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Original Article

Effect of IL-1β on migration, invasion and senescence of bile duct epithelial cells

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Abstract: Objective: To investigate the effects of interleukin-1β (IL-1β) on migration, invasion and senescence of Human intrahepatic bile duct epithelial cells (HIBECs). Methods: HIBECs were stimulated with different concentrations of IL-1β (0, 10, 20 and 30 ng/mL), and their migration, invasion and senescence were evaluated by scratch and Transwell tests, and β-galactosidase staining respectively. Real-time fluorescence quantitative PCR and western blot were used to detect epithelial-mesenchymal transition (EMT) related markers such as E-cadherin, β-catenin, CK19, fibronectin, vimentin, N-cadherin and invasion-related MMP-1 mRNA and proteins respectively. Results: After continuous stimulation of HIBECs with IL-1β for 24 h, migration and invasion were significantly increased compared with the untreated cells (all $P<0.05$). In addition, the number of β-galactosidase-positive senescent HIBECs also significantly increased upon IL-1β stimulation (all $P<0.05$). Under high levels of IL-1β, the expression of interstitial cell markers (fibronectin, vimentin, N-cadherin) and invasion-related genes (MMP-1) significantly increased (all $P<0.05$), and that of epithelial markers (E-cadherin, β-catenin and CK19) significantly decreased (all $P<0.05$). Conclusion: IL-1β stimulation can induce EMT in HIBECs and increase their invasion and migration, as well as promote malignant transformation and senescence.

Keywords: Interleukin-1β, epithelial-mesenchymal transition, human intrahepatic bile duct epithelial cell, cell migration, cell invasion, cell senescence

Introduction

The proportion of intrahepatic bile duct epithelial cells in the human hepatocytes is approximately 3% to 5%, and is present in the interconnected hepatic ducts, capillary bile ducts, and interlobular bile ducts [1-3]. Human intrahepatic bile duct epithelial cells (HIBECs) are involved in transporting water and electrolytes, and play a causative role in immunomodulatory bile duct disease by secreting certain bioactive factors [4, 5].

As a pro-inflammatory cytokine, interleukin-1β (IL-1β) plays an important role in malignant transformation and tumor progression [6]. A Study has shown that overexpression of IL-1β can accelerate tumor growth and metastasis by inducing interleukin-8, vascular endothelial growth factor, matrix metalloproteinase etc. [7, 8]. Clinical study has shown a close association of HIBECs with biliary diseases such as primary biliary cirrhosis, cholangitis on account of autoimmune responses [9]. Exogenous IL-6, another pro-inflammatory cytokine, also stimulates epithelial-mesenchymal transition (EMT) in HIBECs [10]. However, the specific pathogenesis underlying HIBECs mediated bile duct diseases is still unclear. In this study, we investigated the effects of IL-1β on HIBECs EMT, and the specific pathogenesis of migration, invasion and metastasis.

Materials and methods

Cell and culture

The primary HIBEC cell line P5100 was purchased from Shanghai Jining Industry Co. Ltd. The cells were cultured in a 1:1 mix of Ham's F12 and DMEM media including 5% fetal bovine serum (FBS), 10 ug/mL recombinant human hepatocyte growth factor, 5 ng/mL epidermal growth factor, 3 nM triiodothyronine, and 4 μg/mL insulin at 37°C under 5% CO_2.

Table 1. qRT-PCR primer sequence

Gene	Primer sequence (5'-3')
E-cadherin	F: CATTTCCCAACTCCTCTTCTGGC
	R: ATGGGCCTTTCTCATTTTCTGGG
	R: AGAGCAGAGCAATGGAGGAA
β-catenin	F: CACAAGCGGAGTGCTGAAGGTG
	R: GATTCCTGAGAGTCCAACGACAG
CK19	F: TCGCGTCGCCTCCTACTT
	R: GCTCCTCAGTGCAGTAATT
Fibronectin	F: TTACGGTTGCAACTCAAA
	R: GGTCATGCTGCTGATCCC
Vimentin	F: AGATTGCCCTTGACATTGAG
	R: TGGACGAGGCAGAGAAATTC
	R: TGGATAGAAGTCTGAGGAAGG
N-cadherin	F: GGTTGAGGAGAAGAAGACCAG
	R: GCATTAGGCTCCACAGT
MMP-1	F: TGGCTAAAGCCTTTCATTCTG
	R: TCCTGGCCTAATATTCTTAATCC
	R: TTGATTGAGTGAGCCTTT
GAPDH	F: ACCACAGTCCATGTCATCAC
	R: TCCACCACCGTGTTGCTGTA

Stimulation of HIBECs with different concentrations of IL-1β

HIBECs were seeded into 96-well culture plates at a density of $1*10^4$/100 μL/well. IL-1β (50 μL) was then added at varying concentrations of 10, 20 or 30 ng/mL, along with an untreated control, and the cells were cultured for 24 hours. The experiment was repeated three times.

Scratch test

After stimulating with IL-1β, the medium was replaced with serum-free Opti-MEMI (Invitrogen), and the cells were incubated for 12 h under serum starvation. The cells were then washed with PBS, and re-suspended in serum-free medium. The cells were seeded in 6-well plates at a density of $5*10^5$/well, and when they reached about 90% confluency, the monolayer was gently scratched with a sterile pipette tip along the middle of the well. The dislodged cells were removed with PBS washing, and the remaining cells were incubated for 0.5-1 h in serum-free medium. After recovery of the cells, they were imaged at 0 h and 24 h respectively, and the cell migration distance was measured with Image-Pro Plus Analysis software (Media Cybernetics Inc., USA). The experiment was repeated three times.

Transwell assay

Matrigel (BD Company, USA) was dissolved overnight at 4°C and diluted 1:3 with serum-free medium. The upper chambers of each 8 μm Transwell (in 24-well plates) was coated with 50 μL Matrigel, equilibrated at 37°C for 30 min, and then washed once in serum-free medium. The HIBECs were digested and washed 3 times in serum-free medium, and 200 μL of the suspension was seeded into each upper Transwell chamber at the density of $1*10^5$ cells/mL. Medium containing 10% FBS was dispensed into the lower chambers, and the plates were incubated at 37°C for 24 hours. The Transwell inserts were removed, washed twice with PBS for 5 min each, fixed with 5% glutaraldehyde at 4°C, stained with 0.1% crystal violet for 30 min, and again washed twice with PBS. The inserts were observed under an inverted microscope, and five clear fields were randomly selected. The number of cells that migrated through the Matrigel was used as the index to evaluate the invasive ability. The experiment was performed 3 times and the mean number of migrated cells was calculated.

Detection of cell senescence by β-galactosidase positive staining

After stimulation with different concentrations of IL-1β, HIBEC senescence was analyzed with β-galactosidase staining using a commercial kit as per manufacturer's instructions (Beyotime, C0602). The β-galactosidase was present in the senescent cells, and not quiescent, pre-aging, immortalized and tumor cells, converting the substrate X-Gal to a dark blue product. The stained cells were mounted on glass slides and observed under a light microscope. Ten clear fields were randomly selected and the number of positively stained cells were counted.

Detection of mRNA expression by fluorescence quantitative RT-PCR

Total RNA was extracted from cells treated with different concentrations of IL-1β with Trizol, and its concentration and purity were measured as absorbance at A260 and A280 respectively using NanoVue™ Plus ultraviolet spectrophotometer. The RNA was reverse transcribed with Rimescript™ RT Kit (Beijing Nobleryder Technology Co., Ltd.) according to the manufacturer's instructions. The primer sequences are shown in **Table 1**. The reaction mix included 2 μL DNA

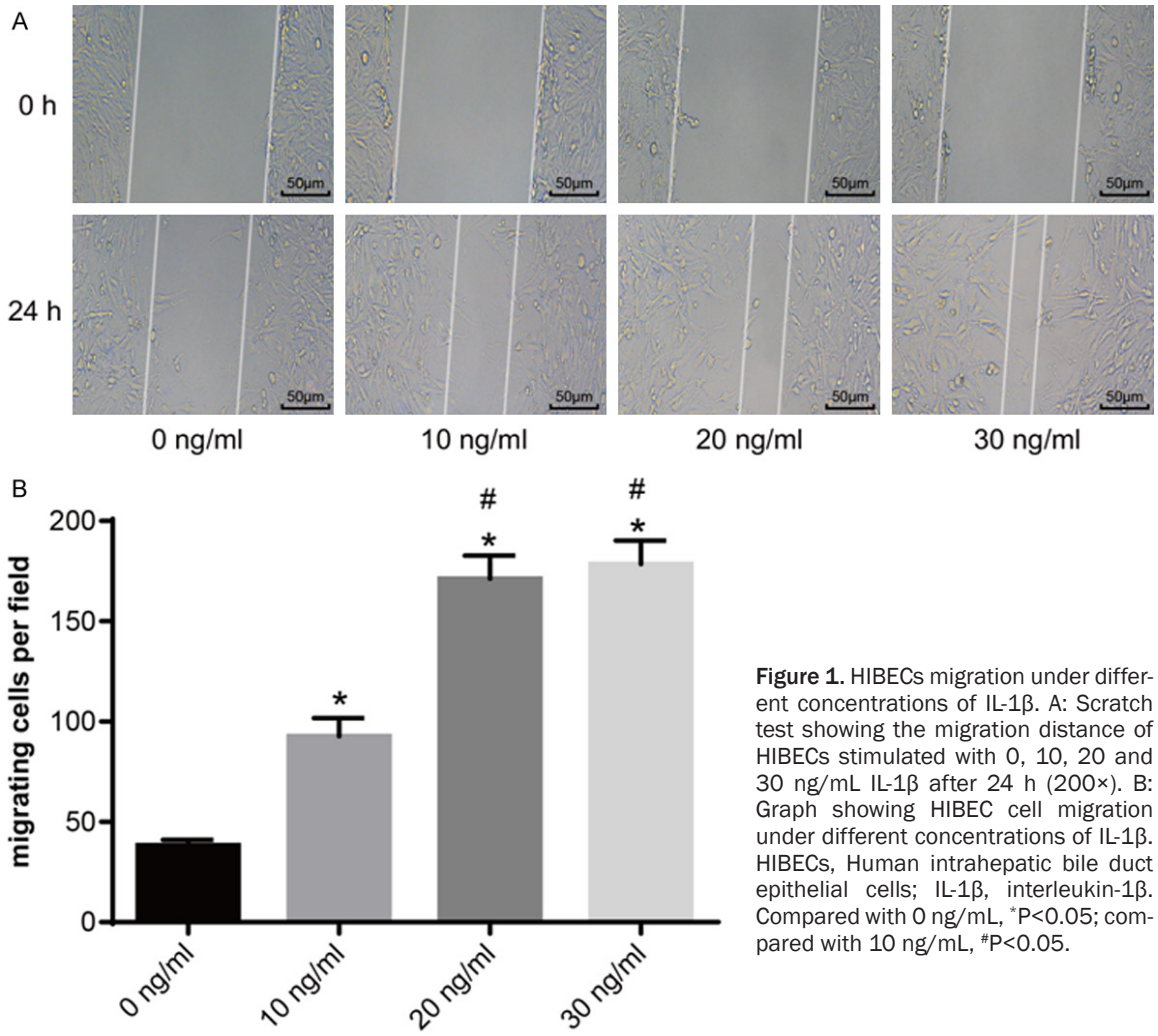

Figure 1. HIBECs migration under different concentrations of IL-1β. A: Scratch test showing the migration distance of HIBECs stimulated with 0, 10, 20 and 30 ng/mL IL-1β after 24 h (200×). B: Graph showing HIBEC cell migration under different concentrations of IL-1β. HIBECs, Human intrahepatic bile duct epithelial cells; IL-1β, interleukin-1β. Compared with 0 ng/mL, *P<0.05; compared with 10 ng/mL, #P<0.05.

(<100 ng), 1 μL of each primer, SYBR® Premix Ex Taq (2X), 5 μL DEPC-treated water, 10 μL ROX plus, with a total reaction volume of 20 μL. The reaction conditions were initial denaturation at 95°C for 30 s, followed by 45 cycles of 95°C for 15 s, 70°C for 30 s and 75°C for 30 s. Amplification C_t values were calculated using the $2^{-\Delta\Delta}$Ct method and the experiment was repeated three times.

Detection of protein by western blot

Total protein was extracted with RIPA buffer, separated through a 10% SDS-polyacrylamide gel by electrophoresis, and then transferred to PVDF membrane. The membranes were blocked with 5% skim milk powder for 1 hour, followed by overnight incubation with the following primary antibodies: mouse monoclonal (HECD-1) anti-E-cadherin (ab1416, 1:50), rabbit polyclonal anti-ZO1 tight junction protein (ab59720, 1:100), anti-β catenin (E247) (ab32572, 1:5,000), rabbit monoclonal (EP1580Y) anti-cytokeratin 19 (ab52625, 1:45,000), rabbit polyclonal anti-fibronectin (ab2413, 1:100), mouse monoclonal (RV202) anti-vimentin (ab8978, 1:1000), rabbit monoclonal (EPR4430) anti-Gemin 1 (ab108424, 1:1,000), or rabbit polyclonal anti-N-cadherin (ab12221, 1:100) at 4°C. The membranes were equilibrated with PBS for 1h at room temperature on the next day, and rinsed with PBST for 5 min 3 times. After incubation with the HRP-conjugated goat polyclonal secondary antibody (ab181658, 1:10,000) for 1 h at room temperature, the membranes were again rinsed with PBST for 5 min 3 times. Color was developed using an Amersham ECL™ advance Western blotting detection kit (Amersham Pharmacia Biotech, Uppsala Sweden). The protein bands were

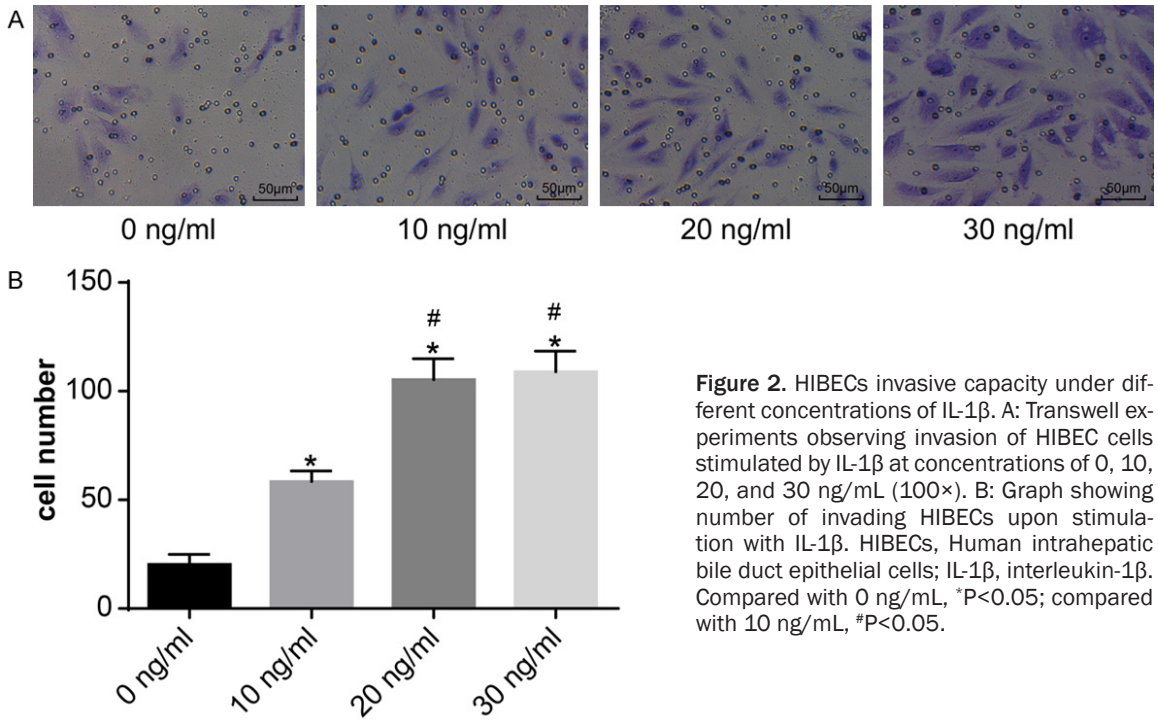

Figure 2. HIBECs invasive capacity under different concentrations of IL-1β. A: Transwell experiments observing invasion of HIBEC cells stimulated by IL-1β at concentrations of 0, 10, 20, and 30 ng/mL (100×). B: Graph showing number of invading HIBECs upon stimulation with IL-1β. HIBECs, Human intrahepatic bile duct epithelial cells; IL-1β, interleukin-1β. Compared with 0 ng/mL, *P<0.05; compared with 10 ng/mL, #P<0.05.

scanned using the Gene GeniusBio Imaging System (Synegene Ltd, Cambridge, MA, USA), and density analysis was performed using Gene-Tools Analysis Software version 3.03.03 (Syngene, Cambridge, UK).

Statistical analysis

SPSS21.0 software was used for statistical analysis. Measurement data are expressed as mean ± standard deviation. The t-test was used for comparison between the two groups, and one-way ANOVA was used for multiple group comparison. The pairwise comparison of multiple groups/means was performed by LSD-t test. P<0.05 is considered statistically significant.

Results

Migration of HIBECs under varying concentrations of IL-1β

The migration distance of HIBECs stimulated with 10, 20 or 30 ng/mL IL-1β after 24 h of scratching was significantly higher than that of the untreated cells (all P<0.05). While the migration distance of cells treated with 20 or 30 ng/mL IL-1β was significantly higher compared with those treated with 10 ng/mL (all P<0.05), no significant difference was seen between the migration distance of cells treated with 20 and 30 ng/mL (P>0.05). See **Figure 1**.

Invasiveness of HIBECs under varying concentrations of IL-1β

The number of HIBECs invading the Transwell membrane after 24 h stimulation with varying concentrations of IL-1β were significantly higher than the untreated cells (all P<0.05). HIBECs treated with 20 or 30 ng/mL IL-1β had significantly greater invasive capacity compared with cells treated with only 10 ng/mL IL-1β (all P<0.05), while no significant difference was seen in the number of invading cells between the 20 and 30 ng/mL treated HIBECs (P>0.05). See **Figure 2**.

Senescence levels in HIBECs after IL-1β stimulation

β-galactosidase staining was used to detect the senescence of HIBECs stimulated with different concentrations of IL-1β. The number of β-galactosidase-positive cells increased with higher concentrations of IL-1β. A significantly higher proportion of positive cells were seen in the 20 and 30 ng/mL stimulated HIBECs compared with those treated with 10 ng/mL (all

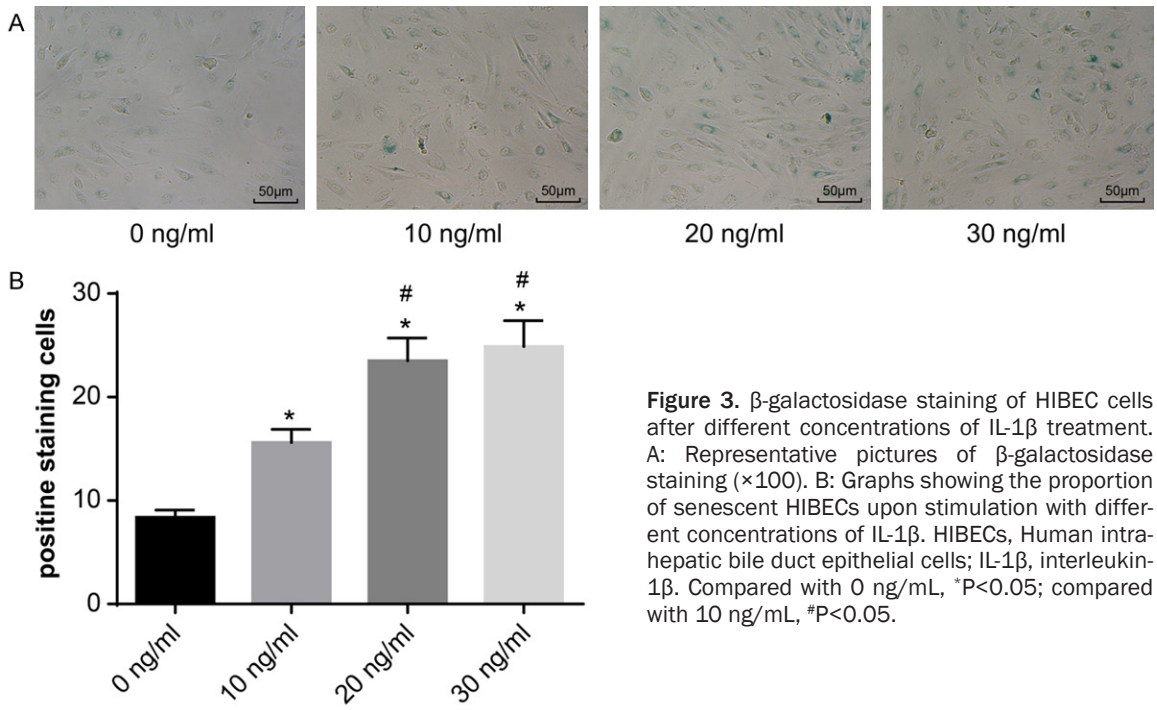

Figure 3. β-galactosidase staining of HIBEC cells after different concentrations of IL-1β treatment. A: Representative pictures of β-galactosidase staining (×100). B: Graphs showing the proportion of senescent HIBECs upon stimulation with different concentrations of IL-1β. HIBECs, Human intrahepatic bile duct epithelial cells; IL-1β, interleukin-1β. Compared with 0 ng/mL, *P<0.05; compared with 10 ng/mL, #P<0.05.

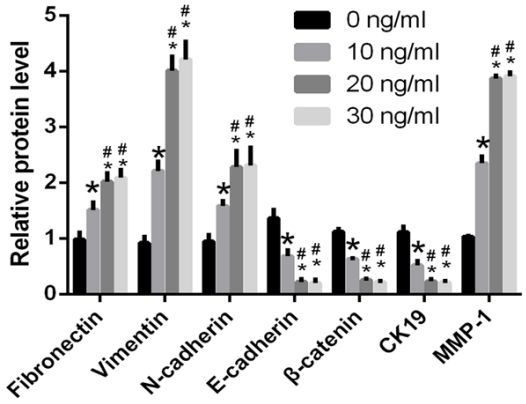

Figure 4. Detection of mRNA levels of EMT-related genes and the invasion-related gene MMP-1 by real-time fluorescence quantitative PCR. HIBECs, Human intrahepatic bile duct epithelial cells; EMT, epithelial-mesenchymal transition. Compared with 0 ng/mL, *P<0.05; compared with 10 ng/mL, #P<0.05.

P<0.05), but no significant difference was seen between the 20 and 30 ng/mL treated HIBECs (P>0.05). See **Figure 3**.

EMT and invasion-related gene mRNA expression after IL-1β stimulation

RT-PCR was used to determine the expression of EMT and invasion-related genes. mRNA levels of fibronectin, vimentin, N-cadherin and MMP-1 were significantly higher, and that of E-cadherin, β -catenin and CK19 were significantly lower in the IL-1β treated cells compared with the untreated cells (all P<0.05). No significant differences were seen in the expression levels of any of these markers between the 20 and 30 ng/mL treated HIBECs (P>0.05), they were significantly different when compared with the 10 ng/mL treated cells, showing the same trends when compared with the untreated cells (all P<0.05). See **Figure 4**.

EMT- and invasion-related protein levels after IL-1β stimulation

Western blot showed significantly higher protein levels of fibronectin, vimentin, N-cadherin and MMP-1, and significantly lower expression of E-cadherin, β-catenin and CK19 in IL-1β treated cells compared to the untreated cells (all P<0.05); similar differences were seen between the 20 or 30 ng/mL treated and 10 ng/mL treated HIBECs (all P<0.05). No significant differences were seen between the 20 and 30 ng/mL treated HIBECs (P>0.05). See **Figure 5**.

Discussion

Bile duct epithelial lesions are an important cause of peripheral cytopathia, invasion and

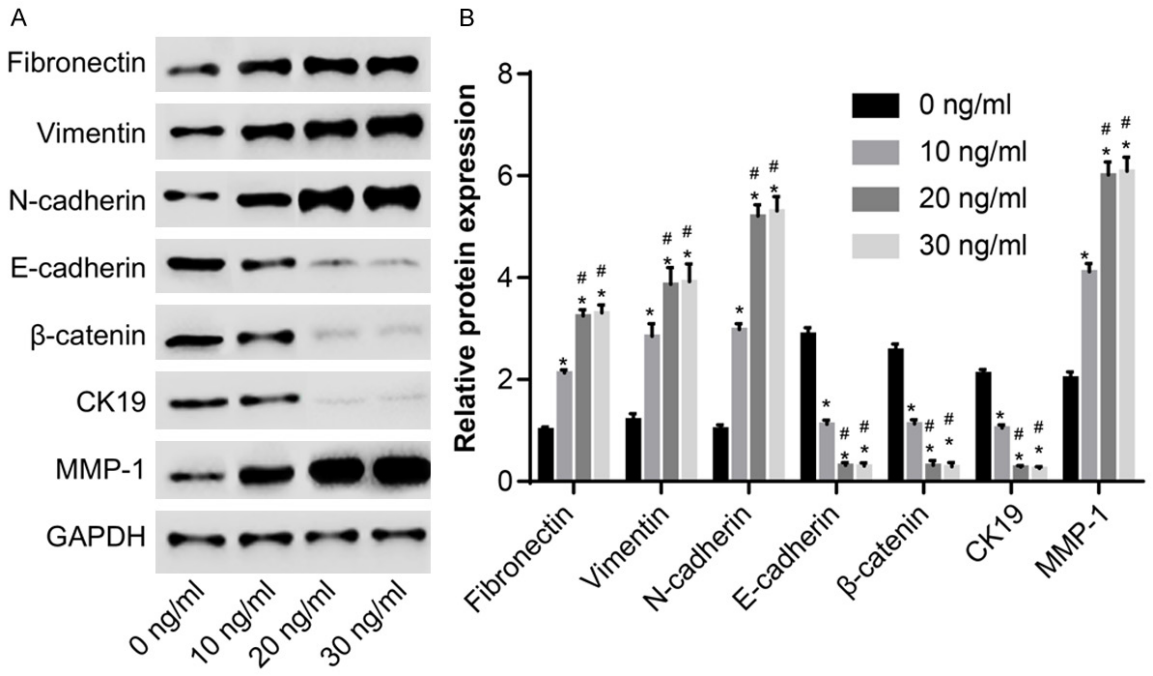

Figure 5. Western blot analysis of EMT proteins (A) and invasion-related protein MMP-1 (B) in HIBECs treated with different concentrations of IL-1β. HIBECs, Human intrahepatic bile duct epithelial cells; IL-1β, interleukin-1β; EMT, epithelial-mesenchymal transition. Compared with 0 ng/mL, *P<0.05; compared to 10 ng/mL, #P<0.05.

metastasis [11]. Under normal physiological conditions, low levels of IL-1β and other inflammatory factors are secreted, and excessive secretion of these factors tend to result in peripheral cytopathic lesions [12, 13]. Studies have shown that IL-1β protein and mRNA levels increase significantly in the liver during pathological conditions [14, 15]. After stimulating HIBECs with different concentrations of IL-1β, we found a significant, concentration dependent increase in their invasiveness and migration. Furthermore, IL-1β stimulated HIBECs also showed an increase in the proportion of β-galactosidase positive cells with increasing concentrations of the cytokine (all P<0.05). These results suggest that exogenous IL-1β stimulation can promote the invasion and migration of HIBECs, and accelerate cell senescence. Our findings are consistent with those of Saeki et al. who found elevated IL-1β levels in chronic cholecystitis and gallbladder carcinoma tissues but very low levels in normal gallbladder tissue, indicating a pathogenic role of IL-1β in cholecystitis and gallbladder carcinoma [16].

To further confirm our hypothesis, we analyzed the mRNA and protein levels of EMT-related markers such as E-cadherin, β-catenin, CK19, fibronectin, vimentin, N-cadherin, and the invasion-associated gene MMP-1 in HIBECs. IL-1β stimulation significantly up-regulated the interstitial markers (fibronectin, vimentin and N-cadherin) and invasion-associated MMP-1, and down-regulated the epithelial cells markers (E-cadherin, β -catenin and CK19) in a concentration-dependent manner. Labernadie et al. showed that up-regulation of interstitial proteins such as N-cadherin was associated with lymph node invasion, malignant differentiation and cancer metastasis [17-19]. In addition, the epithelial markers are mainly present at the junctions of endothelial cells and play important roles in the cytoskeleton. Down-regulation of epithelial marker proteins affects the integrity of the entire cell epithelium and accelerates EMT [20, 21]. The increase in invasion-associated genes further confirms that IL-1β promotes cell invasion. Based on the above findings, we conclude that IL-1β can accelerate the migration and invasion of bile duct epithelial cells, and promote cell senescence.

Our study has certain limitations. We tested a very narrow range of IL-1β concentrations, and did not study the signaling pathways regulating

these proteins. These concerns should be addressed in future studies. In conclusion, exogenous IL-1β stimulation can accelerate the invasion and migration of HIBECs, and promote their malignant transformation and senescence. Our study provides an experimental basis for further clinical studies on the mechanism of HIBEC migration, invasion and metastasis.

Disclosure of conflict of interest

None.

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