Resolvin D1 inhibits the proliferation of lipopolysaccharide-treated HepG2 hepatoblastoma and PLC/PRF/5 hepatocellular carcinoma cells by targeting the MAPK pathway

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Abstract. Hepatocellular carcinoma (HCC) and hepatoblastoma are common malignant tumor types in China. The aim of the present study was to evaluate the effects of resolvin D1 (RvD1) on inflammatory factor levels and mitogen-activated protein kinase (MAPK) signaling in lipopolysaccharide (LPS)-treated liver cancer cells. First, HepG2 hepatoblastoma and PLC/PRF/5 HCC cells were cultured and treated with LPS with or without various concentrations of RvD1 (0.025, 0.05, 0.1 and 0.2%). Subsequently, ELISA was performed to measure the protein levels of tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6 in the culture medium. In addition, cell proliferation of the liver cancer cells was assessed by MTT assay. Reverse transcription-quantitative polymerase chain reaction and western blotting were performed to detect the expression of TNF-α, IL-1β and IL-6 in the cultured cells. Western blotting was also performed to assess the protein expression of phosphorylated extracellular signal-related kinase (p-ERK), p-c-Jun N-terminal kinase (p-JNK) and p-p38. Compared with the control group, LPS treatment increased the protein levels of TNF-α, IL-1β and IL-6 in the culture medium, and RvD1 inhibited this increase in a concentration-dependent manner. RvD1 also reduced the LPS-induced increase in TNF-α, IL-1β, IL-6, p-ERK, p-JNK and p-p38 expression levels in liver cancer cells. LPS promoted the proliferation of liver cancer cells, while RvD1 attenuated this effect. In summary, the current findings suggest that RvD1 inhibits cell proliferation and the expression of inflammatory cytokines in LPS-treated liver cancer cells by targeting the MAPK pathway.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common types of liver cancer. It is the fourth most commonly occurring cancer and the third most common cause of cancer-associated cases of mortality in China (1). HCC is usually caused by the hepatitis C virus, the hepatitis B virus, alcohol abuse and other causes of cirrhosis (2). The main treatment for patients with hepatocellular carcinoma include surgical resection, liver transplantation, interventional therapy, radiotherapy, chemotherapy and immunotherapy (3). Another type of liver cancer is hepatoblastoma, which is specifically formed by immature liver cells (4). Hepatoblastoma is the most common liver cancer of early childhood (5). The treatment of hepatoblastoma is mainly based on platinum-based chemotherapy combined with complete surgical resection of the masses (6). Approximately 15% of the cases require liver transplantation (7). The main treatments for HCC and hepatoblastoma include resection, transplantation, radiofrequency ablation, chemomobilization and sorafenib (8,9). Unfortunately, current treatment approaches for HCC and hepatoblastoma are far from satisfactory as the cancers continue to have a high recurrence and metastasis rate (10,11), and there is an urgent need for novel therapeutic strategies and targets.

Resolvins are active substances with specific lipid structures (12). They are derived from ω-3 unsaturated fatty acids, primarily eicosapentaenoic acid and docosahexaenoic acid (DHA) (13). Functionally, resolvins promote the resolution of the inflammatory response back to a non-inflamed state (12,14). Recent studies using various different models have demonstrated that resolvin D1 (RvD1) may exert a liver-protective effect (15-17). Furthermore, evidence indicates that RvD1 is able to regulate Toll-like receptor 4-mediated inflammatory responses (18,19) in numerous diseases, including HCC. The present study aimed to determine whether RvD1 could inhibit the proliferation of lipopolysaccharide (LPS)-treated liver cancer cells, and to elucidate the possible underlying mechanism of its effect. To the best of our knowledge, no other reports on this topic have been published to date. If the hypothesis is verified, RvD1 may have potential for clinical use in liver cancer therapy.

Mitogen-activated protein kinase (MAPK) is a serine/threonine-specific protein kinase that is stimulated by extracellular
molecules, including cytokines, neurotransmitters, hormones and tumor-promoting substances. There are three types of MAPK signal transduction pathway in mammalian organisms: The extracellular signal-regulated kinase (ERK) signaling pathway regulates cell growth and differentiation (20); while the c-Jun N-terminal kinase (JNK) (21) and p38 MAPK (22,23) signaling pathways serve key roles in inflammation and apoptosis in response to stress. MAPK pathways are typically activated following an inflammatory response; however, resolvins, particularly RvD1, are able to inhibit this signaling transduction (24). Therefore, it was speculated that RvD1 may inhibit cell proliferation and the inflammatory response in LPS-treated liver cancer cells by targeting MAPK. In the present study, the effect of RvD1 on inflammatory cytokine production and MAPK signaling was evaluated in LPS-treated liver cancer cells.

Materials and methods

Cell culture and treatment. The liver cancer cell lines HepG2 (hepatoblastoma) and PLC/PRF/5 (hepatocellular carcinoma) were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). The liver cancer cells were cultured in minimum essential medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin), and incubated in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. Cells were untreated or treated with 100 ng/ml LPS (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 24 h (25) at 37°C with or without various concentrations (0, 0.025, 0.05, 0.1 and 0.2%) of RvD1 (Cayman Chemical Company, Ann Arbor, MI, USA) administered as a pretreatment for 30 min (26) at 37°C. RvD1 was prepared in PBS before use. Untreated cells were used as controls.

ELISA assay. Cells were collected and centrifuged at 500 x g for 5 min at room temperature. The protein levels of inflammatory cytokines [tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6] in the culture media of the liver cancer cells were detected with corresponding Human ELISA kits (cat. nos. PT518, PI305 and PI330; Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer's protocols.

MTT assay. Cell proliferation were determined by MTT assay at 12, 24, 48 and 72 h. Following the various treatments, cells (5x10⁴ cells/well) in 96-well plates were incubated with MTT solution (0.5 mg/ml; Sigma-Aldrich; Merck KGaA) for 4 h at 37°C. The resultant formazan crystals were dissolved in dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA), and the absorbance in each well was measured with a microplate reader (Molecular Devices LLC, Sunnyvale, CA, USA) at a wavelength of 570 nm.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). TRIzol Reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used to extract total RNA from the cells in different groups. cDNA was synthesized using RNA PCR kit (AMV; version 3.0; Takara Biotechnology Co., Ltd., Dalian, China) following the manufacturer's protocols. For cDNA synthesis, samples were incubated at 42°C for 60 min, 70°C for 5 min and 4°C for 60 min. qPCR was performed using an Applied Biosystems StepOne™ Real-Time PCR system (Thermo Fisher Scientific, Inc.) with the SYBR Premix Ex Taq kit (Takara Biotechnology Co., Ltd.). The PCR conditions were as follows: 95°C for 10 min, followed by 40 cycles of initiation at 95°C for 30 sec, annealing at 55°C for 30 sec and elongation at 72°C for 30 sec, and then 4°C for 60 min. The expression levels of TNF-α, IL-1β and IL-6 mRNA were defined based on the threshold cycle (Cq), and the relative expression levels were calculated as 2^-ΔΔCq (27) following normalization by GAPDH. The sequences of the PCR primers were as follows: GAPDH forward, 5'-GGGAGCAGATCCCTCCTAAT-3' and reverse, 5'-GGCTGTGTGTACATCTCTCTGG-3'; TNF-α forward, 5'-GAGGGAACAAGCCCTGTATG-3' and reverse, 5'-CGGCAGATGAATCTCAGC-3'; IL-1β forward, 5'-AGCTAC GAATCTCGACACC-3' and reverse, 5'-GTGTTATCATGTTGAAAGA-3'; IL-6 forward, 5'-ACTCACCTCTTCTAGAACGATTG-3' and reverse, 5'-CCATCCTTGGAGGTTCGCGTTCGGTG-3'.

Western blot analysis. Cells (1x10⁶) were washed with cold PBS and lysed in radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology). The concentration of protein was measured by NanoDrop™ 2000 (Thermo Fisher Scientific, Inc.). Equal amounts of protein (40 µg/lane) were separated by 10% SDSPAGE and electrotransferred to nitrocellulose membranes. The membranes were then blocked in PBS containing 0.1% Tween-20 and 5% (w/v) non-fat dried milk at room temperature for 30 min, and incubated overnight at 4°C with primary antibodies against TNF-α, IL-1β, IL-6, p-ERK, p-JNK and p-p38 (cat. no. 3707, cat. no. 12073, cat. no. 12153, cat. no. 4370, cat. no. 9251 and cat. no. 9211, respectively; dilution, 1:2,000; Cell Signaling Technology, Inc., Danvers, MA, USA). The blots were incubated with HRP-conjugated secondary antibodies (cat. no. A0208; dilution, 1:1,000; Beyotime Institute of Biotechnology) at room temperature for 2 h, then bands were detected by BeyoECL Plus kit (Beyotime Institute of Biotechnology). GAPDH (cat. no. S174; dilution, 1:2,000; Cell Signaling Technology, Inc.) was used as an internal control. The density of the western blot bands was quantified with ImageJ 1.43 software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. Results are expressed as the mean ± standard deviation. Statistical analyses were conducted using SPSS 22.0 software (IBM Corp., Armonk, NY, USA). Data were analyzed by one-way analysis of variance followed by Dunnett's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

RvD1 inhibits the upregulation of TNF-α, IL-1β and IL-6 protein levels in the culture medium of LPS-treated liver cancer cells. ELISA was performed to identify the protein levels of inflammatory cytokines in the supernatant of treated cells. As shown in Fig. 1, compared with the control group, 100 ng/ml
LPS significantly increased the protein levels of TNF-α, IL-1β and IL-6 in the culture medium. Pre-treatment of the cells with RvD1 (0.05, 0.1 and 0.2%) significantly repressed the LPS-induced increases in TNF-α, IL-1β and IL-6 levels. This effect appeared to be in a concentration-dependent manner.

**RvD1 reduces the proliferation of LPS-treated liver cancer cells.** To clarify the role of RvD1 in liver cancer, cells were treated with LPS and an MTT assay was performed to determine the effects of RvD1 on cell proliferation. Compared with the control group, 100 ng/ml LPS significantly promoted cell viability and proliferation (Fig. 2). Compared with the 100 ng/ml LPS group, RvD1 pre-treatment (0.05, 0.1 and 0.2%) significantly reduced cell proliferation. This effect appeared to be in a concentration-dependent manner.

**RvD1 inhibits the mRNA and protein expression of TNF-α, IL-1β and IL-6 in LPS-treated liver cancer cells.** RT-qPCR and western blot analyses were performed to evaluate the effects of RvD1 on the levels of inflammatory factors in liver cancer cells. The results indicated that 100 ng/ml LPS significantly upregulated the mRNA and protein expression levels of the inflammatory cytokines TNF-α, IL-1β and IL-6 compared with the control group (Figs. 3-5). The addition of RvD1 (0.05, 0.1 and 0.2%) significantly attenuated the LPS-induced upregulation. This effect appeared to be in a concentration-dependent manner.

**RvD1 inhibits the expression of p-ERK, p-JNK and p-p38 protein in LPS-treated liver cancer cells.** To verify whether RvD1 plays a role in the MAPK signaling pathway, western
Figure 3. Effect of RvD1 on the expression of TNF-α, IL-1β and IL-6 mRNA in LPS-treated liver cancer cells. The expression levels of TNF-α, IL-1β and IL-6 mRNA in (A) HepG2 and (B) PLC/PRF/5 cells were determined by reverse transcription-quantitative polymerase chain reaction. *P<0.05, **P<0.01, ***P<0.001 vs. control group. #P<0.05, ##P<0.01, ###P<0.001 vs. LPS group. RvD1, resolvin D1; LPS, lipopolysaccharide; TNF, tumor necrosis factor; IL, interleukin.

Figure 4. Effects of RvD1 on the expression of TNF-α, IL-1β and IL-6 protein in LPS-treated HepG2 hepatoblastoma cells. (A) Western blot bands representing TNF-α, IL-1β and IL-6 protein. Relative protein levels of (B) TNF-α, (C) IL-1β and (D) IL-6 were calculated. *P<0.05, **P<0.01, ***P<0.001 vs. control group. #P<0.05, ##P<0.01, ###P<0.001 vs. LPS group. RvD1, resolvin D1; LPS, lipopolysaccharide; TNF, tumor necrosis factor; IL, interleukin.
Figure 5. Effects of RvD1 on the expression of TNF-α, IL-1β and IL-6 protein in LPS-treated PLC/PRF/5 hepatocellular carcinoma cells. (A) Western blot bands representing TNF-α, IL-1β and IL-6 protein. Relative protein levels of (B) TNF-α, (C) IL-1β and (D) IL-6 were calculated. *P<0.05, **P<0.01, ***P<0.001 vs. control group. #P<0.05, ##P<0.01, ###P<0.001 vs. LPS group. RvD1, resolvin D1; LPS, lipopolysaccharide; TNF, tumor necrosis factor; IL, interleukin.

Figure 6. Effects of RvD1 on the expression of p-ERK, p-JNK and p-p38 protein in LPS-treated HepG2 hepatoblastoma cells. (A) Western blot bands representing p-ERK, p-JNK and p-p38 protein. Relative protein levels of (B) p-ERK, (C) p-JNK and (D) p-p38 were calculated. *P<0.05, **P<0.01, ***P<0.001 vs. control group. #P<0.05, ##P<0.01, ###P<0.001 vs. LPS group. RvD1, resolvin D1; LPS, lipopolysaccharide; ERK, extracellular signal-related kinase; JNK, c-Jun N-terminal kinase.
 blotting was performed to measure the expression levels of p-ERK, p-JNK and p-p38 proteins in liver cancer cells. As indicated in Figs. 6 and 7, 100 ng/ml LPS significantly enhanced the expression of p-ERK, p-JNK and p-p38 protein compared with the control group. The addition of RvD1 (0.05, 0.1 and 0.2%) significantly attenuated the LPS-induced upregulation. This effect appeared to be in a concentration-dependent manner.

**Discussion**

RvD1 is a type of resolvin that is derived from ω-3 polyunsaturated fatty acids, including DHA. It has been demonstrated to serve a key function in acute inflammation in certain animal disease models (28). In the present study, an MTT assay indicated that LPS (100 ng/ml) significantly increased the proliferation of liver cancer cells, while RvD1 inhibited this increase, suggesting that RvD1 may inhibit the proliferation of LPS-treated liver cancer cells. Therefore, RvD1 may be useful in therapies against liver cancer.

Recent studies have demonstrated that inflammation is associated with the development and progression of multiple cancer types (29,30). Tumor-associated inflammation can promote angiogenesis and metastasis, and a persistent inflammatory microenvironment can trigger tumorigenesis by inducing certain genetic mutations (31).

TNF-α, IL-1β, IL-6 are pro-inflammatory cytokines, which may serve key functions in the pathological mechanisms of cachexia in cancer (32). These cytokines are involved in cell proliferation, differentiation and apoptosis (33). TNF-α has been implicated in a number of cancer cachexia-associated processes, including anorexia and body weight loss, metabolic alterations and systemic inflammation (34). IL-1β and IL-6 are members of the interleukin family of cytokines and are activated through proteolytic processing. They play critical roles in many chronic diseases, including atherosclerosis, diabetes and various cancer types (35,36).

In the present study, the effect of RvD1 on the aforementioned inflammatory cytokines was evaluated by ELISA, RT-qPCR and western blotting. The results identified that RvD1 treatment markedly decreased the mRNA and protein levels of these inflammatory cytokines in LPS-treated liver cancer cells, and restrained their LPS-induced release. Collectively, these results demonstrate that RvD1 treatment can significantly suppress the LPS-induced expression and release of TNF-α, IL-1β and IL-6, as representative inflammatory cytokines, in liver cancer cells.

MAPK signaling, including the ERK, JNK and p38 pathways, is responsible for transducing extracellular signals to influence numerous cellular processes, including cell proliferation, survival and death (37). In order to investigate whether RvD1 exerted its effects via MAPK, western blotting experiments were performed. RvD1 significantly suppressed
the LPS-induced increases in p-ERK, p-JNK and p-p38 in liver cancer cells.

In summary, the present study demonstrated that RvD1 inhibits the proliferation of LPS-treated liver cancer cells, and reduces the expression of TNF-α, IL-1β and IL-6 at the protein and mRNA levels, as well as their release, in LPS-treated liver cancer cells. In addition, RvD1 decreases p-ERK, p-JNK and p-p38 levels in LPS-treated liver cancer cells. Therefore, RvD1 may suppress liver cancer cell proliferation by targeting MAPK signaling. However, further research is required to confirm this hypothesis. In conclusion, RvD1 may be a novel and effective anti-inflammatory mediator, and could form the basis of a novel therapy for hepatoblastoma or hepatocellular carcinoma.

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Authors’ contributions

YL was a major contributor in writing the manuscript and interpreted the data. QX analyzed the data and revised the manuscript. GY collected the data. WX helped analyse the data. HJ designed the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

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Competing interests

The authors declare that they have no competing interests.

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