JBUON 2021; 26(2): 435-443
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
Email: editorial_office@jbuon.com

ORIGINAL ARTICLE

Activation of human hepatic stellate cells enhances the metastatic ability of hepatocellular carcinoma cells via up-regulation of interleukin-1β

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Summary

Purpose: *The purpose was to investigate the effect of activated human hepatic stellate cell (HSC) microenvironment on the metastatic capacity of hepatocellular carcinoma (HCC) cells and its underlying mechanism.*

Methods: *LX-2 HSCs were stimulated with Human Transforming Growth Factor-Beta 1(TGF-β1), and protein expression of a-smooth muscle actin (a-SMA) and filamentous actin (F-actin) were determined to verify the activation of LX-2 cells. Next, SMMC7721 HCC cells were cultured in the conditioned medium originating from activated LX-2 cells. Wound healing and Transwell assays were performed to examine cell migration and invasion. The expression of metastasis-related genes Matrix Metalloproteinase9 (MMP9), N-cadherin, and Vascular endothelial growth factor (VEGF) was detected. ELISA was carried out to determine the interleukin (IL) -1β level. Finally the inhibitors of TGF-β1 and IL-1β were employed to investigate the roles of LX-2 activation and IL-1β in the metastasis-related gene alterations.*

Results: *TGF-β1 activated LX-2 cells, as evidenced by up-regulated a-SMA and F-actin expression. Compared with the control medium, the conditioned medium derived from LX-2 cells significantly promoted the migration and invasion of SMMC7721 cells. And it also up-regulated mRNA and protein expression of the metastasis-related genes in SMMC7721 cells. Furthermore, it resulted in a significant increase in the IL-1β level in SMMC7721 cells. Importantly, TGF-β1 inhibitor and IL-1β inhibitor either individually or synergistically abolished the up-regulated expression of conditioned medium-induced metastasis-related gene in SMMC7721 cells.*

Conclusions: *The conditioned medium generating from TGF-β1-activated LX2 cells can enhance the metastatic ability of SMMC7721 cells through up-regulating IL-1 expression.*

Key words: *Human hepatic stellate cells, Hepatocellular carcinoma cells, Interleukin 1β, metastasis, MMP9, VEGF*

Introduction

Hepatocellular carcinoma (HCC) is the 4th most common cancer and the 2nd leading cause of cancer-related deaths worldwide, contributing to approximately 811,000 global deaths in 2019 [1,2]. Nearly 80% of patients with HCC have been in the advanced stage at the time of diagnosis and are therefore ineligible for surgical resection, which contributes to the poor 5-year overall survivals in HCC patients. Advanced stage HCC often grows invasively into neighboring tissues and vessels, leading to metastasis [3,4]. A better understanding of the mechanisms underlying HCC metastasis may identify potential therapeutic targets to prevent HCC metastasis and therefore to improve the overall survivals in HCC patients.

Accumulating evidence has indicated that chronic inflammation is associated with high incidence of HCC. Inflammation-inducing factors,

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Received: 03/01/2021; Accepted: 25/01/2021

such as hepatitis B and C viruses and excessive alcohol consumption that contribute to fibrosis and cirrhosis, may change the liver microenvironment, thereby driving hepatocarcinogenesis and tumor survival [5-7]. Chronic inflammation drives hepatocarcinogenesis through the crosstalk among different cell types, such as Hepatic Stellate cells (HSCs) and fibroblasts, in the liver microenvironment. In normal liver, HSCs exist in a quiescent non-proliferative state. During liver injury, transforming growth factor (TGF)-β is expressed and released from various cell types to activate and transdifferentiate HSCs into microfibroblasts characterized by proliferative and migratory phenotypes as well as increased alpha-smooth muscle actin (α-SMA) expression and extra cellular matrix (ECM) production [8]. The activated HSCs also enhance the production and release of matrix metallopeptidases (MMPs) [9-11], vascular endothelial growth factor (VEGF), interleukins (ILs), and angiopoietin 1 [12-14], playing a critical role in HCC development. In addition, HCC cells promotes the activation, migration, and invasion of HSCs *in vitro* co-culture model [15], suggesting that the crosstalk between tumor cells and their microenvironment facilitates the progression of HCC. Targeting activated HSCs has emerged as a promising strategy to improve treatment outcomes for HCC [16].

Activating HSCs promotes the production of inflammatory cytokines, including IL-1β [17]. A recent study has shown that lipopolysaccharide or palmitic acid activates HSCs and promotes IL-1β production from the activated HSCs. The activated HSC-conditioned medium facilitates HCC cell proliferation and invasion [18]. However, whether IL-1β mediates the crosstalk between HSCs and tumor cells remains unknown. Considering that IL-1β has been shown to contribute to HCC invasion [19,20], we hypothesized that IL-1β might mediate the crosstalk between HSCs and tumor cells to facilitate HCC metastasis.

To test our hypothesis, we cultured human HCC cell line SMMC7721 in the conditioned medium derived from TGF-β1-activated human HSC cell line LX-2 to explore the role of activated HSC-generated microenvironment in HCC cell migration and invasion. TGF-β1 inhibitor and IL-1β inhibitor were employed to reveal the roles of HSC activation and IL-1β in HSC/HCC cell crosstalk. Our study suggests that targeting HSC activation and IL-1β production is a promising therapeutic strategy to overcome HCC metastasis.

Methods

Cell lines and cell culture

Human hepatic stellate cell line LX-2 and HCC cell line SMMC7721 were kindly provided by Mengchao Hepatobiliary Technology Key Laboratory of Fujian Province, Mengchao Hepatobiliary Hospital of Fujian Medical University (Fujian, China). Cells were maintained in high-glucose Dulbecco's modified eagle medium (DMEM) (Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), 100 IU/mL penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2.

To prepare the conditioned medium from the activated LX-2 cells, LX-2 cells were seeded in a 6-well plate at a density of 3×10^5 cells/well and cultured in high-glucose DMEM containing 10% FBS for 12 h, followed by 10 μg/L TGF-β1 stimulation [21] for 24 h. The medium was then removed and replaced with serum-free DMEM. After an additional 24-h incubation, the medium was collected and centrifuged at 1,000 g for 10 min. The supernatant was collected as the conditioned medium [22]. SMMC7721 cells were cultured in the conditioned medium for 48 h before treatment with 50 μg/mL disitertide (#HY-P0118; MCE, Monmouth Junction, NJ, USA) or 0.5 ng/mL IL-1β inhibitor (#p00050; Solarbio, Beijing, China) for an additional 24 h.

Immunofluorescence staining

A total of 2×10^5 LX-2 cells were seeded in a confocal dish and cultured in high-glucose DMEM containing 10% fetal bovine serum (FBS) for 12 h, followed by treatment with or without 10 μg/L TGF-β1 for an additional 24 h. After washing with phosphate-buffered saline (PBS), cells were fixed with 4% paraformaldehyde for 15 min, washed with PBS again, and incubated with blocking buffer (PBS containing 3% bovine serum albumin (BSA) and 0.5% Tween20) for 1 h at room temperature. Cells were then incubated with anti-F-actin antibody (#ab130935, 1:500; Abcam, Cambridge, MA,

Table 1. Primers

Gene	*Forward (5' 3')*	*Reverse (5' 3')*
α-SMA	CCACTGCTGCTTCCTCTTC	CGCCGACTC CATTCCAAT
VEGF	GTGGTGAAGTTCATGGATGTCTAT	ACACAGGATGGCTTGAAGATG
MMP9	GTACTCGACCTGTACCAGCG	TTCAGGGCGAGGACCATAGA
N-cadherin	TATACTGATGTTTGTGGTATGG	CTCAAGTCATAGTCCTGGTCTTCT
GAPDH	ATGGGGAAGGTGAAGGTCG	TCGGGGTCATTGATGGCAACAATA

USA) for 1 h at 4°C, followed by rinsing with PBS. After incubation with a Cy3-conjugated secondary antibody (ABclonal AS008 1:250; Jackson ImmunoResearch, West Grove, PA, USA) for 1 h at room temperature, F-actin expression and organization were examined using a LSM 780 confocal microscope (Zeiss, Oberkochen, Germany) at magnification 100×.

Transwell assay

Cell migration and invasion assays were performed using 24-well plates with Transwell inserts (Corning, Corning, NY, USA). For invasion assay, the Transwell insert was coated with Matrigel (BD, Franklin Lakes, NJ, USA). 2×10^5 SMMC7721 cells were resuspended in 500 µL serum-free DMEM and loaded into the upper chamber, while 500 µL DMEM (negative control) or the conditioned medium were added into the lower chamber. Cells were allowed to migrate or invade for 20 h. The cells remaining in the upper chamber were removed using cotton swabs. The migrating or invading cells on the lower membrane surface were fixed in 4% paraformaldehyde and then stained with 0.1% crystal violet. The numbers of cells were counted in five randomly-selected fields and images were acquired using a microscope (Nikan Ri-S&NT-88C3, Tokyo, Japan) at magnification 200×.

Wound healing assay

Wound healing assay was performed to examine cell migration. SMMC7721 cells were seeded into a 6-well plate at a density of 4×10^5 cells per well and cultured overnight. The 200 µL micropipette tip was used to generate a 2 mm-wide scratch line in the cell monolayer. After washing with PBS, cells were incubated in serum free medium or activated LX-2-conditioned medium. Images were captured at 0 or 24 h after scratching using an inverted light microscope. The wounded area was measured using Image-J software (NIH, Bethesda, MD, USA). Wound healing percentage was calculated as

$$\frac{\text{wounded area } (T_0\text{-}T)}{\text{Wounded area } T_0} \times 100\%.$$

(T_0, 0 h after wound formation; T, time after wound formation).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was harvested from SMMC-7721 cells at 48 h after incubation with or without the conditioned medium using RNAiso Plus (TaKaRa, Tokyo, Japan), followed by complementary deoxyribose nucleic acid (cDNA) synthesis using a PrimeScript™ RT reagent kit (TaKaRa, Tokyo, Japan), according to the manufacturer's instructions. PCR was performed using a ChamQ™ SYBR® qPCR master mix (Vazyme, Nanjing, China) following the manufacturer's instruction. The primers were summarized in Table 1. Glyceraldheyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. Gene expression was quantified using the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis

SMMC-7721 cells were lysed at 48 h after incubation with or without the conditioned medium using radioimmunoprecipitation assay (RIPA) buffer (Keygen, Nanjing, China) on ice. Cell lysates were centrifuged at 1, 3000 rpm for 15 min at 4°C. The supernatant was collected, and the protein concentration was measured suing a BCA kit (Thermo Fisher Scientific, Waltham, MA, USA). A total of 30 µg of protein samples were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride (PVDF) membrane. After blocking in 5% skim milk for 90 min, the membrane was incubated with anti-α-SMA (#A7248, 1:10000; ABclonal, Woburn, MA, USA), anti-VEGF(#19003-1- AP, 1:1000; Proteintech, Rosemont, IL, USA), anti-N-cadherin (#ab18203, 1:1000; Abcam, Cambridge, MA, USA), anti-MMP9 (#ab38898, 1:1000; Abcam, Cambridge, MA, USA), or anti-GAPDH (#60004-1-Ig, 1:1000; Proteintech, Rosemont, IL, USA) antibody at room temperature for 2 h. After washing with tris buffered saline with tween 20, the membrane was incubated with the secondary antibody (SA00001-1, SA00001-2, 1:5000; Proteintech, Rosemont, IL, USA) at room temperature for 1 h. The protein bands were visualized using an electrochemiluminescence (ECL) kit (USeverbright, Suzhou, Jiangsu, China).

Enzyme-linked immunosorbent assay (ELISA)

SMMC7721 cells were cultured in the conditioned medium or regular DMEM. LX-2 cells were cultured in regular DMEM. At 48 h after incubation, the IL-1β levels in the cells were measured using a human IL-1β ELISA kit (Boster Biological Technology, Wuhan, Hubei, China) following the manufacturer's instruction.

Statistics

Data are expressed as mean ± standard deviation. Statistical analysis was performed using SPSS 19.0 software (IBM, Armonk, NY, USA). The t-test was used for analyzing measurement data. Differences between two groups were analyzed by using the Student's t-test. Comparison among multiple groups was done using One-way ANOVA followed by *post hoc* test (least significant difference). $P<0.05$ indicated significant difference.

Results

TGF-β1 stimulation enhanced α-SMA and F-actin expression in LX-2 cells

To examine the effect of TGF-β1 stimulation on LX-2 cells, we determined the expression of α-SMA, a marker of the activated LX-2 cells. Western blot analysis and qRT-PCR showed that TGF-β1 stimulation significantly enhanced α-SMA protein and mRNA expression in LX-2 cells (Figures 1A–1C), suggesting that TGF-β1 activates LX-2 cells.

In addition, compared with untreated LX-2 cells (Figure 1D), TGF-β1-stimulated LX-2 cells had significantly increased F-actin expression and displayed linearly arranged F-actin bundles (Figure 1E), further confirming that TGF-β1 activates LX-2 cells.

LX-2 activation promoted cell migration and invasion of SMMC7721 cells

To explore whether activated LX-2 affects the metastatic ability of HCC cells, we cultured SMMC7721 cells in the conditioned medium from TGF-β1-activated LX-2 cells and examined cell migration and invasion. Transwell migration and invasion assays showed that incubation with the conditioned medium resulted in significant increases in the migration and invasion of SMMC7721 cells compared with incubation with regular DMEM (Figures 2A-2F, Table 2). Consistent results were observed in the wound healing assay (Figures 3A and 3B). These data suggest that LX-2 activation enhances the metastatic ability of HCC cells.

LX-2 activation up-regulated the expression of N-cadherin, MMP9, and VEGF in SMMC7721 cells

Then, we sought to explore the possible mechanisms involved in the enhanced metastatic ability of SMMC772 cells cultured in the conditioned medium from activated LX-2 cells. Western blot analysis and qRT-PCR revealed that SMMC772 cells cultured in the conditioned medium had significantly increased protein and mRNA levels of MMP9, N-cadherin, and VEGF compared with LX-2 or SMMC772 cells cultured in regular medium (Figures 4A and 4B). This finding suggests that activated LX-2 enhances the metastatic ability of HCC cells possibly by up-regulating these metastasis-related genes.

Table 2. SMMC7721 cell migration and invasion assays

Assay	*Group*	*n*	*Migrating/invading cell numbers (x± s)*
Migration#	DMEM	5	1.80 0.84
	LX-2-CM	5	368.60 55.04
Invasion*	DMEM	5	1.40 1.14
	LX-2-CM	5	82.20 6.42

#vs. DMEM, t = -14.768, p<0.001; *vs. DMEM, t = -30.239, p<0.001. CM: conditioned medium.

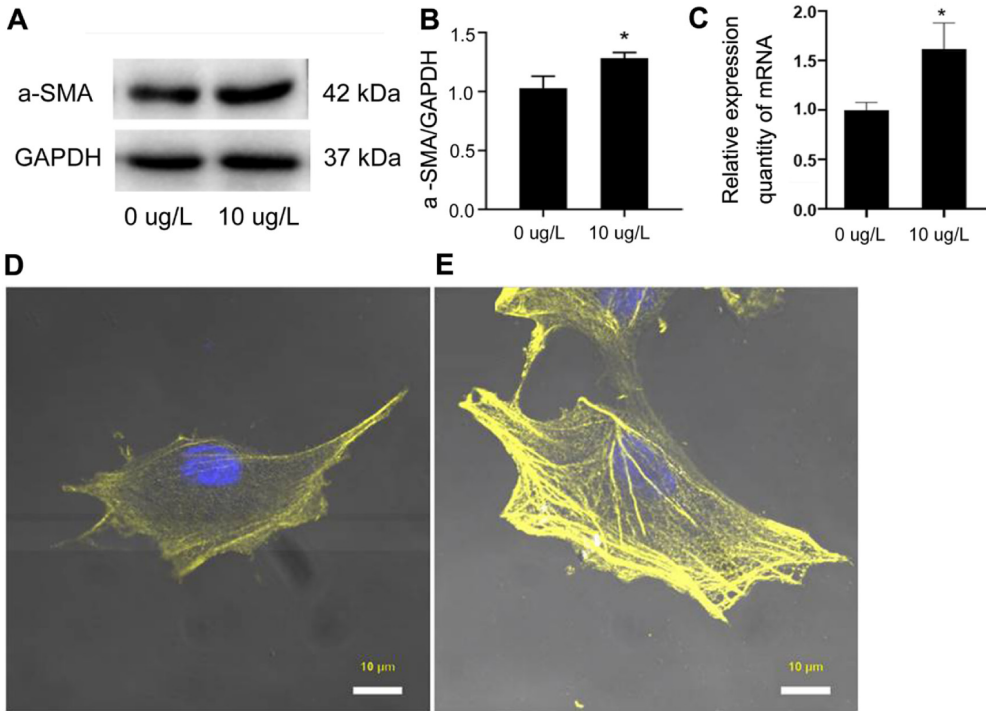

Figure 1. TGF-β1 enhanced protein expression of α-smooth muscle actin (α-SMA) and filamentous actin (F-actin) in LX-2 cells. LX-2 cells were stimulated with or without TGF-β1 (10 μg/L) for 24 h. **A:** Western blot analysis was performed to determine α-SMA protein expression in LX-2 cells. GAPDH was used as an internal control. **B:** Quantification of **(A)**. **C:** Quantitative real-time PCR (qRT-PCR) was performed to determine α-SMA mRNA expression in LX-2 cell. Data are expressed as mean ± standard deviation (SD). *p<0.05 *vs.* untreated cells. **D** and **E:** Immunofluorescence staining was performed to examine F-actin protein expression LX-2 cells without **(D)** or with TGF-β1 **(E)** stimulation. The cells were observed under a confocal microscope. Scale bar=10 μm.

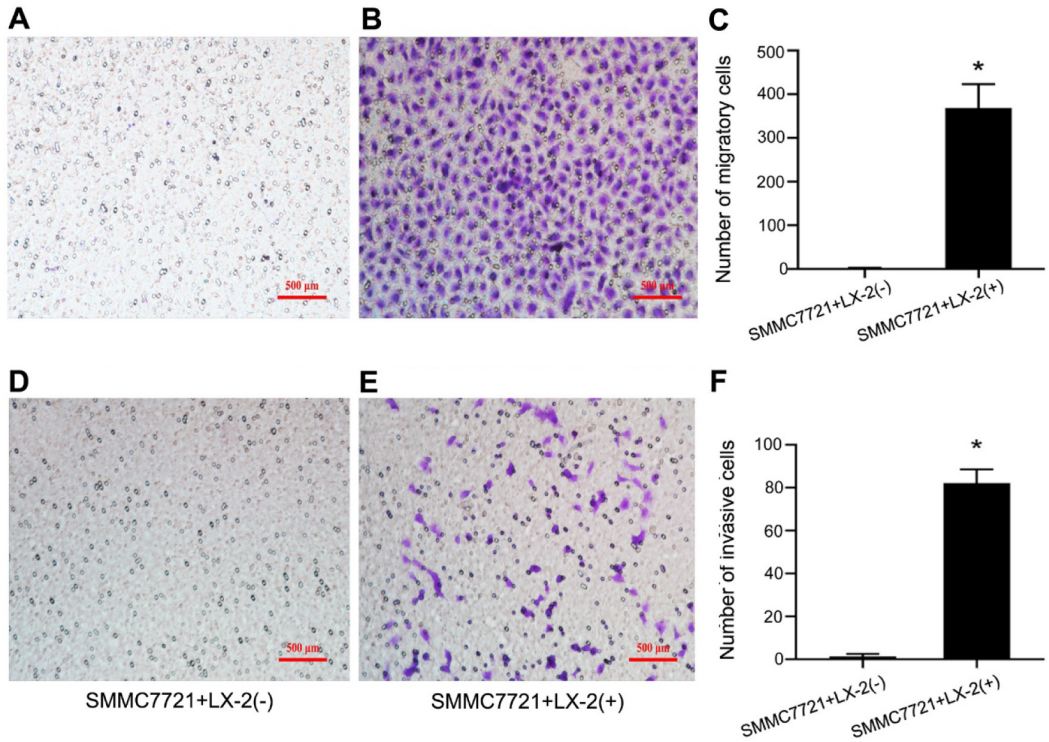

Figure 2. Cell migration and invasion assay. SMMC-7721 cells were cultured in conditioned medium from TGF-β1-stimulated LX-2 cells or regular DMEM for 20 h. The numbers of crystal violet-stained migrating **(A-C)** and invading **(D-F)** cells were counted in randomly selected five fields. Representative images are shown. Magnification 200×. Data are expressed as mean ± SD. *p<0.05 *vs.* cells cultured in regular DMEM (n=3).

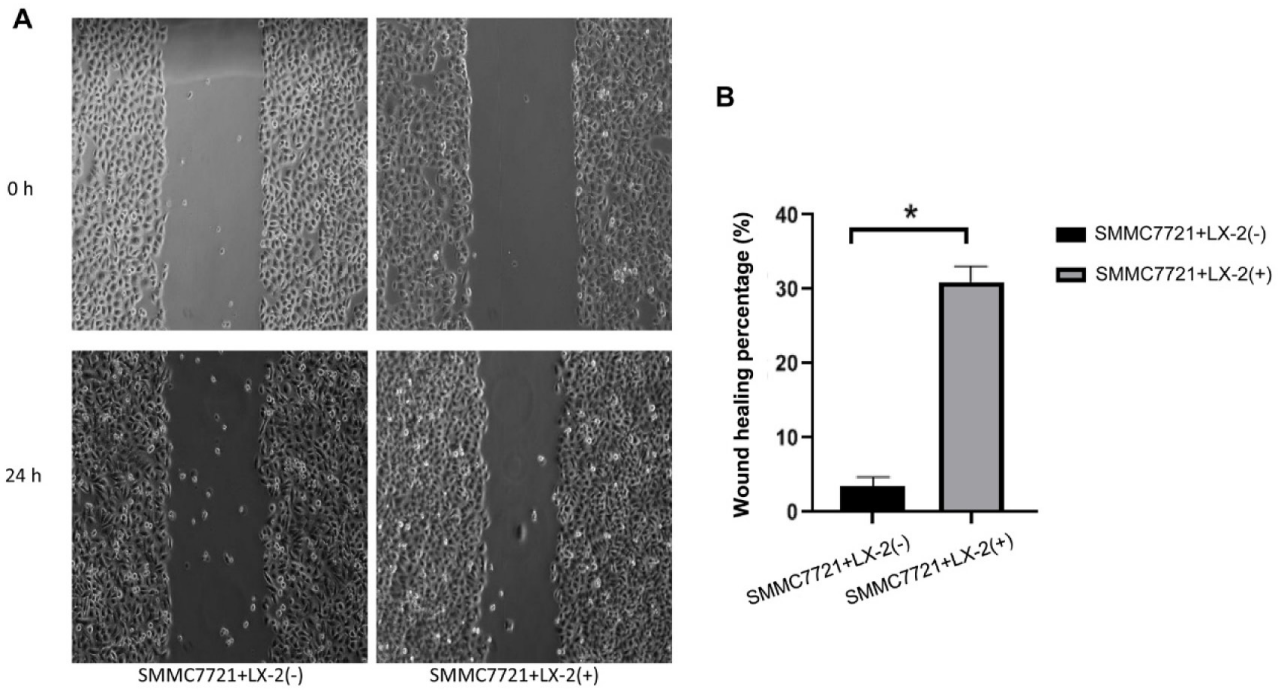

Figure 3. Wound healing assay. SMMC7721 cells were cultured overnight. **A:** 200 μL micropipette tip was used to generate a 2 mm-wide scratch line in the cell monolayer. After washing with PBS, cells were incubated in serum-free medium or activated LX-2-conditioned medium. A: Images were captured at 0 or 24 h after incubation. Representative images are shown. Magnification 200×. **B:** Wound healing percentage was calculated as $\frac{\text{wounded area } (T_0\text{-}T)}{\text{Wounded area } T_0} \times 100\%$.

T_0, 0 h after wound formation; T, time after wound formation. Data are expressed as mean±SD. *p< 0.05 *vs.* cells cultured in serum-free DMEM (n=3).

LX-2 activation promoted IL-1β production in SMMC7721 cells

To investigate the effect of LX-2 activation on the inflammatory response in HCC cells, we measured the IL-1β levels in the cells. The results of ELISA showed that SMMC772 cells cultured in the conditioned medium had significantly increased IL-1β levels compared with those cultured in control medium (Figure 5A), suggesting that LX-2 activation induces inflammatory response in HCC cells.

TGF-β1 inhibitor and IL-1β inhibitor synergistically abrogated LX-2 activation-induced up-regulation of N-cadherin, MMP9, and VEGF expression in SMMC7721 cells

Then, we sought to investigate whether TFG-β1 or IL-1β mediates the up-regulation of N-cadherin, MMP9, and VEGF expression in HCC cells induced by LX-2 activation. As shown in Figure 5B, compared with vehicle treatment, treatment with TGF-β1 inhibitor disitertide or IL-1 inhibitor alone remarkably abrogated the conditioned medium-induced up-regulation of N-cadherin, MMP9, and VEGF protein expression in SMMC7721 cells. Combined treatment with TGF-β1 inhibitor and IL-1 inhibitor further attenuated N-cadherin, MMP9, and VEGF protein expression compared with treatment with each inhibitor alone. These data indicate that inhibition on TGF-β1 and IL-1β synergistically abrogates LX-2 activation-induced up-regulation of N-cadherin, MMP9, and VEGF expression in SMMC7721 cells, suggesting that both TGF-β1 and IL-1β mediate LX-2 activation-induced enhancement of the metastatic ability of SMMC7721 cells.

Discussion

Activated HSCs are the main components of the HCC tumor environment, contributing to HCC initiation and progression directly or via secreting products such as MMPs, ILs, and VEGF [23]. In this study, to explore the role of activated HSC-generated microenvironment in HCC metastasis, we cultured SMMC7721 cells in the conditioned medium from TGF-β1-activated LX-2 cells. We demonstrated that SMMC7721 cells cultured in the conditioned medium exhibited significantly increased metastatic ability compared with those cultured in regular medium. The conditioned medium also up-regulated the expression of metastasis-related genes, including MMP9, N-cadherin, and VEGF in SMMC7721 cells. Furthermore, the conditioned medium promoted IL-1β production

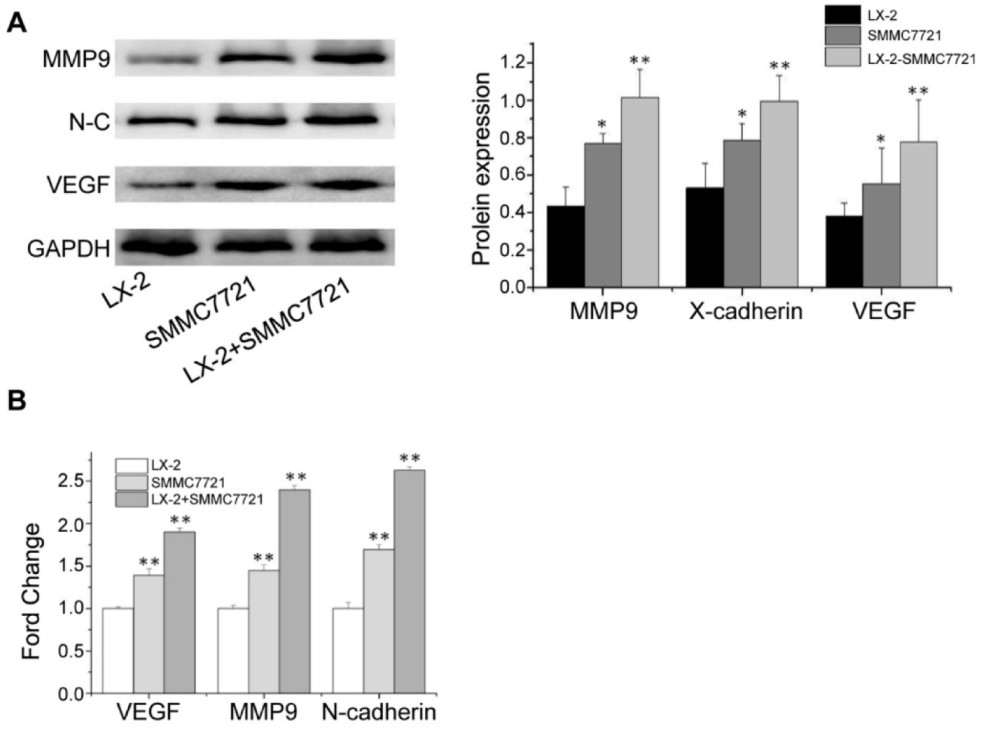

Figure 4. TGF-β1-activated LX-2 cells upregulated the expression of N-cadherin, MMP9, and VEGF in SMMC7721 cells. LX-2 cells were cultured in regular DMEM for 48 h. SMMC7721 cells were cultured in regular DMEM or in the conditioned medium from TGF-β1-activated LX-2 cells for 48 h. Western blot analysis **(A)** and qRT-PCR **(B)** were conducted to measure the protein and mRNA levels of MMP9, N-cadherin, and VEGF in the cells. GAPDH was used as an internal control. Data are expressed as mean ± SD. *p<0.01, **p<0.01 *vs.* SMMC7721 cells cultured in regular DMEM (n=3).

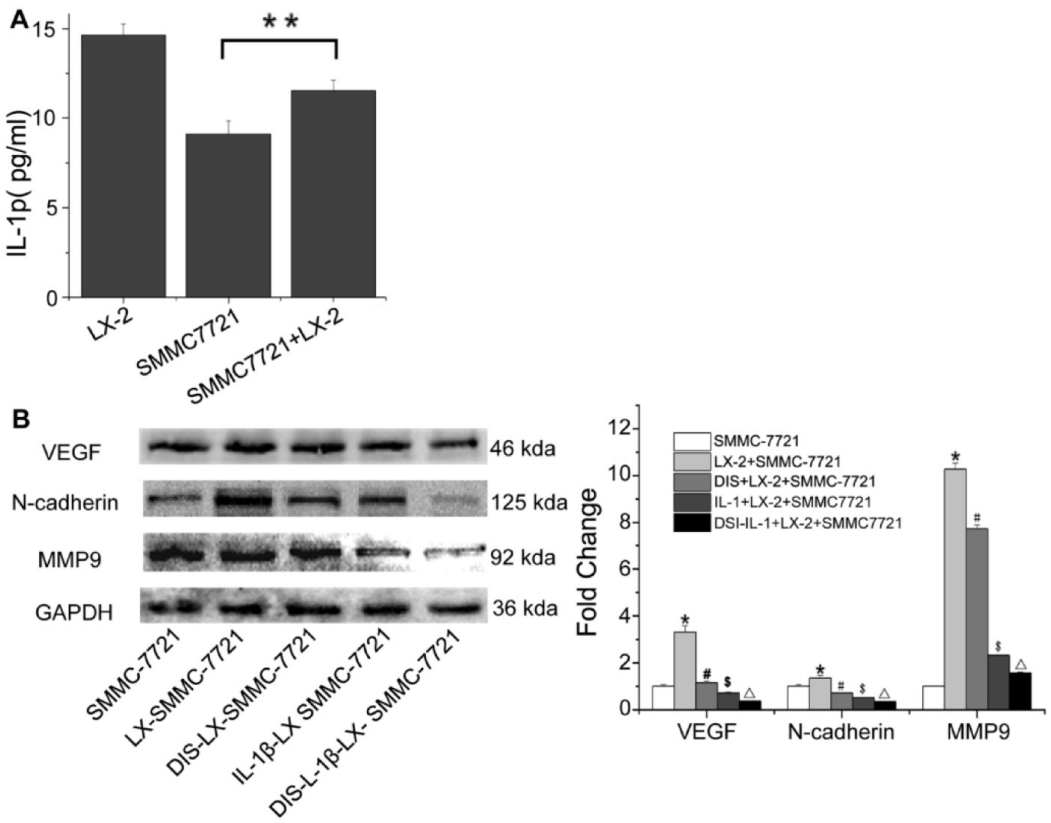

Figure 5. Inhibition of TGF-β1 and IL-1β synergistically abrogated LX-2 activation-induced enhancement of the metastatic ability of SMMC7721 cells. SMMC7721 cells were cultured in regular DMEM or in the conditioned medium for 48 h. Cells cultured in the conditioned medium were treated with TGF-β1 inhibitor disitertide and IL-1β inhibitor individually or in combination for an additional 48 h. **A:** ELISA was performed to measure the protein level of IL-1β in SMMC7721 cells. Data are expressed as mean ± SD. **P<0.01 *vs.* SMMC7721; n=3. **B:** Western blot analysis and qRT-PCR were conducted to measure the protein and mRNA levels of MMP9, N-cadherin, and VEGF in the cells. GAPDH was used as an internal control. Data are expressed as mean ± SD. *p<0.05 *vs.* SMMC-7721; #p<0.05 *vs.* LX-2+SMMC-7721; $p<0.05 *vs.* LX-2+SMMC-7721; △p<0.05 *vs.* DSI-IL-1+LX-2+SMMC-7721 (n=3).

in SMMC7721 cells. Importantly, TGF-β1 inhibitor and IL-1β inhibitor synergistically abolished the conditioned medium-induced up-regulation of metastasis-related gene expression in SMMC7721 cells. Our results suggest that activated HSC-generated microenvironment enhances the metastatic abilities of HCC cells possibly via inducing IL-1β overproduction in HCC cells.

In this study, we stimulated LX-2 cells with TGF-β1 and detected α-SMA and F-actin expression to verify the activation of LX-2 cells. We found that SMMC7721 cells cultured in the conditioned medium from activated LX-2 cells exhibited significantly increased migration and invasion abilities compared with those cultured in regular medium. Consistent with our findings, Yang et al [24] demonstrated that the conditioned medium from activated rat HSC-T6 cells promotes migration of ML1 murine HCC cells and that HSC-T6 cells enhance ML1 metastasis *in vivo*. Similarly, Han et al [25] showed that the conditioned medium from activated human primary HSCs promotes migration and invasion of Hep3B and HepG2 HCC cells. These data collectively indicate that activated HSCs produce a microenvironment favorable to HCC metastasis.

Epithelial-mesenchymal transition (EMT), the conversion of adherent epithelial cells into migratory mesenchymal cells, plays an essential role in HCC invasiveness and metastasis. EMT is characterized by the up-regulation of mesenchymal markers, such as N-cadherin, vimentin, and α-SMA, and the downregulation of epithelial markers, such as E-cadherin [26]. In addition, ECM degradation is also critical for HCC invasion and metastasis. The increased release and activation of MMPs leads to ECM breakdown and enables tumor cells to infiltrate and migrate in the process of EMT [27]. MMP9 is one of the most studied MMPs in HCC EMT. Overexpression of MMP-9 in HCC is correlated with a higher tumor stage, increased lymph node invasion and metastasis, and poorer differentiation and prognosis [28]. Moreover, VEGF is a potent inducer of angiogenesis, facilitating EMT and tumor metastasis in HCC by synergistically work-

ing with other mediators, including ILs, TGF-β, and MMPs [29]. To reveal the mechanism underlying the enhanced metastatic abilities of HCC cells exposed to the conditioned medium, we detected the expression of MMP9, N-cadherin, and VEGF. We found that culture in the conditioned medium up-regulated the expression of MMP9, N-cadherin, and VEGF in SMMC7721 cells, suggesting that activated HSC-generated microenvironment elicits EMT to promote metastasis in HCC.

It has been reported that MMP9 activates pro-IL-1β into active IL-1β [30]. In the early phase of tumor development, VEGF and IL-1β induce each other, playing essential roles in blood vessel growth [31]. In addition, IL-1β is a critical cytokine in hepatic inflammation, triggering the transition from non-alcoholic fatty liver disease to fibrosis, and finally HCC [32]. IL-1β generated within the tumor microenvironment promotes tumor metastasis via different mechanisms [33]. Thus, we determined the IL-1β level in SMMC7721 cells exposed to the conditioned medium from activated LX-2 cells. We found that compared with regular medium, the conditioned medium resulted in a significant increase in the IL-1β levels in SMMC7721 cells, suggesting that IL-1β enhances the metastatic ability of HCC cells. Importantly, TGF-β1 inhibitor and IL-1β inhibitor synergistically blunted the up-regulation of MMP9, N-cadherin, and VEGF caused by the conditioned medium from activated LX-2 cells, suggesting that LX-2 activation and IL-1β overproduction are both essential for EMT and subsequent metastasis in HCC.

Conclusions

In conclusion, activated LX-2-generated microenvironment promotes cell migration and invasion in SMMC7721 cells possibly through up-regulating EMT-related genes, which is reversible by TGF-β1 and IL-1β inhibitors individually or synergistically. This finding suggests that LX-2 activation and IL-1β overproduction play essential roles in HCC metastasis. However, the downstream signaling pathways need further investigation.

Funding acknowledgments

Human hepatic stellate cell line LX-2 and HCC cell line SMMC7721 were kindly provided by Mengchao Hepatobiliary Technology Key Laboratory of Fujian Province, Mengchao Hepatobiliary Hospital of Fujian Medical University (Fuzhou, China). This work was supported by Fuzhou Health and Family Planning Commission (#2017-S-wt1).

Conflict of interests

The authors declare no conflict of interests.

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