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Original Articles

Lipopolysaccharide supports maintaining the stemness of CD133+ hepatoma cells through activation of the NF-κB/HIF-1α pathway

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ABSTRACT

Due to the existence of cancer stem cells (CSCs), persistence and relapse of human hepatocellular carcinoma (HCC) are common after treatment with existing anti-cancer therapies. Emerging evidence indicates that lipopolysaccharide (LPS) plays a crucial role in aggravating HCC, but information about the effect of LPS on CSCs of HCC remains scant. Here, we report that the stemness of CD133+ CSCs sorted from the human HCC cell line Huh7 was maintained well when cells were cultured with LPS. The reduction of CD133 expression was much lesser in cultured CSCs in the presence of LPS. In response to LPS stimulation, CSCs showed an increase in their activity of clonogenesis and tumorigenesis. LPS also supported maintaining CSC abilities of migration, invasion, and chemo-resistance. Treatment with HIF-1α-specific siRNA significantly reduced CD133 expression by CSCs at both mRNA and protein levels. Further, the expression of HIF-1α and CD133 was reduced in LPS-stimulated CSCs when the NF-κB inhibitor was added to the cell culture. HIF-1α-specific siRNA also effectively counteracted the effect of LPS on maintaining CSC abilities of migration and invasion. These data indicate that LPS, an important mediator in the liver tumor microenvironment, supports the maintenance of CSC stemness through signaling of the NF-κB/HIF-1α pathway. Our current study highlights LPS as a potential target for developing new therapeutic approaches to eliminate CSCs during the treatment of HCC.

Introduction

Liver cancer is the second leading cause of cancer-associated death in men worldwide [1]. Hepatocellular carcinoma (HCC), accounting for 70%–85% of the total liver cancer burden [2], is one of the most common malignancies. Despite advances in diagnosis and treatment of HCC during the last several decades, recurrence and mortality of this disease remain high because of advanced stages when the disease is usually diagnosed and strong resistance to existing anti-cancer therapies. Efforts are continually demanded for exploring novel strategies to treat HCC.

Recent studies have shown that a subpopulation of cancer cells, termed cancer stem cells (CSCs) or tumor initiating cells (TICs) with characterizations of self-renewal, plasticity, dormancy, and metastasis, is responsible for tumor relapse after therapy [3,4]. The presence of CSCs in resected specimens of HCC is associated with poor prognosis in patients treated with radical resection [5,6]. Hence, CSCs are considered to be a pivotal target for eradication of HCC. Due to cancer cell plasticity, cancer can survive from many commonly employed cancer therapies [4,7,8]. Factors influencing the stemness maintenance of CSCs should be taken into consideration for the treatment of cancer. Studies have shown that complex interactions among stromal, inflammatory, and cancer cells in the tumor microenvironment may be correlated with the stemness maintenance of CSCs [9–11]. At the present time, however,

Abbreviations: HCC, hepatocellular carcinoma; CSCs, cancer stem cells; TICs, tumor initiating cells; LPS, lipopolysaccharide; EMT, epithelial–mesenchymal transition; FBS, fetal bovine serum; 5-Fu, 5-fluorouracil; TLR4, toll-like receptor 4; PAMPs, pathogen-associated molecular patterns.

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mechanisms underlying the maintenance and enhancement of CSC stemness remain unclear. CD133 (also known as prominin-1 or AC133), in addition to serving as a marker of liver CSCs, is also important in functionally conferring liver CSC-like features and the associated malignancy. $CD133^+$ liver CSCs represent an important subpopulation of cells responsible for developing HCC [12,13]. Studies have shown that when sorted $CD133^+$ HCC cells or $CD133^-$ HCC cells are cultured *in vitro*, they are capable of generating $CD133^-$ HCC cells or $CD133^+$ HCC cells, respectively, in a time-dependent manner [14]. The ratio of CD133 positive to CD133 negative cells eventually reaches a steady state. Expression of CD133 by cultured cells then returns to the level seen in the original cell line from which these cells are sorted [14]. These observations indicate that liver CSCs possess a highly plastic character. Further studies on the mechanism regulating the stemness maintenance of CSCs will be helpful for developing effective therapy to treat HCC.

Lipopolysaccharide (LPS or endotoxin), a cell wall component of Gram-negative bacteria, plays a crucial role in aggravating HCC [15,16]. In patients with chronic liver diseases, elevated levels of LPS in the liver and systemic circulation are common due to the increase in intestinal permeability and bacterial translocation [17,18]. At the present time, little is known about the effect of LPS on the plasticity of liver cancer stem cells. Our previous studies have shown that LPS induces metastasis and invasion as well as epithelial–mesenchymal transition (EMT) of HCC cells [19]. And we also determined that LPS promotes CXCR4-dependent migration and transition to a mesenchymal-like phenotype of colorectal cancer cells [20]. Therefore, we have postulated that LPS plays an important role in the tumor microenvironment of HCC, including the regulation of CSC stemness.

In this study, we found that LPS regulated the activity of $CD133^+$ CSCs. With LPS stimulation, the abrogation of stemness in CSCs was slower in the *in vitro* culture system. This action of LPS on the maintenance of CSC stemness was mediated through the activation of the NF-κB/HIF-1α pathway. These discoveries identify a novel role of LPS in promoting HCC progression through the regulation of CSC activity.

Materials and methods

Reagents and antibodies

Both the Dulbecco modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Invitrogen (Carlsbad, USA). Lipopolysaccharides (LPS) were purchased from Sigma (Saint Louis, Missouri, USA). For isolation of $CD133^+$ HCC cells and flow cytometric analysis, the MACS CD133 kit and anti-human CD133 antibody, purchased from Miltenyi Biotech (Bergisch Gladbach, Germany), were used. Antibodies used in this study included rabbit anti-human GAPDH (Bioworld Technology, USA), rabbit anti-human CD133 polyclonal antibody (Proteintech, USA), rabbit anti-human HIF-1α (Abcam, UK), mouse anti-human HIF-1α (Santa Cruz Biotechnology, CA, USA), goat anti-rabbit IgG antibody (Bioworld Technology, USA), goat anti-mouse IgG antibody (Bioworld Technology, USA), rabbit anti-human IκB-α and rabbit anti-human p-IκB-α (Cell Signaling Technology, USA). BAY11-7082 was obtained from Selleck Chemicals (USA). HIF-1α siRNA (h), control siRNA, and siRNA transfection medium were purchased from Santa Cruz Biotechnology (Carlsbad, CA, USA).

Cell culture and isolation of $CD133^+$ populations by magnetic-activated cell sorting (MACS)

To isolate $CD133^+$ cells from human HCC line Huh7, magnetic-activated cell sorting was performed according to the manufacturer's instructions as described before [21]. Briefly, Huh7 cells were dissociated into single-cell suspension and centrifuged for 10 min at 300 *g*. Cells were then suspended in phosphate-buffered saline (PBS) solution (pH 7.2, 0.5% BSA, 2 mM EDTA) at a concentration of 10^8 cells per 300 μl. The blocking reagent consisted of FcR (100 μl/10^8 cells), and CD133 microbeads (100 μl/10^8 cells) were added and mixed at 4 °C for 30 min. Cells were washed three times in PBS solution and then added to a prewashed magnetic separation (LS) column on the magnetic holding device. The positive fraction of cells was collected after the column was washed three times. Again, the positive fraction was run through another fresh LS column and washed three times. The quality of sorting was verified with flow cytometry. $CD133^+$ cells were maintained in DMEM containing 10% FBS with PBS or LPS (10 μg/ml) at 37 °C in a humidified incubator containing 95% air and 5% CO_2.

Flow cytometry

Sorted $CD133^+$ cells were cultured with PBS or LPS for 4 weeks. Expression of CD133 by the cells was measured by flow cytometry at time points of weeks 0, 1, 2, and 4. Briefly, cells were labeled with the PE-conjugated anti-CD133 antibody. The percentage of $CD133^+$ cancer cells was measured with a MACSQuant Analyzer 10 flow cytometric system (Miltenyi Biotech, Germany).

Real-time reverse transcription polymerase chain reaction (real-time RT-PCR)

Total RNA was isolated using TRIZOL (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA was quantified using an ND-2000 spectrophotometer (Nanodrop Technologies, Wilmington, DE) and complementary DNA synthesis was performed using the PrimeScript RT reagent Kit (Takara, Kyoto, Japan). Standard RT-PCR was conducted using SYBR Green PCR Kit (Applied BI) according to the manufacturer's instructions. The sequences of PCR primers are shown in Supplementary Table S1.

Western blotting

As previously described [22], cells were washed with PBS and lysed in cell lysis buffer for western blotting with 1 mM PMSF. Equal amounts of proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. After transfer, the membrane was blocked in 5% fat-free milk/1 × TBS/0.1% Tween-20 for 1 h at room temperature and incubated with primary antibodies with gentle agitation overnight at 4 °C. The membrane was then incubated with the secondary antibody of goat anti-mouse or anti-rabbit IgG antibody (1:10,000) for 1 h at room temperature, followed by extensive washing with 1 × TBS/0.1% Tween-20. Immunoblots were developed by using the BeyoECL (Beyotime) and Tanon 5200 system.

Clone formation assay

Sorted $CD133^+$ cells were cultured with PBS or LPS for 1, 2 and 4 weeks. Collected cells from the cultures were then seeded in a six-well plate (5×10^2 cells/well). After incubation at 37 °C for 14 days, the cells were washed twice with PBS and stained with 0.1% crystal violet solution. The number of colonies containing more than 20 cells was counted under a microscope [22].

Sphere formation assay

$CD133^+$ cells, which were cultured with PBS or LPS for 1, 2 or 4 weeks, were collected and plated at 500 cells per well in an ultra-low attachment six well plate, then cultured under condition as described previously [21]. The culture medium was serum-free DMEM/F12 supplemented with 20 μg/ml human recombinant basic fibroblast growth factor, 20 μg/ml recombinant human insulin-like growth factor-1, 20 μg/ml epidermal growth factor, heparin sodium salt, 1% MEM non-essential amino acids, 1,2-mercaptoethanol and 1% GlutaMAX-I. 7 days later, spheres were quantified by using inverted contrast microscopy and sphere numbers were counted.

In vivo *tumorigenicity experiments*

Six-week-old male athymic BALB/c nu/nu mice were obtained from Shanghai Experimental Animal Center, Chinese Academy of Science. Mice were maintained under a pathogen-free condition and treated in accordance with the institutional animal welfare guidelines of the Second Military Medical University. For assay of tumorigenicity, $CD133^+$ cells cultured with PBS or LPS (10 μg/ml) for a week were collected, washed, and then suspended in Matrigel/PBS (1:1) at a concentration of 5×10^6 live cells/ml. The cell mixture (0.1 ml/mouse) was injected subcutaneously into the left back of mice. At the end of 4 weeks, the mice were sacrificed. The weight and volume of the tumor developed were calculated as described [23]. For details, the tumor growth was monitored with electronic calipers using the formula: Volume = $\pi ab^2/6$, where a is the length of the tumor and b is the tumor width.

Real-time cell migration assay

Cell migration assay was performed using an xCELLigence Real-Time Cell Analyzer (RTCA) DP Instrument equipped with a CIM-plate 16 (ACEA Biosciences Inc. San Diego, CA, US) [24]. Cells (5×10^4) were added in duplicate to the upper chambers without serum. FBS (10%) was used as a chemoattractant. Migration was monitored every 15 min for 36 hours.

Transwell invasion assay

To conduct the transwell assay, matrigel invasion chambers installed with 8.0 μm PET membrane in 24 well plates (Corning, USA) were used. Matrigel invasion chambers was pre-treated with 50 μl DMEM without serum and incubated at 37 °C for 30 minutes. Cells (5×10^4) in the 15 μl DMEM without serum were plated in each