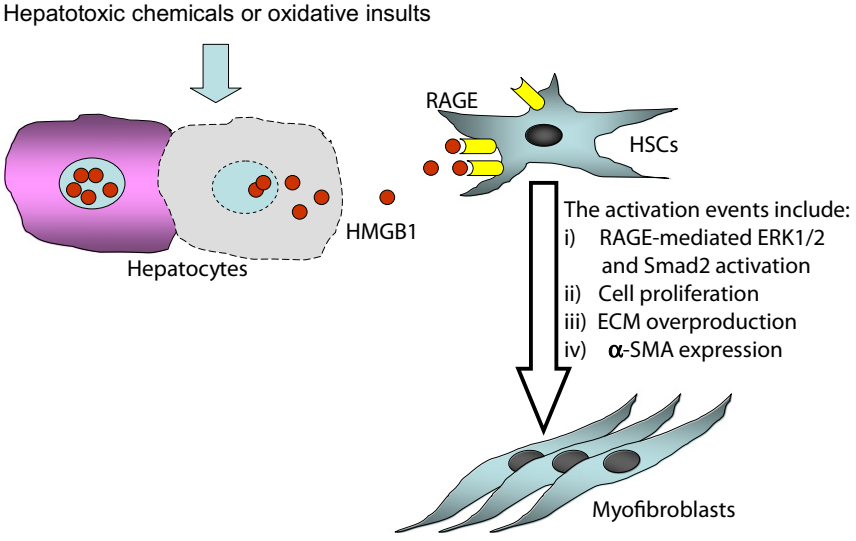

Fig. 9. Hypothetical scheme showing the pathogenetic role of HMGB1 released from injured hepatocytes. When the liver encounters the insults from hepatotoxic chemicals or oxidative stresses, nuclear HMGB1 peptides were mobilized through cytoplasm into extracellular environment as an alarmin. This released HMGB1 molecule may, in a paracrine manner, trigger hepatic stellate cell (HSC) activation through upregulating the signal pathways involving RAGE-dependent Smad2 and EKR1/2 phosphorylation. Under exposure to HMGB1, HSCs altered their behaviors, including an increase in cellular proliferation; ECM over-production; and transformation into myofibroblasts.

and COL1A1 expression (Fig. 5), consistent to the previous report showing that HMGB1 promotes proliferation and migration of HSCs [43]. Additionally, we previously reported that exogenous HMGB1 peptides remarkably decreased gelatinolytic activity of MMP-2 in cultured HSC-T6 cells, which also implicates its tissue remodeling capacity in livers [44]. On the other hand, HMGB1 siRNA treatment suppressed synthesis of α-SMA and collagen types I and III in HSC-T6 cells [45], supporting its pro-fibrogenic role in liver fibrosis. Due to the fact that HMGB1 functionally possesses binding ability to either TLR2, TLR4, or RAGE [12], the pro-fibrogenic effect of HMGB1 has been mechanistically linked to the signaling activities driven by multiple cognate receptors. Controversially, the HMGB1-elicited HSC activation evidently involves the signal pathways mediated not only by TLR4 [33,43,46] but also RAGE [8,34,47].

In the context of the HSC activation governed by TGF-β-elicited signaling, Lindert et al. [48] reported that the phenotypic changes of transdifferentiating HSCs (i.e., α-SMA expression) and the ECM overproduction are two independent processes, while the latter could be stimulated by both Smad-dependent and MAPK-dependent TGF-β1 signaling. Since TGF-β1 canonical Smad and non-canonical p38 MAPK are two important intracellular signaling pathways governing pro-inflammatory cytokine production and ECM biosysnthesis, the cross-talk between MAPK- and Smad-mediated signaling pathways has been known to enhance TGF-β-dependent responses in mesangial cells [49] and carcinogenesis [50]. In fact, the HMGB1-triggered p38 MAPK activation in rodent macrophages [51] and human neutrophils [52] has been demonstrated to be primarily mediated through the RAGE pathway. Other lines of evidence indicate that the HMGB1/RAGE signaling axis plays an important role in lung fibrogenesis [22,31]. The data in the present study support that HMGB1 triggers a TGF-β-independent Smad2 phosphorylation, its subsequent nuclear translocation (Fig. 6), and a concomitant ERK1/2 activation (Fig. 7). Consistent to our findings, the RAGE-driven ERK1/2-dependent Smad2/3 phosphorylation has been manifested not only in HSCs [8], but also in liver myofibroblasts [7] and kidney tubular epithelial cells [53]. Our data further support the notion that the HMGB-driven α-SMA upregulation in cultured HSCs was RAGE-dependent and involved ERK activity (Fig. 7). Supportively, the higher and restrictive expression of RAGE on the HSCs of fibrotic liver highlights the association of exogenous HMGB1 peptides with the HSC activation and its subsequent transition to myofibroblasts [54]. These findings suggest that exogenous HMGB1 peptides might convey activating signal via the RAGE on HSCs. Moreover, recent evidence indicated that HMGB1 acts through RAGE and triggers dynamin-dependent signaling and subsequent HMGB1 endocytosis, which eventually results in cell pyroptosis of macrophages [28]. Consistent with our data showing the RAGE-dependent cellular uptake of HMGB1 by HSC-T6 cells (Fig. 4C) and based on the effectiveness of prophylactic anti-RAGE therapy (Fig. 8), the mechanism of RAGE-mediate HMGB1 endocytosis may play an important role in the HSC activation and the pathogenesis of liver fibrosis. However, this issue awaits further clarification in the future.

In conclusion, this study demonstrated extracellular release of nuclear HMGB1 peptides from the parenchymal cells of the experimentally induced fibrotic livers. The release of HMGB1 peptides from injured tissues not only modulates immune responsiveness, but also participates in tissue remodeling and fibrogenesis. A paracrine action of the soluble HMGB1 peptides between hepatocytes and HSCs, at least in part, contributes to the development of liver fibrosis mainly through activating a TGF-β1-independent pro-fibrogenic signal pathway in HSCs, including RAGE/ERK-mediated Smad2 cascade. Blockade of the bioavailability of extracellular HMGB1 and downstream RAGE-mediated signaling in targeted cells may constitute a therapeutic strategy for the treatment of liver fibrosis.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2014.06.017.

Acknowledgments

The authors thank Ms. Chun-Pzu Hung, Shang-Chieh Lu, Chia-Wei Lin and Ming-Chu Hsieh for their excellent technical assistance. This study was supported in part by the grants from EDA Hospital (EDAHT-99011 to Y.H.K.) and Kaohsiung Chang Gung Memorial Hospital (CMRPG-860191 to B.J.).

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