Cancer Letters 306 (2011) 34–42

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Combination of interferon-α and 5-fluorouracil induces apoptosis through mitochondrial pathway in hepatocellular carcinoma in vitro

Haipeng Yin [a,b,1], Fengxiang Xie [a,1], Jiao Zhang [c], Yongmei Yang [a], Biping Deng [a], Jintang Sun [a], Qingjie Wang [a], Xun Qu [a], Haiting Mao [a,*]

[a] Institute of Basic Medical Sciences, Qi Lu Hospital, Shandong University, 107# Wen Hua Xi Road, Jinan, PR China
[b] Institute of Basic Medical Sciences, Shandong Academy of Medical Sciences, 66# Jing Shi Road, Jinan, PR China
[c] Department of Hepatopathy, Shandong Provincial Hospital, 324# Jing Wu Wei Qi Road, Jinan, PR China

ARTICLE INFO

Article history:
Received 11 January 2011
Received in revised form 14 February 2011
Accepted 15 February 2011

Keywords:
Interferon-α
5-Fluorouracil
Hepatocellular carcinoma
Proliferation
Mitochondrial apoptosis
Intracellular calcium

ABSTRACT

Many clinical reports have proven that the combination therapy of interferon-alpha plus 5-fluorouracil is remarkably effective for advanced hepatocellular carcinoma (HCC). However, the mechanism of this therapy is not well understood. Here, we demonstrated that the combination therapy synergistically inhibited the growth of Fas-negative HCC cells, arrested cell-cycle progression and induced apoptosis. Moreover, the combination therapy significantly increased the protein expression of caspase-8, activated Bid and cytochrome c. Meanwhile, the expression of anti-apoptotic gene Bcl-xL was reduced and intracellular calcium elevated obviously during the early stage of treatment. Therefore, mitochondrial pathway was involved in the apoptosis of Fas-negative HCC cells induced by IFN-α/5-FU and Ca^{2+} partially promoted the beneficial effect against HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancy worldwide and the third leading cause of cancer death due to its poor prognosis [1]. It is a tumor frequently associated with chronic hepatitis or cirrhosis caused by chronic infection with the hepatitis B virus (HBV) or hepatitis C virus (HCV) [2,3]. Current treatment strategies, such as liver transplantation, surgical resection, or regional therapy for advanced HCC are unsatisfactory. Chemotherapy is commonly used for the treatment of various malignancies. However, systemic cytotoxic chemotherapy agents are minimally effective for HCC because of its resistance to anti-cancer drugs. The tumor recurrence rate of patients with HCC remains high and most patients die within several months after diagnosis [4]. Thus, efforts have been made within the past decades to find a new effective therapy.

Immunochemical therapy combining cytokines and chemotherapeutic agents is expected to be effective for treating advanced HCC. Interferon-alpha (IFN-α), originally discovered by its anti-viral activity, belongs to the group of type I interferons. In addition to its anti-viral properties, IFN-α also shows several other actions in vitro and in vivo, such as direct inhibitory effects on tumor cell growth; enhancement of immunogenicity of tumors and modulation of the immune system [5]. IFN-α induces its biological effects by high affinity cell-surface receptors, the JAK/STAT signaling pathway and the mitogen-activated protein kinase (MAPK) are involved in IFN-induced biological actions [6,7]. IFN-α is also used as an anti-cancer drug for renal cell carcinoma, chronic myelogenous leukemia, and malignant melanoma. Recently, the combination therapy of IFN-α and chemotherapeutic agents has begun to be

* Corresponding author. Address: Institute of Basic Medical Sciences, Qi Lu Hospital, Shandong University, 107# Wen Hua Xi Road, Jinan 250012, Shandong Province, PR China. Tel.: +86 531 82169252; fax: +86 531 82169251.
E-mail address: haitingmao@yahoo.com (H. Mao).
[1] These authors contributed equally to this work.

0304-3835/$ - see front matter © 2011 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.canlet.2011.02.017

applied to several types of human malignancies [8–11]. The combination therapy of IFN-α and 5-fluorouracil (5-FU) was firstly proposed to apply to colon cancer cell lines in 1988 [12]. Later, this treatment strategy was experimented in various tumors in vitro, in vivo and in clinical study. In patients with colorectal carcinoma, esophageal carcinoma or gastric carcinoma, satisfactory results were obtained [13,14]. Some clinical studies have suggested that the combination therapy of IFN-α and 5-FU is remarkably effective for patients with advanced HCC and results in excellent clinical outcome [8,9,15–18]. The in vitro studies have provided some explanations about the synergistic effects of the combination of IFN-α/5-FU in HCC, including anti-proliferation, anti-angiogenic effects, and inducing apoptosis [15,16,19]. Several researchers recently reported that IFN-α acted on the metabolic pathway of 5-FU and Fas/FasL apoptosis pathway might be involved in anti-tumor effects of IFN-α/5-FU against HCCs [16,20]. However, the exact mechanism of 5-FU/IFN-α combination therapy has not yet been fully investigated.

The present study was designed to further explore the underlying mechanism of IFN-α/5-FU combination therapy against HCC from the standpoint of the proliferation inhibition and apoptosis signaling pathway. Since HBV is thought to be a main causative agent and most liver cirrhosis and HCC are developed from patients with chronic HBV infection. Therefore, in this study, we used HepG2.2.15, a representative HCC cell line which was stably transfected with the HBV genome and expressed all HBV proteins, to investigate the mechanism underlying the apoptosis-enhancing effect of IFN-α/5-FU that acts via the mitochondrial apoptosis pathway.

2. Materials and methods

2.1. Reagents

Recombinant human IFN-α (IFN-α 1b) was obtained from Kexing Biotech Co. Ltd, (Shenzhen, China). 5-FU and 3-(4-,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma–Aldrich (Beijing, China). Mouse anti-human Fas agonistic monoclonal antibody CH-11 and its isotypical control antibody were products of Upstate Biotechnology Inc (Massachusetts, USA). FITC-labeled anti-human Fas antibody (clone: DX2) was purchased from BioLegend (Beijing, China). Rabbit anti-caspase-8 polyclonal antibody was obtained from NeoMarker (California, USA). Rabbit anti-Bid polyclonal antibody and rabbit anti-cytochrome c antibody were all products of Cell Signaling Technology (Massachusetts, USA). Anti-human β-actin antibody was purchased from Sigma–Aldrich (Missouri, USA). Annexin V-FITC Apoptosis Detection Kit was product of BD Corporation (Shenzhen, China).

2.2. Cell culture

Human hepatocellular carcinoma cell line HepG2.2.15 and human leukemic T cell line Jurkat were obtained from Chinese Academy of Medical Sciences. HepG2.2.15 was derived from human HCC cell line HepG2 and the parental cells were stably transfected with four copies of the HBV genome. HepG2.2.15 cells were maintained in DMEM supplemented with 10% FBS and 0.4 mg/ml G418 (Invitrogen, USA) in a humidified 5% CO_2 atmosphere at 37 °C. The complete growth medium without G418 would be changed 2 or 3 days before experiment.

2.3. Flow cytometric analysis of Fas expression and apoptosis induced by agonistic anti-Fas antibody

HepG2.2.15 cells were characterized for their surface expression of Fas by flow cytometry. Cells (1×10^6) were incubated with anti-Fas antibody (DX2) for 30 min at 4 °C. After washing with PBS, analysis was performed using a Becton Dickenson FACScan and Cell Quest software. In the apoptotic analysis, three different groups were set up. Experimental group: anti-Fas agonistic antibody CH-11 was added to HepG2.2.15 cells and the final concentration of CH-11 was 100 ng/ml. Negative control group: HepG2.2.15 cells were incubated with the isotypical control IgM. In positive control group, Fas-positive Jurkat cells were incubated with CH-11. All of the above cells were cultured for 24 h, and then, the extent of apoptosis was evaluated using the Annexin V-FITC kit according to manufacturer's instructions. Briefly, 1×10^6 cells from each treatment group were incubated with Annexin V-FITC solution at 4 °C for 10 min and then stained with propidium iodide (PI) at room temperature for 10 min. Finally, the stained cells were analyzed with flow cytometer.

2.4. Cell growth assay

Cell growth was assessed by MTT assay. Briefly, 5×10^3 cells were seeded on a 96-well plate in 100 μl of medium and left overnight to adhere. Subsequently, the medium was replaced with 200 μl of fresh medium containing different concentrations of IFN-α (0–8000 U/ml) or 5-FU (0–80 μg/ml), followed by incubation at 37 °C and 5% CO_2 for 48 h. Afterward, HepG2.2.15 cells were treated with single drug or two-drug combination using the chosen fixed concentrations for 24, 48 and 72 h. After treatment, 10 μl of MTT solution (5 mg/ml) were added to each well and the plates were incubated for another 4 h at 37 °C. After 15 min of centrifugalization at 2000 rpm, culture medium was discarded and then was replaced with 150 μl dimethylsulfoxide (DMSO) per well to dissolve the resultant formazan crystals. Absorbance (A) was measured spectrophotometrically in a microplate reader (Bio-Rad, USA) using 570 nm as test wavelength and 630 nm as reference wavelength.

$$\text{Inhibition ratio } (\%) = (\text{1-experimental group } A_{570\text{-}630} / \text{control group } A_{570\text{-}630}) \times 100\%.$$

2.5. Cell cycle analysis

After treatment for 24 h, cells were washed twice with cold PBS. Subsequently, cells were treated with PBS (pH 7.4) containing 1% RNase, and were stained with PI at 100 mg/ml (final concentration). The percentages of cells in G0/G1, S or G2/M phase were calculated from a contour plot obtained for the flow cytometric analysis.