Short Communication

Lipopolysaccharide promotes angiogenesis in mice model of HCC by stimulating hepatic stellate cell activation via TLR4 pathway

Yi Lu†, Jianliang Xu†, Shuxian Chen, Zheng Zhou, and Nan Lin*

Department of Hepatobiliary Surgery, The 3rd Affiliated Hospital of Sun Yat-sen University, Guangzhou 510630, China

†These authors contributed equally to this work.
*Correspondence address. Tel/Fax: +86-20-85253178; E-mail: drlinnan@163.com

Received 5 June 2017; Editorial Decision 24 July 2017

Abstract

Angiogenesis plays a key role in the progression of hepatocellular carcinoma (HCC). This study aimed to investigate whether lipopolysaccharide (LPS) could promote HCC angiogenesis and the role of hepatic stellate cell (HSC) in this process. In vivo orthotopic HCC model and the effect of LPS on HSC in vitro were studied. Our results demonstrated that LPS-induced HSC activation during the promotion of HCC growth and angiogenesis in mice. The LPS-TLR4 (Toll-like receptor 4) pathway in HSC is responsible for HCC angiogenesis. LPS-induced secretion of pro-angiogenic factors from HSC could promote endothelial cell migration and tubulogenesis. This study suggests that LPS acts with HSC in tumor stroma and promotes the secretion of pro-angiogenic factors that increase angiogenesis in HCC.

Key words: lipopolysaccharide, hepatic stellate cell, angiogenesis, hepatocellular carcinoma

Introduction

Hepatocellular carcinoma (HCC) is a severe malignant tumor and the third most common cause of death from cancers worldwide [1]. Although ~20% of HCC patients have the option of curative resection, there is still a high incidence of postoperative recurrence and metastasis for this fatal disease [2]. The liver microenvironment contributes to HCC progression [3,4]. It is well-known that the majority of HCC arises from cirrhotic liver [5]. Hepatic stellate cell (HSC) is an identified feature of liver cirrhosis and plays a key role in HCC microenvironment [6–8]. It has been found that HSC is associated with HCC angiogenesis [9]. HSC secretes angiogenic factors that play a critical role in HCC initiation, progression and metastasis, as well as in the creation of new vessels [10–12], but the mechanism is still unclear.

Portal hypertension induced by liver cirrhosis is associated with increased inflammatory stimuli via translocation of intestinal bacterial [13]. Recent studies suggested that the intestinal microflora, as a main source of portal vein lipopolysaccharide (LPS), plays a critical role in portal hypertension and carcinogenesis [14]. Development of portal hypertension promoted by LPS is linked with angiogenesis and vascular remodeling of the hepatic sinusoids. HSC highly responsive to even low concentration of LPS is a key driver during this process [15,16]. It has been postulated that HSC may also be influenced by LPS in the initiation and progression of HCC, and play a key role in tumor angiogenesis.

Toll-like receptor 4 (TLR4) acts as a receptor of LPS and is expressed in various types of cells in liver, including HSCs [17]. LPS-TLR4 signaling mediates HSC activation in liver fibrosis and cirrhosis. A previous study also demonstrated that TLR4 is involved in hepatic angiogenesis [18]. In the present study, we tested the effect of LPS on the HCC growth and angiogenesis. The mechanism of LPS-TLR4 pathway responsible for the secretion of pro-angiogenic factors in HSC and for HCC angiogenesis were also explored. Our results showed that LPS leads to tumor growth and angiogenesis in mice model of HCC through TLR4 receptor. Furthermore, we also demonstrated that LPS-induced HSCs...
promote endothelial cell (EC) migration and tubulogenesis by pro-angiogenic factors.

Materials and Methods

Cell lines and siRNA transfection
The murine hepatic cancer cell lines H22, HSCs and the rat colon microvascular ECs were purchased from the Animal Laboratory of Sun Yat-sen University (Guangzhou, China). The H22 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; Hyclone, Logan, USA) supplemented with 10% fetal bovine serum (FBS; Life Technologies, Carlsbad, USA). HSC-T6, an immortalized rat liver stellate cell line and ECs were grown in RPMI-1640 (Hyclone) containing 10% FBS. Transfection of TLR4-siRNA (sequence: CCGUGGUAUCAUGACAGGAATAUUUCUGUCAGUACCAAG-GTT) or control siRNA in HSC-T6 was performed according to the manufacturer’s protocol using 10 nM siRNA (Qiagen, Valencia, USA). The siRNA-transfected HSC-T6 cells were used in the subsequent in vivo and in vitro studies.

In vivo orthotopic HCC model
BALB/c nude mice (aged 4–5 weeks) were purchased from the Animal Laboratory of Sun Yat-sen University and maintained under pathogen-free conditions and cared for according to the Experimental Animal Care Guidelines. The hepatic lobe of BALB/c-nu mice (n = 10 per experiment group; mean bodyweight, 20 g) were injected with a 100 μl cell suspension of either a mixture of 1 × 106 H22 cells plus 1 × 106 control siTLR4-HSC-T6 cells; or a mixture of 1 × 106 H22 cells plus 1 × 106 siTLR4-HSC-T6 cells, as outlined in our previous report [12]. Seven weeks later, the mice were injected with 50 μg LPS or saline for a duration of 1 week, and then sacrificed. The volume of the tumors was measured and the tissue was examined histologically.

Immunohistochemistry
Primary orthotopic liver tumors were sectioned (4–5 μm) and immunostained for the detection of α-smooth muscle actin (α-SMA) and CD34 protein expression using goat anti-mouse α-SMA and CD34 antibodies (Santa Cruz Biotechnology, Santa Cruz, USA), respectively. α-SMA and CD34 positive cells were visualized using FITC-conjugated anti-goat secondary antibody (Santa Cruz Biotechnology). In addition, α-SMA and CD34 immunostaining was quantified by counting the number of positive cells within a field (×200 magnification) in five randomly selected tumor positions, and the percentage of positive cells was determined. Meanwhile, the nuclei were counterstained with 4’,6’-diamidino-2-phenylindole dihydrochloride.

ELISA
The concentration of VEGF, PDGF, and Ang-1 in the HSC-H6 or stTLR4-HSC-H6 supernatant was analyzed using a commercial ELISA kit (eBioscience, San Diego, USA) according to the manufacturer’s instructions.

HSC-CM and EC migration analysis
To prepare the conditioned medium (CM), cells were washed twice with serum-free DMEM one day after being seeded into T75 flasks (1 × 106 cells). Then cells were incubated with serum-free DMEM and 5 μg/ml LPS for 24 h. In the control group, CM from LPS-treated HSC-T6 cells was pre-incubated with a VEGF neutralizing antibody at a concentration of 5 μg/ml. The experiments were performed in triplicate and repeated three times with consistent results.

Migration assay was performed using Transwells chambers containing polycarbonate filters with 8-μm pores (Corning Costar, USA). The lower compartment contained LPS-treated HSC-CM with or without VEGF antibody or medium containing VEGF as positive control. ECs were harvested, resuspended in DMEMWitout FBS at a concentration of 5 × 105 cells/ml and placed in the upper chamber. After incubation at 37°C for 24 h, the filters were collected, and the cells that adhered to the lower surface were fixed, stained and counted. The experiments were performed in triplicate and repeated three times with consistent results.

In vitro tubulogenesis assay with ECs
ECs were serum-starved overnight and seeded on growth factor-reduced Matrigel (2 × 104 cells/well chamber slide) in basal DMEM. The slides were cultured for up to 10 h and tubulogenesis was visualized and images were captured using an inverted microscope equipped with a digital camera. Images were quantified using Image Pro Software. To evaluate the effect of pro-angiogenic factors on tube formation in vitro, VEGF neutralizing antibody (20 μg/ml; BD Biosciences, Bedford, USA) was applied at the time that cells were seeded on Matrigel. ECs were also cultured on HSC-T6 derived decellularized matrix in some tubulogenesis experiments as indicated.

Statistical analysis
Data are expressed as the mean ± SD of at least three independent experiments. Statistical analyses were performed using the SPSS statistical software (version 13.0; Professional Statistic, Chicago, USA). A two-tailed paired Student’s t-test and ANOVA was used to determine the significance between the test and the control conditions. The criterion for significance was set at P < 0.05 for all comparisons.

Results
LPS promotes tumor growth and angiogenesis in mice model of HCC
In order to determine the effect of LPS on HCC growth and angiogenesis, the mice model of HCC was established by using H22 and HSC-T6 cells and intrahepatic mixed injection method, then mice were injected with 50 μg LPS or saline for duration of 1 week. After LPS exposure, tumor was resected and CD34 was detected by immunohistochemistry that was used as a marker of microvascular density (MVD). It was found that tumor size and weight in LPS exposure group were significant higher than in control group. The criterion for significance was set at P < 0.05 for all comparisons.

To investigate whether HCC growth and vascular change are associated with HSC, mice tumor samples in both groups were analyzed by immunohistochemistry. It was found that tumor size and weight in LPS group were higher MVD in the LPS group (P < 0.05). LPS could promote HCC angiogenesis, as evidenced by higher MVD in the LPS group (Fig. 1).

To determine the effect of LPS on HCC growth and angiogenesis, the mice model of HCC was established by using H22 and HSC-T6 cells and intrahepatic mixed injection method, then mice were injected with 50 μg LPS or saline for duration of 1 week. After LPS exposure, tumor was resected and CD34 was detected by immunohistochemistry that was used as a marker of microvascular density (MVD). It was found that tumor size and weight in LPS group were increased when compared with that in the control group (Fig. 1). LPS could promote HCC angiogenesis, as evidenced by higher MVD in the LPS group (Fig. 2).

To investigate whether HCC growth and vascular change are associated with HSC, mice tumor samples in both groups were analyzed for α-SMA, a marker for activated HSC. α-SMA was highly expressed in tumor stroma exposed to LPS (Fig. 2). These results indicated that LPS-induced HSC activation during the promotion of HCC growth and angiogenesis in mice.
Figure 1. Tumor size and weight of mice model of HCC by using H22 and HSC-T6 cells intrahepatic mixed injection method  
LPS or control group: H22 and HSC-T6 mixed injection with LPS or saline injection after tumorigenesis. siTLR4 + LPS or siRNA + control group: H22 and siTLR4-HSC-T6 mixed injection with LPS or saline injection after tumorigenesis. Tumor weight and size in siTLR4 + LPS were less than those in LPS group (*P < 0.05).

Figure 2. Immunohistochemical analysis of CD34 and α-SMA in tumor sample  
LPS or control group: H22 and HSC-T6 mixed injection with LPS or saline injection after tumorigenesis. siTLR4 + LPS or siRNA + control group: H22 and siTLR4-HSC-T6 mixed injection with LPS or saline injection after tumorigenesis. There were higher MVD and α-SMA expressions in tumor stroma exposed to LPS. When TLR4 was silenced in HSC-T6, tumor MVD and α-SMA expressions were decreased in the mice model of HCC. Original magnification, 10×. *P < 0.05.
LPS-TLR4 pathway in HSCs is responsible for mice HCC angiogenesis

TLR4 is the identified receptor for LPS and is expressed both in HCC and stromal cells. We therefore investigate whether TLR4 on HSC is involved in LPS-induced mice HCC growth and angiogenesis. TLR4-siRNA was used to silence TLR4 expression in HSC-T6, then siTLR4-HSC-T6 cells was mixed with H22 cells to build the mice model of HCC by intrahepatic injection. The same method described above was used to detect MVD and HSC markers in tumor after LPS or saline exposure for 1 week. It was found that tumor weight and size in siTLR4 + LPS group were smaller than those in LPS group (P < 0.05; Fig. 1). When TLR4 was silenced in HSC-T6, tumor MVD was decreased in the mice model of HCC. The expressions of HSC markers were also correlated with the results of MVD analysis (Fig. 2). These data showed that LPS-TLR4 pathway in HSC is responsible for mice HCC angiogenesis and LPS may also induce HCC cells to promote tumor growth.

LPS increases the secretion of pro-angiogenic factors in HSC through TLR4 receptor in vitro

Then, the effects of LPS on pro-angiogenic factors secretion from HSC in vitro were investigated. HSC-T6 was cultured in the presence or absence of 5 μg/ml LPS for 24 h, and ELISA was performed to detect VEGF, PDGF, and Ang-1 in HSC-T6 supernatant. Indeed, secretion of pro-angiogenic factors was increased in LPS-treated HSC-T6 cells. Specific siRNA targeting TLR4 was used to block the synthesis of TLR4 in HSC-T6 cells. Secretion of VEGF, PDGF and Ang-1 was then assessed in the LPS or control group. It was shown that inhibition of TLR4 expression significantly suppressed the secretion of pro-angiogenic factors in HSC-T6 exposed to LPS (Fig. 3), suggesting that TLR4 mediated the crosstalk between LPS and HSC-T6. These results indicated that LPS-TLR4 pathway is responsible for the secretion of pro-angiogenic factors in HSC.

HSC-derived pro-angiogenic factors promotes EC migration and tubulogenesis

Since LPS promotes HSC-mediated pro-angiogenic factors secretion through TLR4, further experiments were carried out to evaluate the effect of HSC-derived pro-angiogenic factors on EC angiogenic characteristics in vitro, including cell migration and tubulogenesis. The results showed that there was a 2-fold increase in EC migration toward LPS-HSC-CM in Transwell assay compared with that toward control (Fig. 4A). LPS-HSC-CM was associated with a much greater increase in tubulogenesis of EC when compared with that in the control (Fig. 4B). These data indicated that LPS-induced secretion of pro-angiogenic factors from HSC promotes angiogenesis.

Discussion

A large proportion of HCC develops in the background of liver cirrhosis [19]. Portal hypertension almost occurs at the end stage of liver cirrhosis accompanied with intestinal flora disturbance and elevated LPS in the portal vein system [14]. In this study, we examined the effect of LPS on the HCC growth and angiogenesis. Our results demonstrated that LPS induces HSC activation during promotion of HCC growth and angiogenesis in mice. We also showed that LPS-TLR4 pathway in HSC is responsible for mice HCC angiogenesis. Specifically, we demonstrated that LPS-induced secretion of pro-angiogenic factors from HSC promotes EC migration and tubulogenesis. Identification of the potential role of LPS as a promoter of HCC growth and angiogenesis may provide possible therapy targets for HCC.

LPS has been identified as a ligand for TLR4 that mediates specific effects of bacterial products [17]. However TLR4 is expressed in different types of cells in liver, such as tumor cells, HSC, and Kupffer cells [15,20]. We want to clarify whether HSC is involved in LPS-induced HCC growth and angiogenesis through silencing TLR4 expression in HSCs. The lack of additive pro-angiogenic effect of LPS in HCC with siTLR4-HSC supports the concept that LPS-TLR4 pathway in HSC is responsible for HCC angiogenesis. Interestingly, HCC growth also stagnates when TLR4 in HSC is blocked, indicating that angiogenesis is an important step for the proliferation of tumor cells. But it was also noticed that the negative effect of TLR4-siRNA in HSCs on HCC growth is very weak. So we hypothesize that the negative effect of TLR4-siRNA in HSC is mainly on tumor angiogenesis (Fig. 2). Tumor growth depends on many factors, and angiogenesis is only one factor.

Angiogenesis is a critical step during HCC growth and metastasis [21], as reflected by the efficacy of sorafenib which targets this process. The rapid growth pattern of HCC requires new vessel formation which is stimulated by multiple pro-angiogenic factors. HSC is an important kind of cells in HCC stroma and secretes several categories of factors to affect HCC progression, such as angiogenesis, immune condition and tumor metastasis [7,22]. HSCs are known to secrete some pro-angiogenic factors to promote HCC angiogenesis [9,11]. Our previous study showed that HSC cells induce the secretion of VEGF-A in HSCs under hypoxic condition in vitro [23].
Here we demonstrated that LPS can induce HSC secretion of several pro-angiogenic factors, such as VEGF, PDGF, and Ang-1, through the TLR4 pathway in vitro. These pro-angiogenic factors could be key contributors to HCC angiogenesis, which is correlated with tumor progression.

In summary, we proved that the pro-angiogenesis factors secreted by HSC can induce EC migration and form blood vessel. LPS from intestine bacteria flora is a critical promoter for this process. Reducing the production of LPS, with the use of non-absorbable antibiotics, might be a potential anti-HCC therapy in the future.

**Funding**

This work was supported by the grants from the Medical Scientific Research Foundation of Guangdong Province, China (No. A2015116), Science and Technology Planning Project of Guangdong Province, China (No. 2016A020212004), China...
Postdoctoral Science Foundation (No. 2016M602910), the National Natural Science Foundation of China (No. 81760112), and the Natural Science Foundation of Guangdong Province, China (Nos. 2014A030313067 and 2014A030313144).

References