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Apamin inhibits hepatic fibrosis through suppression of transforming growth factor β1-induced hepatocyte epithelial–mesenchymal transition

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ABSTRACT

Apamin is an integral part of bee venom, as a peptide component. It has long been known as a highly selective block Ca^{2+}-activated K^+ (SK) channels. However, the cellular mechanism and anti-fibrotic effect of apamin in TGF-β1-induced hepatocytes have not been explored. In the present study, we investigated the anti-fibrosis or anti-EMT mechanism by examining the effect of apamin on TGF-β1-induced hepatocytes. AML12 cells were seeded at ~60% confluence in complete growth medium. Twenty-four hours later, the cells were changed to serum free medium containing the indicated concentrations of apamin. After 30 min, the cells were treated with 2 ng/ml of TGF-β1 and co-cultured for 48 h. Also, we investigated the effects of apamin on the CCl_4-induced liver fibrosis animal model. Treatment of AML12 cells with 2 ng/ml of TGF-β1 resulted in loss of E-cadherin protein at the cell–cell junctions and concomitant increased expression of vimentin. In addition, phosphorylation levels of ERK1/2, Akt, Smad2/3 and Smad4 were increased by TGF-β1 stimulation. However, cells treated concurrently with TGF-β1 and apamin retained high levels of localized expression of E-cadherin and showed no increase in vimentin. Specifically, treatment with 2 μg/ml of apamin almost completely blocked the phosphorylation of ERK1/2, Akt, Smad2/3 and Smad4 in AML12 cells. In addition, apamin exhibited prevention of pathological changes in the CCl_4-injected animal models. These results demonstrate the potential of apamin for the prevention of EMT progression induced by TGF-β1 *in vitro* and CCl_4-injected *in vivo*.

1. Introduction

Liver fibrosis is characterized by excessive deposition of extracellular matrix (ECM) in the liver during chronic injury. This disease is initiated when liver injury stimulates cells in the liver to synthesize and secrete proteins and other soluble mediators [1]. It has been well documented that activated fibroblasts are key contributors to liver fibrosis. Also, some evidence suggests that adult hepatocytes play a role by way of epithelial mesenchymal transition (EMT) in the accumulation of activated fibroblasts [2,3].

The EMT is a dynamic cellular program in which polarized epithelial cells lose epithelial properties, undergo morphological changes, and acquire mesenchymal characteristics [4]. This phenotypic change generates functionally distinct cell types and an increased capacity for cell migration [5]. Hepatocytes can transdifferentiate into mesenchymal cells by EMT and deposit collagen in the liver during chronic injury [6]. During EMT, intercellular junctions of epithelial cells are interrupted or decreased by down-regulation of adhesion molecules E-cadherin and tight junction component ZO-1 [7]. EMT is also characterized by morphological changes from epithelial to fibroblast-like with up-regulation of mesenchymal markers, including fibronectin and vimentin [8].

Many cytokines and growth factors are involved in EMT [9]. For example, transforming growth factor (TGF)-β1 is recognized as a major pro-fibrogenic cytokine in liver disease [10]. TGF-β1 triggers EMT primarily via canonical Smad-dependent mechanism, which requires two types of receptor kinases and a family of signal transducers called R-Smads (Smad2 and 3). Upon phosphorylation, R-Smads form complexes with a common partner (Smad4) and subsequently translocate into the nucleus to regulate the transcription of target genes responsible for EMT [11,12]. Furthermore, TGF-β1 has the ability to signal in a Smad-independent manner following pathways most commonly associated with receptor

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tyrosine kinases. It can also cooperate with mitogen-activated protein kinases (MAPKs) and phosphoinositide-3 kinase (PI3K)/Akt signaling pathways in a tissue-specific fashion [13,14]. Likewise, TGF-β1 expression is also associated with morphologic alterations like EMT in fetal and adult hepatocytes [11], and changes in survival signaling pathway [15], but these cellular events have not been fully elucidated in the hepatocytes [16]. Thus, exploring the mechanisms of EMT is of importance in developing new and efficacious therapies for the treatment of liver disease [17]. In addition, strategies aimed at disrupting TGF-β1 production and/or blocking signal transduction with particular proteins or small molecules have important theoretical and practical implications for producing effective treatments for liver disease [18].

Apamin is the smallest neurotoxin integral part of bee venom, comprising about 2–3% of its dry weight [19]. It has long been known as a highly selective blocker of Ca^{2+}-activated K^+ (SK) channels [20]. These channels link intracellular calcium transients to changes of the membrane potential by promoting K^+ efflux following increases of intracellular calcium during an action potential [21]. We previously demonstrated that apamin efficiently inhibited the expression of specific genes in the animal model of atherosclerosis [22]. We also revealed that apamin inhibits oxLDL-induced THP-1-derived macrophage apoptosis via mitochondria-related apoptotic pathway [23]. However, the cellular mechanism and the anti-fibrosis effect of apamin in TGF-β1-induced hepatocytes have not been explored. Therefore, we investigated the anti-fibrosis or anti-EMT mechanism by examining the effect of apamin on TGF-β1-induced hepatocytes. Furthermore, the molecular pathogenesis of anti-fibrosis effects of apamin was investigated by CCl_4-injected animal model.

2. Materials and methods

2.1. Cell culture

AML12 murine hepatocytes were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium/Ham's F-12 medium (Gibco, NY, USA) supplemented with 10% fetal bovine serum and 1% antibiotics (Gibco, NY, USA). Cells were cultured in humidified incubator at 37 °C in a 5% CO_2 atmosphere. AML12 cells were seeded at ~60% confluence in complete growth medium. Twenty-four hours later, the cells were changed to serum free medium containing the indicated concentrations of apamin (0.5, 1 and 2 μg/ml; Sigma, MO, USA). After 30 min, the cells were treated with 2 ng/ml of TGF-β1 (R&D System, MN, USA) and co-cultured for 48 h. Following this, the cells were collected for further experiments.

2.2. Western blotting

Western blotting was performed as previously described [24]. The primary antibodies, which are listed as follows, anti-E-cadherin, anti-ERK1/2, anti-phospho-ERK1/2, anti-JNK, anti-phospho-JNK, anti-p38 and anti-phospho-p38, were purchased from Cell Signaling Technology (MA, USA). Also, anti-vimentin from Abcam BD biosciences (CA, USA), and anti-Smad2/3, anti-phospho-Smad2/3, anti-Smad4, and anti-GAPDH from Santa Cruz (CA, USA) were used.

2.3. Immunocytochemistry

AML12 cells were grown on chamber slides and were fixed with 3.7% paraformaldehyde in PBS for 30 min. Cells were permeated with 0.5% Triton for 15 min and were incubated with primary antibodies against E-cadherin (Cell Signaling Technology, MA, USA) and vimentin (BD biosciences, CA, USA) for 1 h at room temperature. After washing, they were incubated with the secondary antibodies (Alexa Flour 488 and/or Alexa Fluor 594) for 30 min at room temperature. Cells were counterstained with Hoechst 33342. Immunolabeling was examined by an Eclipse 80i microscope (Nikon, Tokyo, Japan).

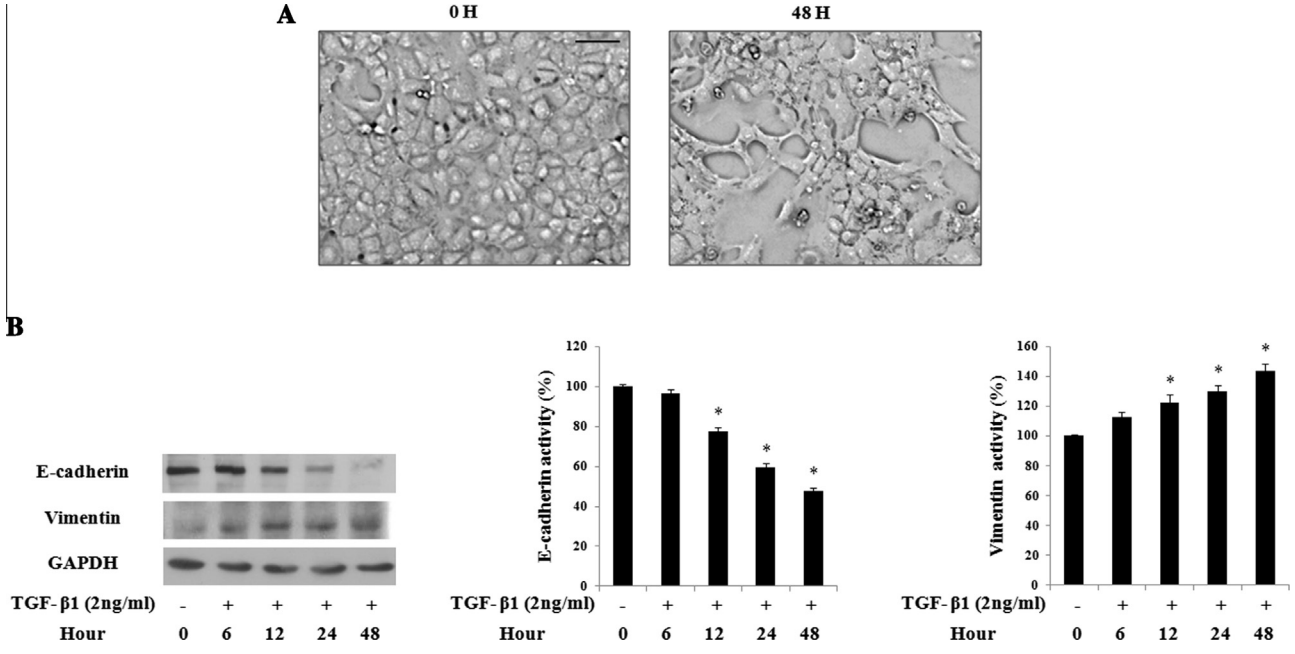

Fig. 1. TGF-β1 was capable of inducing EMT in AML12 cells. (A) Time effects of TGF-β1 on EMT were examined by morphologic changes in 2 ng/ml of TGF-β1-treated AML12 cells. (B) Time effects of TGF-β1 on the expression levels of EMT markers, including E-cadherin and vimentin determined by Western blotting. Magnification ×400, scale bar = 100 μm. GAPDH was used to confirm equal sample loading. Results are expressed as mean ± S.E. of three independent determinations. $^*p < 0.05$ compared to 0 h.

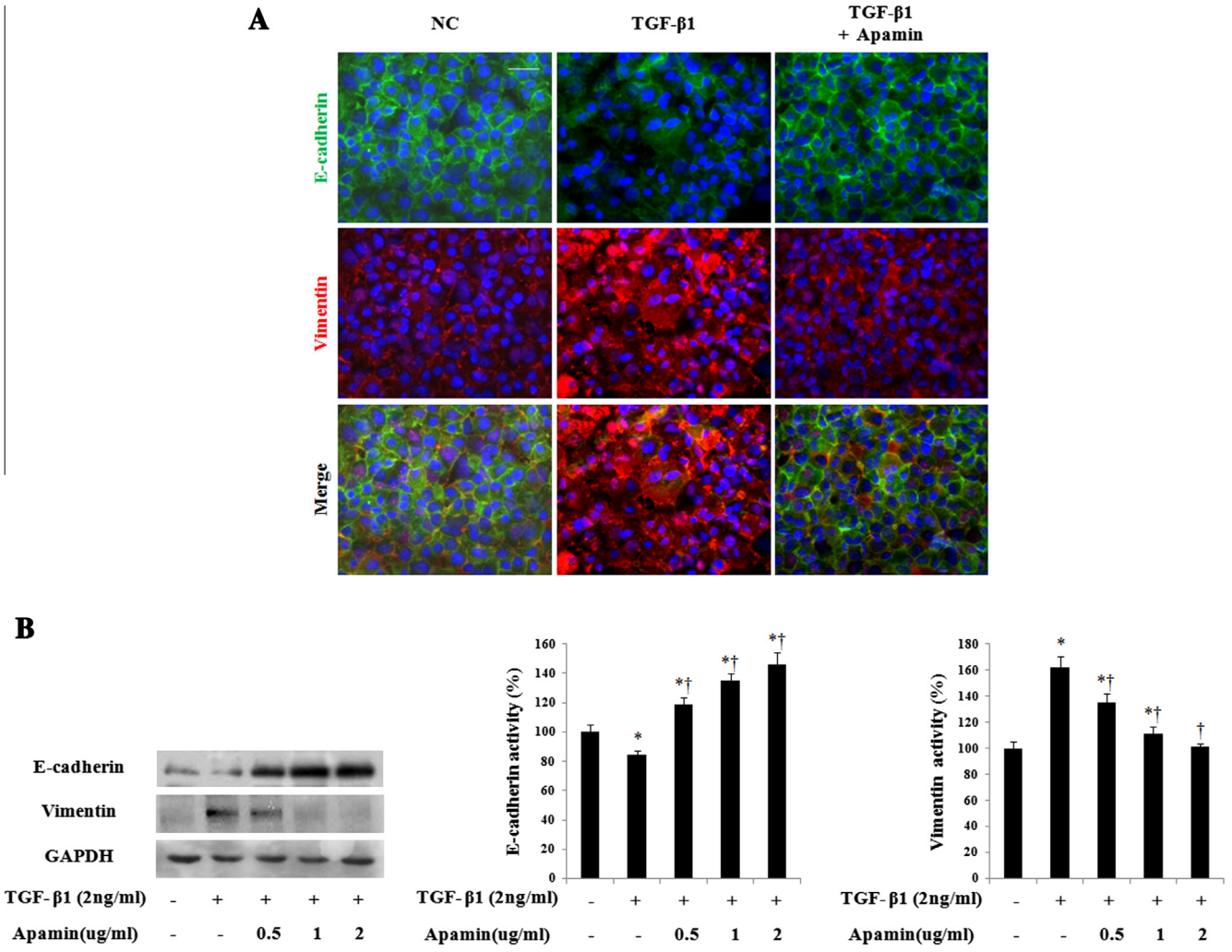

Fig. 2. Apamin suppressed EMT in AML12 cell. AML12 cells were cultured with or without 2 ng/ml of TGF-β1 in the presence or absence of apamin for 48 h. (A) Immunofluorescence staining shows the effects of apamin on the inhibition of TGF-β1-induced changes in EMT markers, including E-cadherin and vimentin. E-cadherin and vimentin immune complexes were detected by anti-mouse FITC (green), anti-rabbit Texas Red (red), and nuclei were stained with Hoechst 33342 (blue). (B) Western blotting results show the effects of apamin on the inhibition of TGF-β1-induced changes in EMT markers, including E-cadherin and vimentin. Magnification ×400, scale bar = 100 μm. GAPDH was used to confirm equal sample loading. Results are expressed as mean ± S.E. of three independent determinations. $^{*}p < 0.05$ compared to normal cells. $^{\dagger}p < 0.05$ compared to TGF-β1-treated cells only. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.4. Animal models

Six-week-old C57BL/6 male mice ($n = 15$) were randomly subdivided into three groups (5 mice/group) and were maintained under various conditions. The normal control group (NC) had free access to standard mouse laboratory chow diet. For hepatic damage group, mice received three intraperitoneal injections of carbon tetrachloride (CCl_4: 2 ml/mg CCl_4 dissolved in corn oil with 1:3 ratio) a week for 8 weeks. Animals in the CCl_4/Apa group were given intraperitoneal injection of CCl_4 three times a week with apamin (0.05 mg/kg) injection into the celiac plexus twice a week for 8 weeks. The animals were sacrificed by cervical dislocation and the tissues were excised. All surgical and experimental procedures used in the current study were approved by the institutional review board committee of Catholic University of Daegu Medical Center.

2.5. Histological and immunohistochemistry

Hematoxylin and eosin (H&E), Masson's trichrome, immunohistochemical and immunofluorescent staining were performed according to the described procedure [24,25]. Sections were stained with H&E and Masson's trichrome. For immunohistochemical analysis, sections were incubated with anti-TGF-beta1 (R&D System, MN, USA), and anti-fibronectin (Santa Cruz, CA, USA) for 1 h at 37 °C, processed by an indirect immuneperoxidase technique using a commercial kit (DAKO, CA, USA).

2.6. Statistical analysis

One-way ANOVA was performed using Tukey's post hoc test for comparison of different groups. Results were expressed as means ± SE. and $p < 0.05$ was considered as statistical significance.

3. Results

3.1. TGF-β1 induced EMT in AML12 murine hepatocyte

We examined whether TGF-β1 was capable of inducing EMT in AML12 murine hepatocyte. AML12 cells were treated with or without TGF-β1 and time-lapse images were collected at 0 and 48 h using a phase-contrast microscopy. Fig. 1A shows that normal

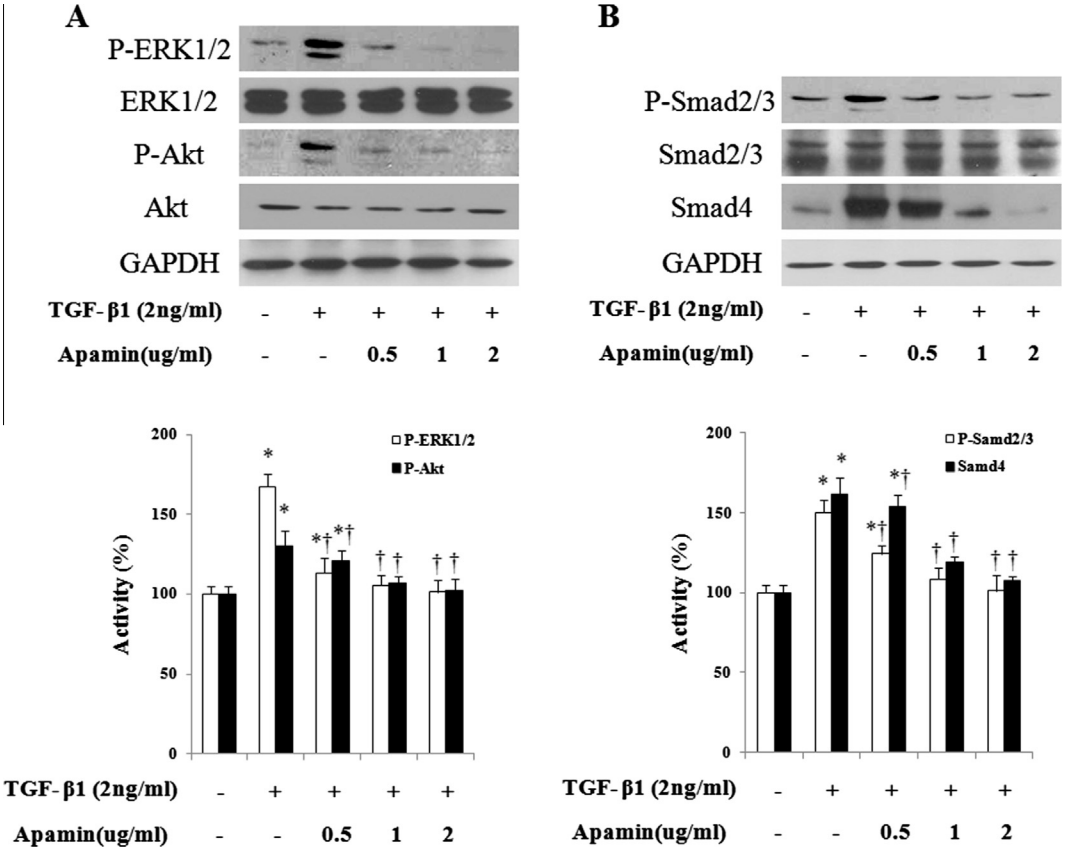

Fig. 3. Apamin suppressed the Smad-independent and Smad-dependent signaling pathways. AML12 cells were cultured with or without 2 ng/ml of TGF-β1 in the presence or absence of apamin for 48 h. (A) Western blotting results show the effects of apamin on the inhibition of Smad-independent signaling pathway, including phosphorylated ERK1/2 and Akt. (B) Western blotting results show the effects of apamin on the inhibition of Smad-dependent signaling pathway, including phosphorylated Smad2/3 and Smad4. GAPDH was used to confirm equal sample loading. Results are expressed as mean ± S.E. of three independent determinations. $^{*}p < 0.05$ compared to normal cells. $^{\dagger}p < 0.05$ compared to TGF-β1-treated cells only.

AML12 cells have a typical epithelial phenotype with polygonal morphology and tight arrangement. However, when exposed to 2 ng/ml of TGF-β1 for 48 h, AML12 cells underwent EMT in which cells lost their epithelial honeycomb-like morphology and obtained a spindle-like shape. We also determined the expression levels of E-cadherin and vimentin in TGF-β1-treated AML12 cells (Fig. 1B). Downregulation of E-cadherin, the well-known EMT marker was observed during TGF-β1 treatment in time-dependent manner. Whereas, TGF-β1 treatment upregulated vimentin expression levels in AML12 cells, indicating that murine hepatocytes acquired a mesenchymal phenotype. These data indicate that TGF-β1 mediates the morphologic changes that are compatible with the induction of EMT in AML12 cell.

3.2. Apamin effectively inhibited the TGF-β1-induced EMT in AML12 murine hepatocyte

To investigate the effect of apamin in TGF-β1-induced EMT in AML12 murine hepatocyte, cells were treated with different concentrations of apamin in the presence of TGF-β1. As illustrated in Fig. 2A, immunofluorescence microscopy in normal heaptocytes demonstrated the localized expression of epithelial marker E-cadherin at the cell border and relatively low levels of expression of the mesenchymal marker vimentin. However, treatment of AML12 cells with 2 ng/ml of TGF-β1 resulted in loss of E-cadherin protein at the cell–cell junctions and concomitant increased expression of vimentin. Cells treated concurrently with TGF-β1 and 2 μg/ml of apamin retained high levels of localized expression of E-cadherin and showed no increase in vimentin. Western blot analysis of expression levels of epithelial and mesenchymal markers corroborated the immunofluorescence data. As shown in Fig. 2B, E-cadherin was downregulated and the expression of vimentin was dramatically elevated in TGF-β1-treated AML12 cells. On the other hand, 2 μg/ml of apamin treatment significantly increased the expression of E-cadherin and reduced the vimentin expression in TGF-β1-treated AML12 cells. These results demonstrate that apamin plays an important role in suppressing TGF-β1-induced EMT and in maintaining the epithelial phenotype of AML12 murine hepatocytes.

3.3. Apamin effectively inhibited the Smad-independent and Smad-dependent signaling pathways

To further explore the intracellular signal mechanism, we examined the effects of apamin on the Smad-independent pathway. MAPK and PI3K signaling pathways play a key role in inducing and maintaining EMT [26]. Accordingly, we hypothesized that apamin might downregulate E-cadherin and/or vimentin expressions by inhibiting MAPK and/or PI3K signaling. Fig. 3A showed that the phosphorylation levels of ERK1/2 and Akt were increased by 2 ng/ml of TGF-β1 stimulation. However, addition of apamin reduced ERK1/2 and Akt phosphorylation in the TGF-β1-treated AML12 cells. Specifically, addition of apamin at the concentration of 2 μg/ml almost completely blocked the phosphorylation of ERK1/2 and Akt following TGF-β1 treatment on AML12 cells. These results indicate that treatment with apamin abrogated the effect of

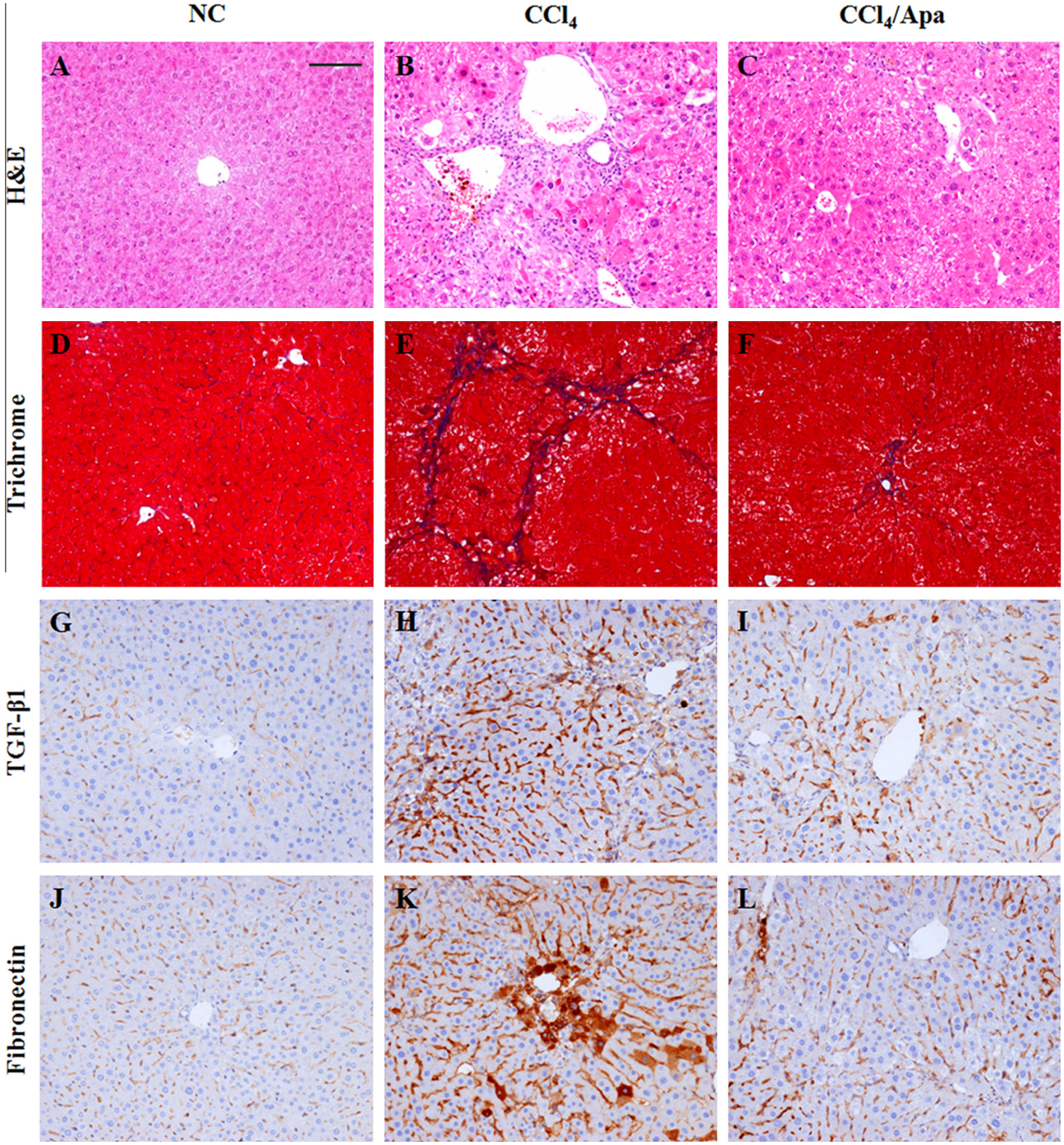

Fig. 4. Effect of apamin on CCl_4-induced liver fibrosis animal model. Apamin attenuated pathological changes and fibrotic cytokine expressions in the liver fibrosis animal model. Representative histological sections of murine liver with hematoxylin and eosin stain (A–C) and Masson's trichrome stain (D–F). Typical examples of immunohistochemical staining of TGF-β1 (G–I) and fibronectin (J–L). NC: Normal control group. CCl_4: CCl_4 (2 ml/mg CCl_4 dissolved in corn oil with 1:3 ratio) was intraperitoneally injected three times a week for 8 weeks. CCl_4/Apa: Intraperitoneal injection of CCl_4 three times a week with apamin (0.05 mg/kg) injection into the celiac plexus twice a week for 8 weeks. Magnification ×200, scale bar = 50 μm.

TGF-β1 stimulation on altering the expression levels of genes, which are relevant to EMT through MAPK and PI3K signaling. We next analyzed the effects of apamin on the Smad-dependent pathway. Fig. 3B demonstrates that phosphorylated Smad2/3 and Smad4 were dramatically increased after treatment with TGF-β1. However, phosphorylated Smad2/3 was significantly decreased after concomitant treatment with apamin in a dose-dependent manner. At the same time, the expressive amount of Smad4 was also decreased in AML12. These results suggest that apamin modulated Smad-independent and Smad-dependent signaling pathways in TGF-β1 induced AML12 cells.

3.4. Apamin suppressed liver pathological changes and fibrotic cytokine expression in the CCl_4-induced liver fibrosis animal model

These *in vivo* observations and further demonstrated that EMT in hepatocyte was seen in CCl_4-injected animal model. Histological analysis was conducted to examine the effects of apamin in the

liver fibrosis animal model. The morphological changes of liver injury and fibrosis caused by CCl_4 were visualized in the sections stained with H&E (Fig. 4A–C) and trichrome (Fig. 4D–F). As shown in Fig. 4A and D, NC group illustrated a normal lobular architecture with central veins. The morphology of the liver parenchymal cell was good with no congestion and inflammation in the sinusoids. However, in the CCl_4 group, it showed severe pathological abnormalities including increased fibrous septa, ballooning changes of hepatocytes and multifocal hepatocellular necrosis (Fig. 4B). Also, collagen fibers visualized by trichrome staining were distinctly deposited in the CCl_4 group (Fig. 4E). Importantly, the area and extent of necrosis were attenuated and the collagen deposition was decreased by apamin treatment in the CCl_4/Apa group (Fig. 4C and F). Subsequently, immunohistochemical stains (Fig. 4G–L) were performed to evaluate the impact of apamin in the regulation of expression of the genes relevant to liver fibrogenesis, including TGF-β1 and fibronectin. As shown in Fig. 4G and J, the expression levels of TGF-β1 and fibronectin were barely detected in the NC group. However, administration of CCl_4 showed significant upregulation of these expression levels observed in CCl_4 group (Fig. 4H and K), while treatment with apamin led to the evident downregulation of TGF-β1 and fibronectin expressions (Fig. 4I and L). These results demonstrated that apamin exhibited prevention of pathological changes in the CCl_4-injected animal models.

4. Discussion

EMT has been recognized as an important process in liver development for a long time, and recent data show that EMT plays substantial roles in liver fibrosis as well [27,28]. It has been shown that certain adult liver cell types are capable of undergoing EMT *in vivo* and excessive EMT during liver repair is fibrogenic which can cause liver fibrosis [29]. Recently, evidence showed that TGF-β1 is a major inducer of liver fibrosis and cirrhosis and studies of TGF-β1-induced EMT and apoptosis in mouse hepatocyte are of great importance for understanding the mechanism of the progress of liver cirrhosis [17]. Our previous reports showed that bee venom and melittin inhibited TGF-β1-induced hepatocyte apoptosis [30,31]. Nevertheless, the protective effect of bee venom components against TGF-β1-induced hepatocyte EMT has not been previously studied. Therefore, the present study is the first attempt to elucidate the effect of apamin in the hepatocyte EMT and CCl_4-induced liver fibrosis animal model.

Previously study has reported a variety of mechanisms regarding the anti-inflammatory effect of bee venom and its constituents [32]. We previously demonstrated that melittin efficiently suppressed the expression of specific genes in the animal model of liver cirrhosis and atherosclerosis [24,33]. Furthermore, our recent evidence showed that several factors, including TGF-β1 and tumor necrosis factor (TNF)-α, produced by activated hepatic stellate cells or myofibroblasts, were associated with liver fibrosis, and that bee venom could suppress liver fibrosis by inhibiting these factors in CCl_4-induced liver fibrosis animal model [34]. Following our previous studies, we focused our attention especially on the anti-EMT or anti-fibrotic actions of apamin *in vitro* and *in vivo*.

EMT can be further determined by examining the changes of cellular localization of E-cadherin or the expression levels of vimentin. The disruption of tight junctions, which is an indicator of loss or attenuation of epithelial polarity, is a hallmark of EMT [35]. Our current results showed that TGF-β1-induced morphologic changes of cells are associated with the delocalization of cell adhesion molecule E-cadherin by increased phosphorylation of ERK1/2, Akt, Smad2/3, and Smad4. Based on these results, we investigated the effects of apamin on TGF-β1-indued AML12 cells. Apamin effectively prevented the cell morphological changes and levels of E-cadherin and vimentin modulation via TGF-β1 treatment. Apamin also inhibited EMT by suppression of Smad-independent and/or -dependent signaling pathways in TGF-β1-induced AML12 cells. Collectively, these data provide *in vitro* evidence that apamin maintains the epithelial properties of hepatic epithelial cell and prevents hepatic epithelial cells from transition to a mesenchymal-like phenotype in response to TGF-β1.

TGF-β1-activated Smad proteins are important in EMT-induction and Smad phosphorylation alone is not sufficient to trigger EMT. MAPK and/or PI3K pathway in cooperation with integrin signaling are required for EMT [26,36]. Several papers showed that EMT through activation of ERK with evidence of changes in tight junctions is likely mediated through transcriptionally-dependent alterations in occluding and claudin-2 expression [37]. Moreover, in a mammary cell line, PI3K/Akt signaling was necessary for EMT and cell migration. These findings suggest that TGF-β1-induces EMT and that these changes may be mediated through the PI3K/Akt and MAPK pathway [26]. In recent years, some signaling molecules other than Smads are found to be involved in TGF-β1 signaling [38]. In this study, our results suggest that TGF-β1 activated Smad and other signaling of ERK1/2 and Akt. In addition, apamin mediates the Smad-independent and Smad-dependent signaling pathways in TGF-β1-induced AML12 cell.

Based on the *in vitro* results, we investigated the effects of apamin on the CCl_4-induced liver fibrosis animal model. CCl_4-injected animal model has been widely used as an experimental tool of chronic damage to the liver that produces fibrogenesis and may mimic the situation of human chronic liver disease [39]. In this study, intraperitoneal injection of CCl_4 induced pathological abnormalities and increased collagen deposition. Genes relevant to liver fibrogenesis, including TGF-β1 and fibronectin were also increased in CCl_4-injected animal model. Subsequently, we examined the effects of apamin on the CCl_4-induced animal model. The major findings of *in vivo* results are that apamin attenuates the manifestation of liver tissue pathologies and decreases the expression of TGF-β1 and fibronectin which are related to fibrosis.

In conclusion, our findings demonstrated that apamin suppressed the TGF-β1-induced hepatocyte EMT *in vitro* and CCl_4-injected fibrosis *in vivo*. Administration of apamin significantly increased the expression of epithelial marker E-cadherin and decreased mesenchymal marker vimentin in the TGF-β1-induced hepatocytes. In particular, apamin suppressed the expression of Smad-independent and Smad-dependent signaling pathways in AML12 murine hepatocyte. In addition, apamin exerted the anti-fibrosis effects against the CCl_4-injected animal model. These results demonstrate the potential of apamin for the prevention of EMT progression induced by TGF-β1 *in vitro* and CCl_4-injected *in vivo*.

Acknowledgments

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