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Full-length Article

Sympathetic nervous system promotes hepatocarcinogenesis by modulating inflammation through activation of alpha1-adrenergic receptors of Kupffer cells

Hong-bo Huan [a], Xu-dong Wen [a], Xue-jiao Chen [b], Lin Wu [a], Li-li Wu [a], Liang Zhang [a], Da-peng Yang [a], Xia Zhang [b], Ping Bie [a], Cheng Qian [b], Feng Xia [a,*]

[a] Institute of Hepatobiliary Surgery, Southwest Cancer Center, Southwest Hospital, Third Military Medical University, Chongqing, China
[b] Institute of Pathology and Southwest Cancer Center, Southwest Hospital, Third Military Medical University, and Key Laboratory of Tumor Immunology and Pathology of Ministry of Education China, Chongqing, China

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ABSTRACT

The sympathetic nervous system (SNS) is known to play a significant role in tumor initiation and metastasis. Hepatocellular carcinoma (HCC) frequently occurs in cirrhotic livers after chronic inflammation, and the SNS is hyperactive in advanced liver cirrhosis. However, it remains unclear whether the SNS promotes hepatocarcinogenesis by modulating chronic liver inflammation. In this study, a retrospective pathological analysis and quantification of sympathetic nerve fiber densities (tyrosine hydroxylase, TH^+) in HCC patients, and diethylnitrosamine (DEN)-induced hepatocarcinogenesis in rats were performed. Our data showed that high density of sympathetic nerve fibers and α1-adrenergic receptors (ARs) of Kupffer cells (KCs) were associated with a poor prognosis of HCC. Sympathetic denervation or blocking of α1-ARs decreased DEN-induced HCC incidence and tumor development. In addition, synergistic effects of interleukin-6 (IL-6) and transforming growth factor-beta (TGF-β) in hepatocarcinogenesis were observed. The suppression of the SNS reduced IL-6 and TGF-β expression, which suppressed hepatocarcinogenesis, and KCs play a key role in this process. After the ablation of KCs, IL-6 and TGF-β expression and the development of HCC were inhibited. This study demonstrates that sympathetic innervation is crucial for hepatocarcinogenesis and that the SNS promotes hepatocarcinogenesis by activating α1-ARs of KCs to boost the activation of KCs and to maintain the inflammatory microenvironment. These results indicate that sympathetic denervation or α1-ARs blockage may represent novel treatment approaches for HCC.

1. Introduction

Liver cancer is the second most common cause of cancer-related death worldwide (Torre et al., 2015). Hepatocellular carcinoma (HCC), as the most common primary liver cancer, frequently occurs in cirrhotic livers after years of chronic inflammation due to persistent liver damage (Naugler et al., 2007) and has an annual incidence of 1%–5% (Prieto, 2008). It is reported that the sympathetic nervous system (SNS) is involved in tumorigenesis (Ondicova and Mravec, 2010), and the SNS is hyperactive in advanced liver cirrhosis (Worlicek et al., 2010). However, the effect of SNS on hepatocarcinogenesis is rarely reported. Therefore, it is necessary to explore whether the SNS takes part in the initiation of HCC and to evaluate the neurobiological mechanism of hepatocarcinogenesis.

Abbreviations: HCC, hepatocellular carcinoma; SNS, sympathetic nervous system; KCs, Kupffer cells; TH, tyrosine hydroxylase; AR, adrenergic receptor; DEN, diethylnitrosamine; 6-OHDA, 6-hydroxydopamine; $GdCl_3$, gadolinium (III) chloride; IL-1α, interleukin-1 alpha; IL-1β, interleukin-1 beta; IL-6, interleukin-6; IL-8, interleukin-8; IL-10, interleukin-10; IL-22, interleukin-22; TNF-α, tumor necrosis factor alpha; IFN-γ, interferon gamma; TGF-β, transforming growth factor beta; MCP-1, monocyte chemotactic protein-1; Pra, prazosin; Pro, propranolol; NE, norepinephrine; ISO, isopropylarterenol; LPS, lipopolysaccharide; 5-Mu, 5-methylurapidil; CEC, chloroethylclonidine dihydrochloride; AFP, a-fetoprotein; ELISA, enzyme-linked immunosorbent assay; RT-PCR, real-time quantitative reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

* Corresponding author at: Institute of Hepatobiliary Surgery, Southwest Cancer Center, Southwest Hospital, Third Military Medical University, Chongqing 400038, China.
E-mail address: txzzxf@163.com (F. Xia).

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The role of the nervous system in cancer pathogenesis has been attracting increasing attention. Previous reports have suggested that tumor tissues are innervated and that neurotransmitters may affect tumor initiation and metastasis (Ondicova and Mravec, 2010; Li et al., 2013; Szpunar et al., 2016). High concentrations of norepinephrine may be involved in the development of various types of cancer (Fitzgerald, 2009). Activation of the SNS promotes malignant cell growth by activating adrenergic receptors in ovarian cancer cells (Thaker et al., 2006). Recent studies have revealed that the SNS plays an important role in the initial phase of prostate cancer by promoting tumor cell survival (Magnon et al., 2013).

HCC is a classic case of cancer that is linked to chronic inflammation. Chronic inflammation has been suggested to be a major contributing factor of carcinogenesis (Karin and Greten, 2005). HCC frequently occurs after years of chronic inflammation, and chemically induced HCC also depends on inflammatory signaling (Naugler et al., 2007). Furthermore, it has been suggested that psychological factors influence the incidence and progression of cancer by regulating immune responses (Ondicova and Mravec, 2010). The SNS is involved in the enhancement of inflammatory and immune responses in cancer (Powell et al., 2013). It has been revealed that the SNS is associated with increased expression of cytokines released from macrophages (Reiche et al., 2004). However, it is unclear whether the SNS modulates liver inflammation to participate in hepatocarcinogenesis.

Kupffer cells (KCs) are specialized resident macrophages in the liver and have multifaceted capabilities that contribute hepatocarcinogenesis (Stauffer et al., 2012). It is known that activation of KCs results in the release of various inflammatory cytokines that modulate chronic liver inflammatory responses and cancer (Heymann and Tacke, 2016; Roberts et al., 2007). The inflammatory function of KCs is affected by activation of multiple types of adrenergic receptors (ARs) (Grisanti et al., 2011; Seelaender et al., 1999). However, it appears that AR activation of KCs influences the immune response in a complicated manner. Activation of α1-ARs (Seelaender et al., 1999) and α2A-ARs (Zhang et al., 2010) of KCs caused a significant increase in the production of the inflammatory cytokines, while the production was significantly reduced upon activation of β2-ARs of KCs (Tiegs et al., 1999). Although KCs participate in liver inflammatory responses and contribute to hepatocarcinogenesis, it remains unclear whether KC activities are enhanced by AR stimulation to promote the immune response in hepatocarcinogenesis, and the crucial type of ARs has not been elucidated in this process.

Here, we tested the hypothesis that the SNS promotes hepatocarcinogenesis by modulating liver inflammation via increasing the activity of KCs. In the present study, we found that a high density of sympathetic nerve fibers was correlated with poor prognoses in HCC patients. Additionally, diethylnitrosamine (DEN)-induced hepatocarcinogenesis was inhibited after sympathetic denervation or blocking α1-ARs. Importantly, the SNS regulated inflammatory responses by activating α1-ARs to enhance the activity of KCs in hepatocarcinogenesis. It indicates that sympathetic innervation is vital for hepatocarcinogenesis, and sympathetic denervation or α1-AR blockage may be a novel treatment approach for HCC.

2. Materials and methods

2.1. Animal studies

Six-week-old male Sprague-Dawley rats weighing 140–160 g were obtained from the Center for Animal Experimentation of Southwest China (Third Military Medical University). Rats were housed in a pathogen-free facility under conditions of controlled temperature (24 ± 2 °C) and humidity (55 ± 5%) and were fed standard rodent chow and tap water *ad libitum*. All animal experiments were approved by the Animal Care Ethics Committee of the Third Military Medical University.

Forty male rats were administered a chemical carcinogen, 0.4% (wt/vol) DEN (Sigma-Aldrich, St. Louis, MO, USA), to induce HCC. DEN was administered in the drinking water from Monday through Saturday, with normal drinking water provided on Sunday. Five rats were randomly selected for euthanasia at 4, 8, 12, 16, 20, and 24 weeks of DEN administration. Ten normal rats were administered normal sterile drinking water as the control.

For sympathetic denervation and blocking of α1-ARs, 45 male rats were administered DEN, as described above, for 24 weeks. After 16 weeks of DEN administration, the rats were randomly divided into three groups (DEN; DEN + 6-hydroxydopamine [6-OHDA]; and DEN + prazosin [Pra]). 6-OHDA (Sigma-Aldrich) was administered intraperitoneally to destroy tyrosine hydroxylase $(TH)^+$ neural fibers in rats of DEN + 6-OHDA group twice per week (Monday and Thursday) at a dose of 25 mg/kg body weight. A specific α1-AR antagonist, prazosin (Sigma-Aldrich), was added to daily to the drinking water of the rats in the DEN + Pra group at a dose of 5 mg/kg. To eliminate KCs *in vivo*, 15 rats were intraperitoneally administered gadolinium (III) chloride ($GdCl_3$, Sigma-Aldrich) at a dose of 10 mg/kg (twice per week on Monday and Thursday) after the 16 weeks of DEN administration. All rats were administered with DEN and other drugs in different groups until they were euthanized after 24 weeks. Nine normal rats were administered normal sterile drinking water as a control. Liver tissues were collected immediately from all rats after sacrifice. The size, weight, and number of liver tumors were measured and recorded, and the livers were weighed and divided into several pieces including tumor and adjacent normal tissue. Some tissues were frozen in liquid nitrogen, while others were fixed and paraffin-embedded for histological analysis.

2.2. Tissue samples of HCC patients

To evaluate the relevance of sympathetic innervation in human HCC, we retrospectively analyzed the density of sympathetic nerve fibers in hepatectomy tissues from 54 HCC patients. Preexisting paraffin-fixed tumor specimens were obtained after acquiring informed consent from all patients. This study was approved by the Institutional Review Board of the Southwest Hospital, Third Military Medical University, and conformed to the ethical guidelines of the Helsinki Declaration. For each patient, pathological evaluation was conducted by two independent pathologists from Southwest Hospital. All 54 patients have completed the follow-up information. The average follow-up time was 24 months, and the longest was 72 months.

2.3. Histology, immunohistochemistry, and immunofluorescence

Rat liver tissues, including tumor tissue and adjacent normal tissue, were paraffin-embedded. Liver sections from the rats were stained with hematoxylin and eosin (H&E). Liver fibrosis was evaluated by Picro-sirius red staining according to the manufacturer's instructions (Abcam, Cambridge, UK). Immunohistochemistry was performed by using a DAKO EnVision Detection System (Agilent Technologies, Santa Clara, CA, USA). Pathological evaluation was conducted by two independent pathologists who were blinded to the sample sources.

For immunofluorescence, sections were deparaffinized with xylene and rehydrated through graded alcohol washes followed by antigen retrieval in sodium citrate buffer. To determine the nine subtypes of ARs on the KC surface, KCs were seeded onto glass