Interferon-α and cyclooxygenase-2 inhibitor cooperatively mediates TRAIL-induced apoptosis in hepatocellular carcinoma

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\textbf{A B S T R A C T}

Hepatocellular carcinoma (HCC) is a leading cause of cancer-related mortality worldwide. Interferon-alpha (IFN-α) has recently been recognized to harbor therapeutic potential in the prevention and treatment of HCC, but it remains controversial as to whether IFN-α exerts direct cytotoxicity against HCC. Cyclooxygenase-2 (COX-2) is overexpressed in HCC and is considered to play a role in hepatocarcinogenesis. Therefore, we aimed to elucidate the combined effect of a COX-2 inhibitor, celecoxib, and IFN-α on in vitro growth suppression of HCC using the hepatoma cell line HLCZ01 and the in vivo nude mouse xenotransplantation model using HLCZ01 cells. Treatment with celecoxib and IFN-α synergistically inhibited cell proliferation in a dose- and time-dependent manner. Apoptosis was identified by 4',6-diamidino-2-phenylindole dihydrochloride and fluorescent staining. IFN-α upregulated the expression of TRAIL, while celecoxib increased the expression of TRAIL receptors. The combined regimen with celecoxib and IFN-α reduced the growth of xenotransplanted HCCs in nude mice. The regulation of IFN-α and COX-2 inhibitor-induced cell death is impaired in a subset of TRAIL-resistant cells. The molecular mechanisms of HCC cells resistant to TRAIL-induced apoptosis were explored using molecular biological and immunological methods. Interferon-α and the COX-2 inhibitor...
Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, with the majority of cases resulting from persistent infection with hepatitis B virus (HBV) or hepatitis C virus (HCV). In particular, chronic HBV infection is a predominant risk factor for HCC in Asia and Africa [1]. It is the second most frequent cause of cancer death in men, and it is the sixth leading cause of cancer death. An estimated 748,300 new HCC cases and 695,900 cancer deaths occurred worldwide [2]. Half of these cases and deaths were estimated to occur in China [3]. Hepatic resection, liver transplantation and radiofrequency ablation are increasingly used for the curative treatment of HCC. However, only about 10–20% of patients with HCC are currently eligible for surgical intervention. Most patients are still diagnosed at advanced stages and can only receive palliative treatment [4]. Thus, developing an effective chemoprevention strategy is critically important, in order to reduce the incidence of this disease.

Interferon-α (IFN-α) has been discovered based on its antiviral activity [5]. IFN-α belongs to the type I interferon family of cytokines, originally identified for their antiviral properties. Further studies revealed the anti-tumor activity of IFN-α against various tumors via direct inhibitory effects on tumor cells, anti-angiogenesis, enhanced immunogenicity of tumors, and immunomodulatory effects [6]. IFN-α is one of the drugs first approved by the FDA for the treatment of chronic HBV infection, and has been clinically applied for 20 years [7]. However, patients often develop resistance to IFN-α, and the mechanisms of resistance are unknown [8]. It is unclear if IFN-α is directly cytotoxic, and indeed, most hepatoma cell lines are resistant to IFN-α-mediated apoptosis [9]. Consequently, combination therapy is necessary to overcome IFN-α resistance.

Overexpression of cyclooxygenase-2 (COX-2) has been associated with HCC carcinogenesis [10]. Its inhibitor, celecoxib, leads to marked growth inhibition of human liver tumor cells, due to the induction of apoptosis and inhibition of proliferation. Thus, celecoxib may offer a therapeutic and preventive potential in human hepatocarcinogenesis [11]. Our previous studies have indicated that the COX-2 inhibitor celecoxib effectively inhibits the growth and angiogenesis of HepG2 cell xenografts in nude mice via suppressing the expression of COX-2 [12].

The tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a member of the TNF superfamily that can initiate apoptosis through the activation of their death receptors. The ability of TRAIL to selectively induce apoptosis of transformed or tumor cells, but not normal cells, promotes the development of TRAIL-based cancer therapy. Importantly, TRAIL, TRAIL-R1 (DR4), and TRAIL-R2 (DR5) can all be induced by chemotherapeutics and/or radiation, which can sensitize cancer cells to TRAIL [13], so TRAIL is an attractive death ligand in targeted cancer therapy. It triggers apoptosis via two of its receptors, TRAIL-R1 (DR4) and TRAIL-R2 (DR5). Upon binding of TRAIL or agonistic antibodies to TRAIL-R1 or TRAIL-R2, death inducing signaling complex (DISC), caspase-8 and -10 are activated, and may drive the subsequent execution of the apoptotic response through mitochondrial-independent and mitochondrial-dependent pathways [14]. Thus, understanding the regulation of the TRAIL apoptosis pathway can help develop more selective TRAIL-based agents for the treatment of human cancer.

The present study was designed to further explore the underlying mechanism of combination therapy with IFN-α and the COX-2 inhibitor celecoxib against HCC from the standpoint of the proliferation inhibition and apoptosis signaling pathway. HBV is thought to be a main causative agent of most cases of liver cirrhosis, and HCC often develops in patients with chronic HBV infection. We previously established a hepatoma cell line, HLCZ01, the first cell line supporting the entire life cycle of both HBV and HCV [15]. Therefore, in this study, we used HLCZ01 to investigate the mechanism of HCC cells resistant to the TRAIL-induced apoptosis effect of IFN-α and celecoxib combination therapy against HCC. Interferon-α and celecoxib synergistically enhanced TRAIL expression-induced apoptosis in hepatocellular carcinoma. In conclusion, IFN-α and celecoxib may offer a novel role with important implications in designing new therapeutics for TRAIL-resistant tumors.

Materials and methods

Reagents and antibody

Recombinant human IFN-α was obtained from Kexing Biotech Co. Ltd., (Beijing, China). Celecoxib was purchased from Cayman Chemical (Ann Arbor, MI, USA). Celecoxib was dissolved in dimethyl sulfoxide (DMSO) and stored at −80 °C. Human recombinant TRAIL and neutralizing monoclonal anti-TRAIL were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and PGE2 was purchased from Cayman Chemical.

HBV infection of cell culture

Human HCC cell line HLCZ01 was grown in Dulbecco’s Modified Eagle’s Medium (DMEM) with 10% fetal bovine serum at 37 °C in 5% CO2. The supernatant of HepG2.2.15 cells derived from human hepatoma cell line HepG2 transfected with the full genome of HBV was collected and filtered. HLCZ01 cells were inoculated overnight with the filtered supernatant at a multiplicity of infection (MOI) of 20 genome equivalents (Geq) per cell. The cells were washed three times with PBS, maintained in DMEM/F12 medium, and harvested at the indicated times. HLCZ01 cells were inoculated with the different strains of sera from hepatitis B patients diluted at 1:20 and were cultured for the indicated time periods. The HLCZ01 cells of HBV infection were isolated and cultured as described previously [15].

Cell growth assay

Cell growth was assessed by MTT assay. Briefly, 5 × 103 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

Apoptosis

HLCZ01

celecoxib synergistically increased TRAIL-induced apoptosis in hepatocellular carcinoma. These data suggest that IFN-α and celecoxib may offer a novel role with important implications in designing new therapeutics for TRAIL-resistant tumors.
fresh medium containing different concentrations of IFN-α (0–8000 U/ml) and/or celecoxib (0–60 μmol/L), followed by incubation at 37 °C and 5% CO2 for 48 h. Afterward, HLCZ01 cells were treated with a single drug or a two-drug combination using the chosen fixed concentrations for 24 and 48 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 centrifugation at 2000 rpm, the culture medium was discarded and then was replaced with 150 μl 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 the test wavelength and 630 nm as the reference wavelength. Inhibition ratio (%) = (1– absorbance sample/absorbance control) × 100%. The effect of soluble TRAIL, TRAIL-neutralizing antibody, and PGE2 on cell growth was also evaluated.

Flow cytometry analysis

Cell cycle analysis was performed by flow cytometry. DNA labeling was performed using the CytoTest Plus DNA Reagent kit (BD Biosciences Pharmingen, San Diego, CA), and the samples were analyzed using a flow cytometer (BD Pharmingen, San Diego, CA). For the detection of apoptotic cells, labeling tests involving both propidium iodide (PI) and annexin-V were performed using an annexin-V staining kit (Invitrogen, USA) according to the manufacturer’s instructions. The data were analyzed with CellQuest software.

Caspase activity measurement

Cells were treated with IFN-α and/or celecoxib for the indicated times in the presence or absence of the different porphyrins (CoPP, SnPP, ZnPP; 10 μM each) for the indicated times. Caspase-3, Caspase-8, and Caspase-10 activity were measured in cell lysates and tissue homogenates. Cells were stimulated with cephalostatin 1 (1 μM) or etoposide (25 μg/ml) for 8 h. Cells (4 × 104) were centrifuged onto glass slides and dried at room temperature overnight. Samples were fixed and permeabilized with 4% paraformaldehyde containing 0.05% saponin in PBS for 15 min at room temperature. After washing with 0.03% saponin in PBS, probes were blocked with 1% BSA, 1% FCS, and 0.1% Tween 20 in PBS for 1 h and washed twice in PBS. Release of free 7-amino-4-fluoromethyl coumarin (AFC) from the synthetic substrate Ac-DEVD-AFC (50 μM) at 37 °C was determined by fluorescence measurement in a SpectraFluor Plus plate reader (Tecan, Crailsheim, Germany) at an excitation wavelength of 390 nm and an emission wavelength of 535 nm.

Western blot analysis

Cells were washed twice with PBS and lysed in buffer containing 20 mM Tris–HCl (pH 7.4), 137 mM NaCl, 10% (wt/vol) glycerin, 1% Triton X-100, 2 mM EDTA, and a protein inhibitor cocktail (Roche) for 30 min on ice. The homogenates were centrifuged at 14,000 rpm for 20 min at 4 °C and the supernatants were transferred to fresh tubes. Protein samples were separated using 10% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto a nitrocellulose membrane. The membranes were blocked in 5% nonfat dry milk containing 0.1% Tween-20 for 1.5 h at 37 °C, and then probed with primary antibodies at 4 °C overnight. After washing, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody followed by ECL detection (Amersham Pharmacia Biotech, USA). The membranes were scanned with a LAS-4000 luminescent image analyzer (Fujifilm, Japan). Subsequently, the membranes were washed in 20 mM glycine (pH 2.3) and subjected to an anti-tubulin antibody (Sigma) for equal protein loading. The bound antibodies were detected by ECL reagent (Pierce, Rockford, IL), according to the manufacturer’s instructions.

Real-time reverse PCR

Total cellular RNA was extracted by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s instructions. Real-time RT-PCR for DR4, DR5, TRAIL, and COX-2 was performed using the ABI PRISM 7700 instrument (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) with gene-specific primers and the SYBR Green I protocol. A total of 1.0 μg RNA was reverse-transcribed to complementary DNA (cDNA) in a total volume of 20 μl, and 1 μl of this mixture was used as a template for Quantitative real-time polymerase chain reaction (RT-PCR). The primers used for amplification are listed in Table 1. Relative expression ratios normalized to that of GAPDH were calculated.

Animal studies

All animal experiments were undertaken in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Hunan Provincial Tumor Hospital Review Board, Changsha, China, and also following the Hunan provincial government’s Animal Care and Use Committee-approved protocols. All animals were housed in a virus-free facility and maintained in a standard temperature- and light-controlled animal facility. HLCZ01 cells were infected with HBV, and 5 × 106 HLCZ01 cells in 200 μl PBS were injected subcutaneously into the lower right flank of 6-week-old male BALBc Nude. Tumor size and body weight were measured three times weekly, and tumor volumes (mm3) were evaluated. The values were transformed into tumor size using the following formula: tumor volume = 0.5 × width2 × length. When the tumor volume reached around 150 mm3, the mice were randomly divided into four groups (n=8 each). Celecoxib was suspended in water with gum arabic and given orally with intubation at a dose of 150 mg/kg every day. IFN-α was intraperitoneally injected at a dose of 5 × 106 U/kg every 3 days until day 24. The mice were sacrificed and tumors were resected and fixed with 10% neutral-buffered formalin.

<p>| Table 1 – List of primers used for real-time PCR. |
|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences</th>
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<tbody>
<tr>
<td>GAPDH</td>
<td>f: 5′-TTTTCGAGCTAAGCTTTCCTG-3′</td>
</tr>
<tr>
<td></td>
<td>r: 5′-TTGTCAGGGTCTTTCCTC-3′</td>
</tr>
<tr>
<td>TRAIL</td>
<td>f: 5′-ACCAAGCTGTTACAGCATG-3′</td>
</tr>
<tr>
<td></td>
<td>r: 5′-TCCTTTATGATCCCTCAGAG-3′</td>
</tr>
<tr>
<td>DR4</td>
<td>f: 5′-CAGAATCTCCCTGAGGCTTAC-3′</td>
</tr>
<tr>
<td></td>
<td>r: 5′-ATGCCCATTGCTGATTTCTTG-3′</td>
</tr>
<tr>
<td>DR5</td>
<td>f: 5′-TGCAAGCCCTTAGCTTCTG-3′</td>
</tr>
<tr>
<td></td>
<td>r: 5′-GCACCAACTGCTGAAACGAT-3′</td>
</tr>
</tbody>
</table>

Applied Biosystems, Foster City, CA, USA), according to the manufacturer’s instructions. Total cellular RNA was extracted by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s instructions. Real-time RT-PCR for DR4, DR5, TRAIL, and COX-2 was performed using the ABI PRISM 7700 instrument (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) with gene-specific primers and the SYBR Green I protocol. A total of 1.0 μg RNA was reverse-transcribed to complementary DNA (cDNA) in a total volume of 20 μl, and 1 μl of this mixture was used as a template for Quantitative real-time polymerase chain reaction (RT-PCR). The primers used for amplification are listed in Table 1. Relative expression ratios normalized to that of GAPDH were calculated.

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and they were cut into 5-μm sections after embedding in paraffin. Tumor sections were determined by immunohistochemistry.

**TRAIL expression by immunohistochemistry**

Immunohistochemical staining was done on formalin-fixed and paraffin-embedded tissue using 5-μm section from tissue microarray blocks. Mounted tissue section were baked at 60 °C for 30 min, deparaffinized in xylene and rehydrated through graded alcohols. Antigen was retrieved by heating in 1 μM sodium citrate (pH 6.0) in a pressure cooker for 2 min. According to the manufacturer’s instructions, anti-TRAIL (dilution 1:500) was incubated on the section overnight at 4 °C after non-specific staining was blocked, followed by incubation with a horseradish peroxidase-conjugated secondary antibody. Staining was visualized using DAB chromogen substrate and counterstained with haematoxylin. The tissue sections were viewed at 400× magnification. Three fields per section were analyzed and TRAIL-positive cells were calculated using Image-Pro Plus software.

**DAPI staining**

Cells treated with IFN-α (0–6000 IU) and/or celecoxib (0–60 μmol/L) for 24 h were fixed in 4% paraformaldehyde and incubated in 0.5 μg/mL 4, 6-Diamidino-2-phenylindole (DAPI, Sigma Chemical Co.) solution for 30 min in the dark at room temperature. The cells were examined by fluorescence microscope.
Statistical analysis

All results were reported as mean ± SD or median (range). The normality of data distribution was tested by the Kolmogorov–Smirnov test. Pearson’s chi-square test, independent-samples t-test, nonparametric Mann–Whitney U or Wilcoxon signed ranks test, logistic regression, and nonparametric Spearman’s rank correlation were used when appropriate. By using the Finner harmonic mean method and Q-analysis to evaluate the synergistic effect of the two drugs used in the combination therapy group. Receiver operating characteristic (ROC) curves were generated to determine the areas under the ROC curves (AUC) and their 95% confidence intervals (CI). Data analysis was performed using SPSS version 16.0 (SPSS, Inc., Chicago, IL, USA). A univariate test was used to examine the influence of each clinical variable on survival. Student’s t-test was applied to determine statistical significance.

P-values of < 0.05 were considered statistically significant.

Results

The cytotoxic effect of TRAIL on a newly developed HCC HLCZ01 cell line with HBV infection

To better understand the virus lifecycle and the interaction between host and virus, we established a novel HCC cell line HLCZ01 with HBV infection. The cells were derived from well-differentiated HCC tissue of a male patient, and the cells expressed the human liver-specific gene and the protein albumin [15].

IFN-α inhibits HBV replication by decreasing the transcription of viral pregenomic RNA (Fig. 1A). We examined the TRAIL sensitivity of different human HCC cell lines SMMC-7721, Huh7, HLCZ01, and HepG2. Cells were exposed to TRAIL and cell death was tested using DNA ladder assay. DNA ladder was abundantly found in TRAIL-treated SMMC-7721 and HepG2 cells, while rare in Huh7 and HLCZ01 (Fig. 1B). PARP was cleaved in SMMC-7721 and HepG2 cells with TRAIL treatment (Fig. 1C). A possible mechanism of different sensitivity of tested cells to TRAIL-induced apoptosis could be due to the variable levels of the death receptors resulting in increased apoptosis signaling in sensitive cells. The functional TRAIL receptor isoforms (DR4 and DR5) had comparable levels in TRAIL-sensitive cells compared to TRAIL-resistant cells (Fig. 1D).

Anti-tumor effects of IFN-α and celecoxib in HBV-infected HLCZ01 cells

We evaluated the effects of IFN-α, celecoxib, and combination therapy on the growth of HLCZ01 cells using the MTT assay. Treatment with IFN-α alone had a moderately inhibitory effect and only less than 30% of cell growth inhibition ratio was achieved, even though a high concentration of IFN-α (6000 IU/mL) was administered to the cells, indicating that the effect of growth inhibition of IFN-α as a single agent was not very effective for HLCZ01 (Fig. 2A). However, the proliferation of celecoxib-treated
cells was strongly reduced at 48 h, and the inhibition was achieved in a dose-dependent manner (Fig. 2B). To examine whether the combination therapy of IFN-α/celecoxib has synergistic effects on cell growth inhibition, the single concentration for IFN-α and celecoxib was carefully chosen to achieve 20–30% of inhibition ratio in HLCZ01 cells. The half maximal (50%) inhibitory concentration (IC) for treatment with IFN-α and celecoxib was 7834.73 IU/mL and 36.17 μM and the IC50 for the combination therapy group was 4714.1 IU/mL for IFN-α and 32.61 μM for celecoxib. Further evaluated the results by Q-analysis, and obtained the Q value = 0.634 (< 1), so that the combination therapy of IFN-α and celecoxib has synergistic effects. Based on the above results, 4000 IU/mL for IFN-α and 40 μM for celecoxib were chosen for the concentrations of combination treatment for subsequent experiments. HLCZ01 cells were exposed to IFN-α, celecoxib, or a combination of IFN-α and celecoxib for up to 48 h. A synergistic effect was observed in a time-dependent manner and more effective inhibition of cell growth was induced in the combination therapy group in comparison with the single-therapy groups (Fig. 2C).

**IFN-α and celecoxib anti-tumor therapy arrests cell cycle and induces apoptosis in HLCZ01 cells with HBV infection**

Since IFN-α and celecoxib have synergistic anti-tumor roles, we next assessed whether the therapy arrests cell cycle and induces apoptosis. For understanding the mechanism of growth inhibition, we investigated the effects of IFN-α and celecoxib on cell-cycle progression (Fig. 3A). The results of cellular DNA contents distribution showed an increased accumulation of G0/G1-phase cells in the combination group, compared to the single-agent groups and no-treatment control. In parallel, a remarkable decrease of cell populations at the S phase was induced. The findings show the synergistic effect of IFN-α and celecoxib on HLCZ01 cells in inhibiting cell-cycle progression into DNA synthesis phase.
We next explored the effects of IFN-α and celecoxib on apoptosis. Flow cytometric results demonstrated that IFN-α as a single agent failed to induce apoptosis (10.00% ± 1.64%) in HLCZ01 cells compared with the control group (7.88% ± 1.13%), while celecoxib resulted in marked apoptosis (17.26% ± 2.37%). The maximum effect was observed in the combination-therapy group (25.52% ± 3.10%). There were significant differences between the combination-therapy group and the single-agent groups (P < 0.05) (Fig. 3B). Moreover, the combination of IFN-α and celecoxib also induced a remarkable increase in cellular apoptosis, detected by DAPI staining, and an increase in TRAIL expression (Fig. 3C).

**Interferon-α and celecoxib synergistically increase TRAIL expression in HLCZ01 cells with HBV infection**

TRAIL is an IFN-targeted gene [16]. However, the underlying regulatory mechanism has not been explored thus far. Real-time RT-PCR showed that IFN-α (4000 IU/ml) remarkably increased the levels of TRAIL mRNA, even at 6 h following treatment with HBV-infected HLCZ01 cells (Fig. 4A). The levels of DR5 mRNA and DR4 mRNA were slightly downregulated in HLCZ01 cells by IFN-α and celecoxib. Furthermore, the transcription of TRAIL receptors and DR5 mRNA levels were lowered by IFN-α or celecoxib. Consequently, combination treatment induced the expression of both TRAIL ligand and TRAIL receptor. IFN-α and celecoxib increased the expression of DR5 and TRAIL, respectively, and combination treatment amplified both expressions (Fig. 4B).

**Interferon-α- and celecoxib-mediated apoptosis in HLCZ01 cells involves activation of caspases**

Treatment of HBV-infected HLCZ01 cells with celecoxib elicited apoptosis in a dose- and time-dependent manner. To better understand this process, we investigated whether the activation of caspase-8 was induced by either IFN-α or celecoxib alone, or by combination therapy. The caspase-8 activity of HLCZ01 cells with HBV infection at 24 h after treatment was about 6-fold higher than that of control cells, followed by the activation of caspase-3. This observation suggests the involvement of caspases in apoptosis induced by IFN-α and celecoxib. Involvement of caspases was further confirmed by fluorometric determination of the increased enzymatic activity of the caspase-3 and caspase-8 class of proteases in HLCZ01 cells following combined treatment with IFN-α and celecoxib (Fig. 5).

**Effects of interferon-α and celecoxib combination therapy on HLCZ01 cells xenografts in nude mice in vivo**

To test whether the synergistic effects of IFN-α and celecoxib have potentially relevant clinical implications, we next assessed the
anti-tumor effects of IFN-α plus celecoxib in vivo. As shown in Fig. 6A,B and C, treatment with IFN-α and celecoxib synergistically suppressed tumor growth and reduced tumor weight in vivo. Examination of the effects of the treatments on tumor cell proliferation by immunohistochemical staining for TRAIL expression is shown in Fig. 6D. The proportion of TRAIL-positive cells in the control group was 29.9 ± 12.9%, while the percentage with IFN-α or celecoxib treatment alone was 45.2 ± 14.2% and 62.4 ± 12.3%, respectively (P < 0.05 versus control). Combination therapy showed a significantly greater inhibition of tumor cell proliferation in comparison with the control and single-therapy groups (the TRAIL expression was 78.7 ± 10.6%; P < 0.01 versus control), and TRAIL expression was negatively correlated with PI (r = -0.5530, P < 0.01). These results imply that treatment with IFN-α and celecoxib inhibits tumor growth and induces apoptosis of HCC in vivo.

Discussion

Worldwide, over 2 billion people have serological evidence of HBV, and approximately 360 million individuals are chronic HBV surface antigen (HBsAg) carriers[17]. Hepatitis B is a main cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma[18]. There is no vaccine against HCV, and many patients who are persistently infected by HBV or HCV do not respond to currently available therapies[19].

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**Fig. 5** – Effect of IFN-α and/or celecoxib on caspase-3 and -8 activity in HLCZ01 cells. HLCZ01 cells were treated with IFN-α (4000 IU/mL) and/or celecoxib (40 μM) for 24 h, followed by a measurement of the activity of caspase-3 and -8 using a colorimetric assay kit. Data represent mean ± SD of 6 samples. *P < 0.05, **P < 0.01, ***P < 0.005 compared with control cells.

**Fig. 6** – Combined anti-tumor effects of IFN-α and celecoxib on HCC in vivo. (A) HLCZ01 were injected into nude mice for 4 weeks and treatment with IFN-α or/and celecoxib, tumor formation was observed in each groups. (B) Xenografts tumor volume was measured at indicated time points, and the statistical significance was analyzed by two-way ANOVA. (C) Tumor weight was measured on the day of harvest, after excision of the tumor from the euthanized mouse. (D) Immunohistochemistry staining was performed to detect TRAIL expression; Scale bar, 50 μm; *P < 0.05, **P < 0.01.
The following mechanisms contribute to the antitumor effect of IFN-α. First, IFN-α can cause the induction of pro-apoptotic genes. Second, IFN has direct effects on malignant cells. Third, IFN-α can inhibit angiogenesis. Last, it can augment antitumor immune responses and improve liver function [20,21].

Some studies have suggested that COX-2 is an inducible enzyme frequently found in inflammatory tissues and is involved in carcinogenesis pathways in many organs. It has been reported that COX-2 expression is correlated with angiogenesis, invasion, relapse, chemoresistance, and tumorigenesis in HCC [22]. Besides, a significant correlation between COX-2 expression and active inflammation in the adjacent non-cancerous liver tissue is associated with shorter disease-free survival in HCC patients [23]. Celecoxib, a selective COX-2 inhibitor and non-steroidal anti-inflammatory drug (NSAID), is widely used for pain and inflammation. Celecoxib attenuates Akt phosphorylation and induces growth inhibition and apoptosis in HCC cells, which can be partially reversed by ectopic COX-2 expression and PGE2 [24]. The chemopreventive effect of celecoxib has been demonstrated in animal models of diethylnitrosamine-induced HCC [25].

However, the therapeutic efficacy of IFN-α and celecoxib for HCC cells with HBV infection has not been tested in immune-competent animals with orthotopic HBV infecting hepatoma. Therefore, we used HBV-infected HLCZ01 cells to investigate the mechanism of combination therapy with IFN-α and celecoxib, a COX-2 inhibitor, against HCC cells resistant to TRAIL-induced apoptosis.

Our studies from MTT showed that IFN-α and celecoxib acted synergistically to inhibit HCC. The molecular mechanism behind this synergy has been explored by some studies. For example, Li et al. [26] found that aspirin not only significantly enhanced IFN-α-induced antiproliferation and apoptosis of HCC in an in vitro study, but also enhanced tumor growth inhibition in nude mice. These findings suggest a novel strategy of using aspirin to overcome tumor resistance and enhance the effectiveness of IFN-α in HCC treatment through activating the STAT1 gene, and have potential implications for improving future IFN-α protein and gene therapy. These results are also supported by our own observation that combined therapy with IFN-α and celecoxib delayed the cell cycle related to the G0/G1 phase and induced a marked accumulation of G0/G1-phase cells. In addition to growth inhibition and cell cycle arrest, the present study also strongly suggested that IFN-α and celecoxib synergistically induce apoptosis of HCC cells. An interesting finding is that HLCZ01 cells support the entire lifecycle of HBV, as previous studies did not show the entire lifecycle of HBV in hepatoma cell lines [27–28]. In the current study, however, we further confirmed the apoptotic effects induced synergistically by IFN-α and celecoxib in HBV-infected HCC cells.

The prognosis of HCC is poor. One of the important factors affecting survival is resistance to therapeutic agents. Owing to its specific toxicity for cancer cells, recombinant TRAIL is among the most promising apoptosis-based antitumor agents. Therapy based on TRAIL is now being tested in phase 2 clinical trials in different types of cancers. However, some HCC cells remain resistant to TRAIL-induced apoptosis. It has been shown that the death ligand TRAIL is a critical IFN-induced apoptosis mediator in this scenario [29]. DR4 and DR5 are the main apoptosis-inducing receptors, and the receptors have an intracellular death domain to transmit the apoptotic signal. Herzer et al. [30] reported that IFN-α exhibits a TRAIL-sensitizing effect in primarily TRAIL-resistant hepatoma cell lines. Their research has shown that the TRAIL receptor/ligand system to IFN-α-induced apoptosis in HCC cells that have acquired TRAIL resistance may even support tumor progression, and that IFN-α induced apoptosis through caspase-9 activation.

Kern et al. [31] reported that celecoxib engages different apoptosis pathways in HCC cells, including the stimulation of DR4 and DR5 signaling, activation of caspases-3, caspases-8, caspases-9, and apoptosis originating from mitochondria. TRAIL is an attractive candidate for future cancer therapies. Several human carcinomas, including HCC, are resistant to TRAIL when used as a monotherapy. Recent studies have shown that IFN-α and celecoxib sensitize HepG2 and Huh7 cells to exogenous TRAIL-induced apoptosis, and that this sensitization can be achieved through several mechanisms, such as the upregulation of TRAIL receptors, activation of caspases-3 and caspases-8 [32]. Our results showed that exogenous TRAIL, when used alone, could not effectively suppress cell growth of HLCZ01 cells, while celecoxib, but not IFN-α, could overcome TRAIL resistance through DR5 upregulation. The combination of exogenous TRAIL and COX-2 inhibitors may also be a useful regimen for HCC.

Yano et al. [33] reported that IFN-α induced strong antitumor effects and the downregulation of IFNAR-2 in HCC cells, and it decreased tumor volume, weight, and IFNAR-2 in vivo. These data suggest the potential clinical application of PEG-IFN-α for the prevention and treatment of HCC. Cao et al. [34] investigated the in vivo growth inhibitory effects of celecoxib on the HCC cell line BEL-7402. Athymic nude mice implanted with BEL-7402 cells were given celecoxib, and the effect of treatment on tumor growth was evaluated. The results suggest that celecoxib would be efficacious for the treatment of HCC. Our animal studies have shown that single treatment with either IFN-α or celecoxib reduced the growth of xenotransplanted HCCs, and that combined treatment with IFN-α and celecoxib showed an even stronger inhibitory effect. Immunohistochemical analysis with DAPI staining revealed that apoptosis plays a role in the inhibition of growth. These results underline and strengthen the effect of combination treatment shown in the in vivo study.

IFN-α has various biological properties, including antiviral response, immune modulation, and antiproliferative activity. It has therefore been widely used for the treatment of chronic hepatitis viral infections and HCC. IFN-α may prolong survival in some patients. However, the response is often not satisfactory because of the limited ability to identify patients who are most likely to benefit from such targeted adjuvant therapies. HCC cells remain resistant to IFN-α treatment, and tumor heterogeneity requires tumor adjuvant therapies [35]. The effects of celecoxib on the recurrence of adenomas and their modification by genetic background and baseline selenium level remain to be determined [36]. In conclusion, the present study has shown that combination therapy with IFN-α and celecoxib exerts synergic effects in anti-proliferation, cell cycle arrest, and apoptosis induction in HLCZ01 cells with HBV infection. In addition, we have demonstrated that the mitochondrial pathway is involved in the apoptosis of HBV-infected HLCZ01 cells induced by IFN-α and celecoxib combination therapy, and that celecoxib sensitizes HBV-infected HLCZ01 cells to IFN-α through upregulating TRAIL expression. Revealing the mechanisms of IFN-α and celecoxib responsiveness of HCCs will contribute greatly to establishing better therapeutic strategies for liver tumors and will, thus, enable a more efficient application of IFN-α in clinical cancer therapy.
Conflict of interest
The authors declare that they have no competing interests.

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