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Original Articles

Hepatic stellate cells activated by acidic tumor microenvironment promote the metastasis of hepatocellular carcinoma via osteopontin

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ABSTRACT

Extracellular pH of solid tumor is generally acidic due to excessive glycolysis and poor perfusion. But whether acidic tumor microenvironment influenced the stromal cells infiltrating in tumor remains unknown. As the predominant progenitor of stromal cells in liver, the number of activated hepatic stellate cells (HSCs) was found positively correlated to the acidification level in the tumor tissues of HCC patients in our study. Whereas, *in vitro* acidic culture condition and *in vivo* co-implanting xenograft model were adopted to study the response of HSCs and its influence on HCC progression. HSCs were activated under acidic culture condition depending on the phosphorylation of cellular signal-regulated kinase (ERK). Acidity-activated HSCs promoted HCC metastasis *in vitro* and *in vivo*. Osteopontin (OPN) excretion from HSCs was increased under acidic condition and proved to promote the migration of HCC cells. Furthermore, the expression level of OPN was significantly associated with myofibroblasts and the combination of α-SMA with OPN was a powerful predictor for poor prognosis of HCC patients. Activation of HSCs in acidic tumor microenvironment represents a novel mechanism for HCC metastasis and provides a potential therapeutic strategy for HCC.

Introduction

To maintain the rapid growth and proliferation, the metabolism of the tumor cells shifts to glycolysis even in the presence of oxygen, which is known as aerobic glycolysis or the 'Warburg effect' [1]. Excessive proton derived from glycolysis is transported to the intercellular space by over-expressed H^+ transporters in tumor cell to maintain relative week basic intracellular pH (pHi) [2,3]. The remarkable prevalence of Warburg effect with the aerobic glycolysis is perpetuating feature of tumor malignancy, leading to consistent acidification of local tumor microenvironment, so called acidic tumor microenvironment, which can reach low pH values approaching 5.8 [4–7]. Hyperexpression and hyperactivation of proton pumps render cancer cells adapting to extracellular acidosis and keeping on proliferating, whereas the normal cells are unable to survive under such condition [1]. In fact, when proton pumps are blocked through specific inhibitors malignant cells undergo massive cell death [5].

Low extracellular pH (pHe) has shown to affect on different stages of cancer progression via promoting angiogenesis [8], facilitating extracellular matrix degradation [9,10], and inducing tumor cell epithelial-to-mesenchymal transition [11]. There are also evidences that low pHe can promote cancer progression by influencing stromal cells. The efficacy of tumor-infiltrating T lymphocytes (TILs) and the cytokine production of monocytes are impaired by acidic pHe [8,12,13]. Myofibroblasts, one type of tumor stromal cells, are the most prominent cancer-associated fibroblasts (CAFs) within hepatocellular carcinoma (HCC) and play a critical role in modulating neighboring cancer cells [14,15]. However, the impact of tumor microenvironment acidification on myofibroblasts during the process of HCC progression is unknown.

Hepatocellular carcinoma (HCC) is the seventh most common malignancy and the fourth leading cause of cancer-related death worldwide (GLOBOCAN 2008). It is generally accepted that HCC originates from hepatocytes but grows and advances while fully embedded in a surrounding microenvironment with fibrosis and cirrhosis [16,17]. Activation of hepatic stellate cells (HSCs) is a key feature of liver fibrosis and cirrhosis. Following liver injury, quiescent HSCs become activated and convert into highly proliferative myofibroblast-like cells, which can be recognized by their expression of α-smooth muscle actin (α-SMA) [18]. HSCs activation is the most dominant pathway that contributes

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to myofibroblast accumulation in liver [19]. HSCs-derived myofibroblasts are found to present within HCC stroma and tend to promote tumor growth and invasiveness, as well as inhibit necrosis, which are more remarkable than peritumoral activated HSCs [15,20,21]. In this study, we evaluated the correlation between tumor acidification and HSCs activation. The acidic tumor microenvironment aggravated the activation of HSCs within HCC stroma. Moreover, acidity-activated HSCs performed the metastasis-promoting function on HCC and were associated with the poor prognosis of patients with HCC.

Materials and methods

Patients, specimens and public databases

Human HCC tissue arrays of 51 patients were provided by Shanghai Biochip (Shanghai, China). Forty-nine cases used in quantitative real-time PCR were collected from HCC patients who underwent surgical resection in the Liver Cancer Institute, Zhongshan Hospital, Fudan University between 2004 and 2005. Ethical approval for human subjects was obtained from the research ethics committee of Zhongshan Hospital, and informed consent was obtained from each patient. All patients were followed up until October 2010. Overall survival was defined as the interval between the dates of surgery or the last follow-up. Disease-free survival was defined from the dates of surgery to recurrence or the last follow-up.

The expression levels of pHi regulators and α-SMA in HCC tissues and normal liver tissues were evaluated using Oncomine datasets (https://www.oncomine.org/resource/login.html). Significance of differences in gene expression was determined by the unpaired Student's *t* test.

Cell culture

The human HCC cell lines, Hep3B and PLC, were purchased from the American Type Culture Collection (ATCC, VA, USA); Huh7 was purchased from RIKEN BRC Cell Bank, Japan; and MHHC-97L was from the Liver Cancer Institute, Zhong Shan Hospital, Fudan University. The human hepatic stellate cell line LX-2 was a gift of Dr. Scott. Friedman. All the cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, CA, USA), with 10% fetal bovine serum (Gibco, CA, USA), in a 5% CO_2 atmosphere at 37 °C. Medium was further supplemented with 25 mM HEPES (Sigma-Aldrich, MO, USA) and 1.5 g/L $NaHCO_3$ buffer system and was adjusted to pH 7.2 under the 5% CO_2 atmosphere at 37 °C. The acidic culture medium (pH 6.2) was prepared just like the pH 7.2 medium except that the medium was supplemented with 25 mM MES (Sigma-Aldrich, MO, USA), 25 mM HEPES, and 1.5 g/L $NaHCO_3$ buffer system.

Xenograft model

MHCC-97L cells (2×10^6) with or without LX-2 cells (2×10^6) were suspended in 40 μL serum-free DMEM/matrigel (1:1) for each mouse. Through an 8-mm transverse incision in the upper abdomen under anesthesia, each nude mouse (12 in each group, female BALB/c-nu/nu) was orthotopically inoculated in the left hepatic lobe with a microsyringe. After 1 week, the mice in each of the two groups were randomly divided into two groups (six mice in each group), with ESOM or saline respectively intraperitoneal injected three times every week. After 3 weeks, mice were sacrificed, and their livers and lungs were dissected, fixed with phosphate-buffered neutral formalin and prepared for standard histological examination, and serums were collected for ELISA assay. All mice were received human care and manipulated according to protocols approved by the Shanghai Medical Experimental Animal Care Commission.

Immunohistochemistry

The tissue array sections or other tissue sections (5-μm) were dehydrated and subject to peroxidase blocking. The primary antibody was added and incubated at room temperature for 30 min on the Dako AutoStainer (Carpinteria, CA, USA) using the DakoCytomation EnVisiont System-HRP (DAB) detection kit. The slides were counterstained with hematoxylin. The stained slides were observed under microscope and images were acquired. Primary antibodies were α-SMA (Sigma, 1:400), HIF1α (Abcam, 1:40), P-Erk1/2 (CST, 1:200) or OPN (Abcam, 1:100). After nuclear counterstaining with hematoxylin, the cytoplasmic immunostaining intensity was categorized semiquantitatively into four groups: negative (score 0); weakly positive (score 1); moderately positive (score 2); and strongly positive (score 3). These scores were determined independently by two senior pathologists.

Microarray data analysis

The total RNA of LX-2 cells cultured in pH 7.2 or pH 6.2 medium separately for 6 days was used in gene expression microarray analysis. Total RNA was isolated from cells at 80% confluence with mirVana™ miRNA Isolation Kit (Ambion, Life Technology, MA, USA) and purified using an RNeasy Mini Kit (Qiagen). Then, 795 ng Cy3-labeled cRNA was used for hybridization on Agilent Human SurePrint G3 genomic expression microarray 8 × 60K (Agilent Technologies). Gene expression data were extracted using Feature Extraction software and were quantile-normalized using GeneSpring software (Agilent Technologies). Genes with a fold change ≥1.5 and *P*-value <0.05 were considered to be differentially expressed. Array files are available on the GEO DataSets database under accession No. GSE 49301. Ingenuity pathway analysis (IPA) was used to examine the functional association between differentially expressed genes and to generate the highest significant gene networks (Ingenuity). Relevant networks were identified using the scoring system provided by IPA.

Real-time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen) and reversely transcribed using the PrimeScript™ RT Reagent Kit (Perfect Real Time) (TaKaRa Biotechnology). The real-time polymerase chain reaction (PCR) was subsequently performed following the manual (TaKaRa Biotechnology). The expression levels were normalized against those of the internal reference genes, β-actin or 18 sRNA as indicated in figures, and the relative expression levels were displayed using the $2^{-\Delta\Delta Ct}$ method. Primers used were listed in Supplementary Table S2.

Western blot

Cell lysates were prepared with the T-PER tissue protein extraction reagent (Pierce, Rockford, IL) with a cocktail of proteinase inhibitors (Roche Applied Science, Switzerland) and a cocktail of phosphatase inhibitors (Roche Applied Science). The total protein concentration was determined by 280 nm OD on NanoDrop 2000 (Thermo Scientific, MA, USA). The western blotting assay was as described before [6]. Primary antibodies used were anti-α-SMA (Sigma), anti-α-tubulin (Calbiochem), anti-Erk1/2, or anti-P-Erk1/2 (CST), respectively.

Immunofluorescence

LX-2 cells cultured in pH 6.2 for 5 days were planted on 3.5-cm dishes (NEST, GBD-35-15, Italy) for another day, then fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 for 10 minutes, and blocked with 5% bovine serum albumin (AMRESCO, OH, USA). Samples were incubated with α-SMA primary antibody (Sigma) at 4 °C overnight, then were incubated with Alexa flour 594 secondary antibody (Invitrogen) and FITC-Phalloidin (1:1000) (Beyotime) at room temperature for 1 h. Nuclei were stained with Hoechst 33342 (Invitrogen) for 5 minutes. Following a final rinse of three times with PBS, the cells were imaged by confocal laser scanning microscopy FluoView FV1000 (Olympus) using appropriate excitation and emission filter sets for trinal fluorophore detection.

In vitro *migration assay*

For the transwell migration assay, cells were trypsinized and resuspended in serum-free DMEM, 5×10^4 cells (200 μL) were planted on the top chamber of each insert (BD Biosciences, NJ, USA) with 8-μm-diameter pores on its membrane. To conduct migration assay of HCC cells, 600 μL of conditioned medium collected from LX-2 cells was injected into the lower chambers. To assess the chemotaxis of LX-2 cells, 600 μL DMEM supplemented with 10% FBS was injected into the lower chambers. After incubation at 37 °C, cells remaining in the top chamber of the inserts were carefully removed. After fixation and staining in a dye solution containing 0.1% crystal violet and 20% methanol, cells adhering to the lower side of the inserts were counted and imaged through an IX71 inverted microscope (Olympus). To examine the involvement of OPN, neutralization antibody of OPN (R & D Systems, Wiesbaden, Germany) was added to the condition medium of the lower chambers with the indicated concentration. The same volume of PBS was added to the other group of chambers with the same conditioned medium as control.

Extracellular pH assay

The proton secretion of HCC cells was determined by measuring pHe. As described previously [6], pHe was measured using pH-sensitive dye BCECF (Sigma Chemical Co., St. Louis, MO). Cells (1×10^5) were seeded per well in 48-well plate and were cultured in 25 mM HEPES and 1.5 g/L $NaHCO_3$-buffered DMEM medium (pH 7.2) containing 10% FBS at 37 °C in 5% CO_2. The medium was removed after the cells attached to the plate, and 200 μL serum-free 25 mM HEPES and 1.5 g/L $NaHCO_3$-buffered DMEM medium (pH 7.4) with ESOM or saline were added into each well. After the cells were cultured with 170 μmol/L or 340 μmol/L ESOM (AstraZeneca, Sweden) for 24 and 48 h at 37 °C in 5% CO_2, respectively, 100 μL supernatant per well was collected and 1 μmol/L BCECF was added into each sample. The sample was excited at 490 and 440 nm, the emitted fluorescence was measured at 535 nm by the Perkin-Elmer LS-50B, and pHe was calibrated with the curve plotted by the fluorescence ratio F490/F440 of standard DMEM medium containing 1 μmol/L BCECF with a series of pH buffered by 25 mM HEPES and 1.5 g/L $NaHCO_3$.