Immunology Letters 188 (2017) 116–123

Contents lists available at ScienceDirect

Immunology Letters

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

ERK pathway activation contributes to the tumor-promoting effects of hepatic stellate cells in hepatocellular carcinoma

Yu-Xiao Xie[1], Rui Liao[1], Long Pan, Cheng-You Du*

Department of Hepatobiliary Surgery, the First Affiliated Hospital of Chongqing Medical University, Chongqing, 400016, China

ARTICLE INFO

Keywords:
Hepatic stellate cell
Epithelial–mesenchymal transition
Liver cancer
Mitogen-activated protein kinase

ABSTRACT

Background: Activated hepatic stellate cell (aHSC) play a critical role in hepatocellular carcinoma (HCC) progression crosstalking with cancer cell via various signaling pathways. The aim of our study is to explore the tumor-promoting effects of aHSCs on HCC via ERK pathway.

Methods: α-SMA, p-ERK and p-JNK expression levels in tumoral and peritumoral tissues of HCC were assessed by immunohistochemical and western blotting. The protein and mRNA expression levels in human hepatoma cell treated with aHSC conditioned medium (CM) were determined by western blotting and real-time quantitative PCR, respectively. Cell migration and invasion abilities were assessed using transwell assays. The proliferation ability of HCC cells induced by aHSCs-CM was detected by CCK-8 assay and cell cycle analysis.

Results: We found that aHSC number was positively correlated with p-ERK expression levels in tumoral tissues and aHSC-CM could time-dependently promote PCNA, p-ERK expression in HCC cells. Moreover, aHSC-CM enhanced HCC cells proliferation via ERK. Additionally, aHSC upregulated c-jun and cyclinD1 expression levels, accelerating the transition from G1 to the S phase of HCC cells, and this effect could be arrested by inhibiting ERK pathway. Furthermore, aHSC-CM promoted migration and invasion of HCC cells via ERK. Epithelial–mesenchymal transitions (EMT) phenomenon could be reversed by ERK suppression.

Conclusion: High expression of p-ERK and aHSCs may be associated with the aggressive behavior of HCC cells. Secretions from aHSCs could promote proliferation and EMT of HCC cells via ERK1/2/c-jun/cyclinD1 axis or ERK pathway.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide [1] and shows the third highest mortality among cancer patients in China [2]. Surgery remains the primary measure for the treatment of HCC patients [3]. However, the 5-year recurrence rate after resection is still very high (50–75%). Two of most significant causes of the high recurrence rate in HCC are the tumor-promoting effects induced by surrounding inflammatory microenvironment and the malignant biological behaviors of cancer cells crosstalking with some hepatic inflammatory/immune cells [4]. Therefore, an in-depth understanding of the cellular heterogeneity and intercellular communications in the tumor microenvironment may provide useful therapeutic strategies against this disease.

The neoplastic transformation of hepatocytes and progenitor cells may be determined and/or influenced by a series of genetic and epigenetic events after interactions between stromal cells and extracellular matrix [5]. Notably, activated hepatic stellate cells (aHSCs) are pivotal members of the stromal cells accelerating this transformation. HSCs activation is the most dominant pathway converting into highly proliferative myofibroblast-like cells and sequencely tend to promote tumor growth and invasiveness [6]. We have previously revealed that triggering receptor expressed on myeloid cells (TREM)-1 derived from aHSCs enhanced the aggressiveness of HCC cells, suggesting the tumor-promoting ability of aHSCs [7]. Moreover, by DNA microarray analysis, we demonstrated that aHSCs and cancer-associated myofibroblasts (C-AMFs) markedly expressed hepatocarcinogenesis related genes [8]. These alterations could be related with the molecular framework of tumor–stromal interaction and the acquirement of malignant phenotypes and behaviors of aHSCs in tumor microenviroment. Furthermore, a number of evidence shows that aHSCs drive the progression of HCC via various ways (e.g. hypoxia, acidic microenvironment, epithelial–mesenchymal transitions, et al) [9–11] and signaling pathways (e.g. TGF-β, HGF/c-Met, TLR4, et al) [12–14]. These studies highlight aHSC-tumor cell interactions as a critical mediator during cancer progression.

* Corresponding author at: Department of Hepatobiliary Surgery, the First Affiliated Hospital, Chongqing Medical University, No. 1 Yixueyuan Rd., Chongqing, 400016, China.
E-mail address: duchengyou@126.com (C.-Y. Du).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.imlet.2017.06.009
Received 29 March 2017; Received in revised form 5 June 2017; Accepted 26 June 2017
Available online 28 June 2017

Mitogen-activated protein kinases (MAPKs) are serine-threonine kinases with three classics pathways including ERK, JNK, P38 that play crucial roles on cellular growth, metastases and invasion after provoked by signaling shift from surface to nucleus [15]. Inactivation of PI3K/Akt and p38-MAPK signaling could eventually induce the reversal of epithelial–mesenchymal transitions (EMT) phenotype and down regulation of MMP-2 in HCC cells via miR-129-3p [16]. Also, aHSCs could induce migration or proliferation via MAPK signaling in HCC cells [17,18], and regulate EMT via TGF-β signaling in HCC cells [19]. There is no doubt that aHSCs play key roles in HCC development via EMT. Therefore, it is urgent to understand the molecular basis of EMT involved in aHSCs responsible for the clinical outcomes in HCC patients.

Our previous studies on gene expression profile indicated that MAPK could react on the interactions between HCC cells and aHSCs [8]. By far, few studies have investigated the molecular mechanisms of EMT via ERK/MAPK signaling in HCC cells after communicating with aHSCs. In this study, we explored whether aHSCs could influence proliferation, invasion and EMT of HCC via ERK pathway which may provide us better understanding the mechanisms of HCC development and potential therapeutic targets in HCC.

2. Materials and methods

2.1. Patients and specimens

A total of 57 pairs of tumoral and matched adjacent tissue samples were acquired from patients with pathologically confirmed HCC atfer surgery in our department of First Affiliated Hospital of Chongqing Medical University. All patients received no radiotherapy or chemotherapy before liver resection. Tumor-nodemetastasis (TNM) classification system of International Union Against Cancer (edition 7) and tumor characteristics, such as tumor capsule formation and vascular invasion are described in Table 1. Tissues were used for immunohistochemistry and western blot, respectively. The study obtained approval by the Ethics Committee at the First Affliated Hospital of Chongqing Medical University and all written informed consents were obtained.

2.2. Immunohistochemical staining

All samples immediately fixed in 4% paraformaldehyde for two days after taking out from liver tissue within 1 h, then processed for gradient alcohol dehydration embedd paraffining, and cut into 4 mm thick for subsequent experiments. Immunohistochemistry was carried out streptavidin peroxdase conjugataed method according to the manufacturer's instruction. Briefly, slides were incubated in diluted primary antibodies at 4 °C overnight after 10% goat blood serum blocking nonspecificity antigen. After incubation with secondary antibody and streptoavidin-labeled horseradish peroxidase, DAB chromogenic agent was employed, and the slides were counterstained in hematoxylin. p-ERK (4370S, 1:400, CST), p-JNK anti-human antibody (4668T, 1:400, CST) and α-smooth muscle actin (α-SMA, M0851, 1:50, DAKO) were used and phosphate buffered solution (PBS) was used for negative controls.

Table 1
Primers sequences of c-jun and cyclinD1 factors.

Factors		Primer sequence
GAPDH	Forword primer	5′- AGAAGGCTGGGGCTCATTTG −3′
	Reverse primer	5′- AGGGGCCATCCACAGTCTTC −3′
c-jun	Forword primer	5′-AGAGCGGACCTTATGGCTACAG−3′
	Reverse primer	5′- ATGTGCCCGTTGCTGGACT −3′
cyclinD1	Forword primer	5′- ATGCCAACCTCCTCAACGAC −3′
	Reverse primer	5′- GGACCTCCTTCTGCACACAT −3′

2.3. Cells culture and collection of conditioned medium

Human hepatoma cell lines SMMC-7721, HepG2 (presented from Academy of Life Sciences of Chongqing Medical University) and HSC cell line LX-2 (as gifts by professor Jin-sheng Guo in Zhongshan hospital) were all cultured in complete Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) (Gibico, USA) at 37 °C in 5% CO_2. These cells were passage cultured while density reached to 70%–80%. Conditioned medium of LX-2 (LX2-CM) was collected as described previously [7,20]. Briefly, after growing aroud 80% in 250 ml culture flasks, LX-2 cells were washed two times with PBS, and then cultured in serum-free DMEM for 24 h. LX2-CM were collected, centrifuged at 1500 rpm for 5 min to remove cellular debris, filtered through a 0.22-μm filter, added 0.5% FBS and stored at −20 °C until use.

2.4. Co-culture of HCC cells and LX-2

SMMC-7721 and HepG2 were cultured in 6-well dishes in DMEM containing 10% FBS for 12 h at 5×10^5 e/well and 3×10^5 e/well respectively and then maintained in serum-free medium for 12 h prior to the co-culture experiment. LX-2 cells were cultured on 3 μm pore size cell culture inserts (BD Biosciences, SanJose, CA, USA) in complete DMEM for 18 h at 1/5 density of hepatoma cells and then starvation treatment for 6 h. The inserts were placed on the hepatoma cells plates respectively for incubation for 0, 12, 24, 36 and 48 h, respectively. Hepatoma cells were collected at each time point.

2.5. Cell proliferation assay

Cell counting kit (CCK-8, Dojindo Laboratories, Japan) was used to determine the proliferative ability of hepatoma cells. SMMC-7721 (5×10^3/well) and HepG2 (3×10^3/well) were seeded in 96-well dishes and cultured for 18 h, treated with serum-free medium for 6 h, then incubated with LX2-CM for 0, 12, 24, 36, 48, 60, 72 h, respectively. In another experiment, LX2-CM and different concentration of U0126 (an inhibitor of MEK1/2, 0, 10, 20, 30, 40 μmol/L, respectively) were added simultaneously for 24, 36 and 48 h, respectively. The control group was added into equivalent volume DMEM and DMSO. About 10 μl of CCK-8 reagent and 100 μl of fresh DMEM mixture were added into each well, and then the 96-well plate was constantly incubated at 37 °C for 2 h. Absorbance was measured at 450 nm.

2.6. Immunofluorescence assay

Hepatoma cells were cultured on 48-well plate for 24 h, washed 3 times with PBS at room temperature, fixed with 4% paraformaldehyde for 20 min, and permeable in 0.1% Triton-100 for 10 min. Goat serum was used to block nonspecific staining. Hepatoma cells were incubated with the anti-p-ERK 1/2 (4370S, 1:200, CST), E-cadherin (3195P, 1:200, CST), Vimentin antibody (5741P, 1:200, CST) overnight at 4° C. After washing with PBS, cells were blotted with goat anti-rabbit IgG (1:300, ZB-2301, ZSGB-BIO) for 2 h at 37 °C. Cells were placed on fluorescence microscope for observation.

2.7. Western blot analysis

Protein extracted from 15 cases of HCC tissues and hepatoma cells treated with LX2-CM or LX2-CM + U0126 in glass and co-cultured flask. Protein concentration was detected by BCA kit. Protein samples with equal volume were separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were blocked with 5% nofatted milk in Tris-buffered saline (TBS) containing 0.1% Tween 20 for 2 h at room temperature. Primary antibody contained ERK1/2, p-ERK1/2, JNK, p-JNK, PCNA, cyclinD1, c-jun, E-cadherin, Vimentin, Snail, Twist (1:1000 for all, CST), GAPDH (1:500), were