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Research Article

Long-term use of indomethacin leads to poor prognoses through promoting the expression of PD-1 and PD-L2 via TRIF/NF-κB pathway and JAK/STAT3 pathway to inhibit TNF-α and IFN-γ in hepatocellular carcinoma

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ABSTRACT

HCC still has a poor prognosis in clinical due to high recurrence and metastasis rates worldwide nowadays. Indomethacin pretreatment is used as a potential chemopreventive agent in cancers for it could assist in anti-tumor functions of other agents and exert anti-tumor effect. Our study aims to discuss the effects and mechanisms of long-term use of indomethacin in HCC. The HepA mouse models were used to observe tumor recurrence, intrahepatic metastasis and remote metastasis. NK cell, αβ T cell and γδ T cell were used to explore the underlying mechanisms for anti-tumor effect of indomethacin. The results showed that long-term use of indomethacin facilitated intrahepatic recurrence, intrahepatic dissemination and lung metastasis, and indomethacin inhibits TNF-α and IFN-γ *in vivo* and *in vitro* in a dose-dependent manner. Furthermore, long-term use of indomethacin increased the expression of PD-1 and PD-L2 in programmed death-1 pathway. Blockade of PD-1 and PD-L2 reversed the reduced production of TNF-α and IFN-γ induced by indomethacin in γδ T cells. In addition, long-term use of indomethacin activates TRIF/NF-κB and JAK/STAT3 pathways, and indomethacin promotes the expression of PD-1 and PD-L2 via TRIF/NF-κB pathway and JAK/STAT3 pathway respectively in γδ T cells. Given these findings, we drew a conclusion that long-term use of indomethacin leads to poor prognoses through promoting the expression of PD-1 and PD-L2 via TRIF/NF-κB pathway and JAK/STAT3 pathway to inhibit TNF-α and IFN-γ in HCC.

1. Introduction

As one of the most common cancers and one of the leading causes of cancer related death worldwide [1], hepatocellular carcinoma (HCC) is often diagnosed at an advanced stage [2], resulting that the five-year survival rate of HCC is only 30–40%. Although liver transplantation is the preferred therapy, the lack of suitable donor organs precludes this option for many patients. Now surgery is still the main method for HCC therapy, but the recurrence rate is high because of the dissemination of malignant cells after surgery [3]. To reduce postoperative recurrence and metastasis, actively explore new methods of cooperate operation is an important task in the clinical.

Non-steroidal anti-inflammatory (NSAIDs) are widely applied in clinical treatment for their anti-inflammatory effects. In addition, NSAIDs have been verified to reduce migration, increase apoptosis, and decrease angiogenesis via altering the tumors or the tumor microenvironment [4]. Therefore, the NSAIDs have been reported to prevent cardiovascular disease, diabetes, disease of central nervous system, and even various types of cancer, such as gastric carcinoma, prostatic cancer, and breast cancer [5]. Arun et al. reported that epidemiologic evidence suggests the incidence of colon, and lung cancers is inversely related to the use of aspirin and NSAID drugs, which are nonspecific inhibitors of COX-1 and COX-2 [6]. Another research based on 1392 confirmed cases of breast cancer indicated that the regular use of aspirin, ibuprofen or other NSAIDs may have a significant chemopreventive effect against the development of breast cancer [7]. However, the long-term use of NSAIDs for cancer prevention and therapy remains controversial, because many clinical studies indicate that the compounds have significant side effects for patients. Indomethacin, a kind of NSAIDs, exerts antibiotic, antipyretic and

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analgesic functions using in adjuvant therapy of HCC, but its effects and mechanism are less reported. Previous study reported that indomethacin enhanced the cytotoxicity of doxorubicin which is a chemotherapeutic drug widely used for the treatment in HCC [8]. It is still unclear whether long-term use of indomethacin has side effects in HCC.

According to a report by Inaoka et al., NSAIDs can selectively inhibit Tumor necrosis factor (TNF-α) and interferon γ (IFN-γ) production in NK cells and γδ T cells while sparing that by acquired immune cells, suggesting that frequent use of NSAIDs should be avoided by patients with viral infections or allergic diseases [9]. TNF-α and IFN-γ inhibit the growth of numerous tumors, and have the potential to be a highly specific anti-cancer therapy and have been tried to use in the clinical management of tumors. TNF-α is produced primarily by macrophages, and also a variety of other cells, including NK cells, T lymphocytes, smooth muscle cells and fibroblasts [10]. A recent research showed that TNF induced manganese superoxide dismutase (MnSOD) and the cellular response to cytotoxicity in breast cancer MCF-7 cells [11]. IFN-γ, a multifunctional cytokine, is produced mainly by T helper cells, cytotoxic T cells, natural killer cells, myeloid cells, dendritic cells and macrophages during the onset of the infection [12]. IFN-γ exerts antiviral, anti-proliferative, immunomodulatory and anti-angiogenesis effects [13]. The research reported that B-cell IFN-γ production inhibits Treg-cell differentiation and exacerbates arthritis. The important practical conclusion is that IFN-γ, specifically derived from B cells, uniquely contributes to the pathogenesis of autoimmunity through prevention of immunoregulatory mechanisms [14].

Programmed death-1 pathway plays an important role in allergy, infectious disease, transplantation immunity and tumors [15]. PD-L1 (CD274) and PD-L2 (CD273) are two ligands of PD-1 (CD279). To enhance antitumor immunity, the therapy targeting the programmed death-1 pathway is under investigation in multiple human cancers [16].

Our study aims to discuss the effects and mechanisms of long-term use of indomethacin in HCC and provide the theoretical and experimental basis for clinical application and development of new drugs for patients with HCC. In present research, the HepA mouse models were used to observe tumor recurrence, intrahepatic metastasis and remote metastasis. Next, the product levels of TNF-α and IFN-γ in serum plus mRNA levels of PD-1, PD-L1 and PD-L2 were measured. Then the mechanism underlying the effects of long-term use of indomethacin on liver tumor development was elucidated. The results of our study demonstrate that long-term use of indomethacin leads to poor prognoses through promoting the expression of PD-1 and PD-L2 via TRIF/NF-κB pathway and JAK/STAT3 pathway to inhibit TNF-α and IFN-γ in HCC.

2. Materials and methods

2.1. The establishment of mice HCC model

To mimic the effects of indomethacin on HCC, the model of hepatoma (HepA) in mice was established. Specifically, the mouse HepA cell line was supplied by the Experimental Animal Center of the Chinese Academy of Medical Sciences. HepA ascites tumor cells from hepatoma mice were used to prepare the cellular suspension of 1.0×10^7 ml^{-1} with saline water under aseptic condition. Forty-eight male BALB/c mice weighing approximately 20 g were selected to establish mice HCC models and 0.1 ml (1.0×10^6 cells) HepA cellular suspension was injected into the caudal vein.

Forty-eight HepA mouse models were used to observe tumor recurrence, intrahepatic metastasis and remote metastasis. All animals were randomly divided into a 1 mg/kg indomethacin group, a 2 mg/kg indomethacin group, a 4 mg/kg indomethacin group and a control group with 12 mice in each group, then were given partial hepatectomy and pneumonectomy to carry on the pathology analysis. Animals were taken the corresponding dose once a day until the mice were sacrificed.

2.2. ELISA for TNF-α and IFN-γ

Cytokines of TNF-α and IFN-γ in the serum were measured with an ELISA kit according to the manufacturer's instructions (BD Biosciences). The limit of detection was 25–30 pg/ml.

2.3. Sorting of NK cells, αβ T cells and γδ T cells by MACS

Human peripheral blood mononuclear cells (PBMCs) donors from two healthy male were used to isolate NK cell, αβ T cell and γδ T cell with the approval of Fudan University Shanghai Cancer Center. PBMCs were cultured in complete RPMI1640 media supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10 mM HEPES (Gibco). Cells were labeled with either anit-γδ TCR antibodies conjugated with hapten or anti-αβ TCR antibodies conjugated with phycoerythrin. Then micro-bead labeled cells were selected using magnetic cell separation system (MACS). NK cells were isolated by MACS NK cell isolation kit II (Miltenyi Biotec).

The separated cells were cocultured in 1 mL of complete RPMI1640 media containing control and 5 μg/ml, 10 μg/ml, 20 μg/ml indomethacin (Wako) in 24-well cell plates. After 48 h the cells were assessed by flow cytometry for analysis of TNF-α and IFN-γ as described previously [9].

2.4. Real-time PCR analysis

DNA was extracted from circulating γδ T cells using Qiagen Maxi DNA isolation kits (QIAGEN Inc.). Specific mRNA levels were measured by real-time PCR starting from 100 ng DNA using a Real-time PCR system (Life Technologies Corporation). Values were normalized to GAPDH mRNA as internal standard and analyzed.

2.5. Western blot

Equal amounts protein of cells was resolved by 10% SDS-PAGE. After blotting on PVDF membranes, the membrane was blocked with 3% fat-free milk for 2 h, which were probed with primary antibody for TRIF, nuclear p65, JAK, STAT3 and β-actin at 4 °C overnight. Then the membranes were incubation with appropriate HRP-conjugated secondary antibody for 2 h at room temperature. The chemiluminescence was detected and acquired on to photographic films by using Femto® enhanced chemiluminescence detection system (Pierce).

2.6. Blockade of PD-1 and PD-L2 in γδ T cell

The anti-human PD-1 mAb and the anti-human PD-L2 mAb have been described and the pecific binding of these antibodies to their respective ligands has been demonstrated [17]. Antibodies were manufactured by Bioexpres Cell Culture Inc.

2.7. Transfection of siRNA oligonucleotides

γδ T cells were transfected with siRNA oligonucleotides (300 nM) directed against TRIF, NF-κB, JAK and STAT3 using Lipofectamine RNAiMAX (Invitrogen) following manufacturer's instructions.