CF mediated G1 arrest is associated with induction of p27Kip1 and inhibition of cyclin D1 expression in human hepatoma HepG2 cells

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Abstract

CF is a kind of diterpenoid which was first isolated and purified from Chinese tropical plants by our laboratory. Our previous works have demonstrated it could inhibit the proliferation of several malignant tumor cell lines and stimulate them to differentiate to normal cells. In this article we investigated the effect of CF on human hepatocellular carcinoma HepG2 cell viability, differentiation, cell cycle distribution and G1 cell cycle related genes expression. We also detected the effect of retinoic acid (RA) which was used as positive control and the effect of combination CF + RA. Our data suggested that CF could be useful to induce growth arrest and differentiation in HepG2 cell lines, and could reverse the transformed phenotype. This anti-tumor effect was due to G1 arrest in cell cycle which was associated with an increase of p27Kip1 and a decrease of cyclin D1 expression, so CF might be a useful targeted therapy strategy for HCC. Results also showed RA has a different mechanism from CF on G1 arrest, and CF has not synergistic anti-tumor effect with RA on HepG2 treatment.

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Keywords: CF; Hepatocellular carcinoma; Cell differentiation; Cell cycle; Retinoic acid

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common neoplasm in the world, and the third most common cause of cancer-related death that 564,000 new cases are diagnosed every year [1]. In China HCC has become the second cancer killer [2]. Every year HCC causes 315,000 deaths in the world and 137,000 (43.7%) deaths in China [3]. Recently, the incidence of HCC has been found to be increasing in some countries [4]. Estimates of the burden of HCC in the USA suggest that its incidence will increase within two decades, probably to equal that currently reported in Japan [5]. Therefore, there was an urgent need to develop mechanism-based approaches for treatment of hepatocellular carcinoma.

The hallmark features of tumor cells are that they have developed a block to normal differentiation and gained unlimited proliferative capacity. The arrest of cell cycle is the first event that occurs at the moment when the fate of mammalian cells is decided to either differentiation or proliferation. The decision of cells to differentiate is commonly made in the G1 phase of the cell cycle, and the induction of differentiation is believed to require cell cycle arrest [6]. Cell cycle progression is regulated by the periodic activation of a family of cyclin-dependent kinases (cdk's). The regulation of cells entering from the G1 phase of the cell cycle into S phase is particularly important, as the cells normally must pass through a restriction point in late G1 to progress to the S phase [7]. The activities of three types of cyclins (cyclin A, cyclin D, and cyclin E) and cyclin-dependent kinases 4 and 6 (cdk4 and

Abbreviations: AFP, alpha-fetoprotein; cdk, cyclin dependent kinase; CKI, cdk inhibitors; γ-GT, γ-glutamyl transpeptidase; HCC, hepatocellular carcinoma; RA, retinoic acid; TAT, tyrosine amino transferase

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CF is a kind of diterpenoid compound which was first isolated and purified from a kind of Chinese tropical plants by our lab. Our previous work has demonstrated it can inhibit the proliferation of several malignant tumor cells and then stimulate them to differentiate to normal cells, and these anti-tumor effects in some cells are more significant than retinoic acid (RA) which is the most biologically active metabolite. More importantly, CF has only a slightly toxic side effect [unpublished work]. RA is able to regulate the reversion of transformed phenotype in different types of tumor [9–11] and induce growth arrest and differentiation by means of different mechanisms in several transformed cell lines [12–15]. It is important to detect whether CF has the same effect of inducing growth arrest and differentiation on HepG2 cells as RA, whether they have a synergetic effect on anti-tumor. In this report, we chose RA sensitive cells human hepatocellular carcinoma HepG2 cell line as a model to investigate the effects of CF on cell viability and differentiation, then analyzed the cell cycle distribution and the possible influence on expression of G1-related cell cycle genes.

2. Materials and methods

2.1. Reagents and antibodies

CF was isolated and purified by our laboratory. It is a kind of white powder which has a molecular weight about 410. The CF concentration was chosen after a series of preliminary experiments as being a significant anti-tumor effect and not being cytotoxic [unpublished work]. CF and RA (Sigma, St. Louis, MO) were dissolved in ethanol at 100 mM and 10 mM concentration respectively and stored in the dark at −80 °C. Applied concentration in medium in all experiments is 100 μM CF and 10 μM RA, respectively, CF + RA was mixture of the equal volume of 100 μM CF and 10 μM RA. Cells treated with serum-free medium were considered as controls, the possible effects of ethanol were preliminary excluded using additional medium containing the same amount of ethanol.

For Western Blot analysis, anti-cyclin D1 (A12), anti-cdk2 (M2), anti-cdk4 (C-22), anti-p16INK4A (F-12) were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. Anti-α-tubulin (mouse IgG1 isotype) was purchased from Sigma, St. Louis, MO, USA. Anti-p27KIP1 was purchased from Bio-LAB Co., Beijing, China, horseradish peroxidase-conjugated goat anti-rabbit/mouse IgG was purchased from Zhongshan LAB Co., Beijing, China.

2.2. Cell culture and treatment with chemicals

The human hepatocellular carcinoma cell lines HepG2 cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD) and were cultured in RPM 1640 medium (Gibco Invitrogen Corp., Grand Island, NY, USA), supplemented with 10% heat-inactivated fetal bovine serum (ChuanYe, Tianjin, China), 2 mM glutamine, gentamicin at 37 °C in a humidified incubator with 5% CO2. The medium was changed every day.

Experiments were performed on cells that had been transferred to serum-free medium 24 h previously, and cells were then supplemented with serum-free medium containing the indicated concentration of CF or RA or CR + RA for the indicated times. Control dishes were treated with an equivalent volume of serum-free RPM 1640 medium.

2.3. Cell proliferation assay

Cell proliferation was determined by a colorimetric assay using MTT. MTT is a water-soluble tetrazolium salt that yields a yellowish solution when prepared in medium or salt solutions that lack phenol red. Dissolved MTT is converted to the colored product formazan in active mitochondria, and then can be solubilized using acid–isopropanol mixture (1N HCl/isopropanol = 4:96, v/v). Dual end optical density reading at 510 nm is directly proportional to the number of cells. The treated cells were washed by PBS and incubated with 20 μl MTT (Sigma, St. Louis, MO, USA) solution (5 mg/ml) in sterile RPM 1640 medium and then incubated at 37 °C in a humidified incubator with 5% CO2 for 5h. After incubation, 10% SDS (dissolved in 0.01 M HCl) solution was added to incubate over night. Transfer to plate reader and measure absorbance at 510 nm.

2.4. Cell differentiation assay

Alpha-fetoprotein (AFP) and γ-glutamyl transpeptidase (γ-GT, E.C.2.3.2.2) were recognized malignant phenotype proteins of human hepatoma cells. On the contrary, tyrosine aminotransferase (TAT, E.C.2.6.1.5) can only be found in well differentiated human hepatocytes. We used these three genes as hepatocyte-differentiation markers to detect the stimulating differentiation effect of CF on HepG2. Cells were harvested from exponentially growing cultures and seeded at dishes and then were treated with the indicated concentration of CF, CF + RA, and RA of serum-free medium for 3 days, and the medium was changed every day. The transcription of AFP mRNA is tightly regulated in a developmental and tissue-specific process, we detected the influence of CF to AFP mRNA expression by RT-PCR, measured the activity of TAT by modified Diamondstone method [16] and the activity of γ-GT by method as described previously [17]. Protein concentration was determined using the method of Bradford [18]. The activity of γ-GT or TAT was presented as ml/mg crude protein.

2.5. Flow cytometric analysis of cell-cycle

HepG2 cells (5 × 104) were plated into 6-well tissue culture dishes and then were treated with the indicated con-
Table 1

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence of primer</th>
<th>Annealing temperature (°C)</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFP</td>
<td>5′-AAAXG CTTTG CTC CTGC TOC-3′</td>
<td>5′-CAGCC TCAAG TGTTT CTCCT-3′</td>
<td>35</td>
</tr>
<tr>
<td>CCND1</td>
<td>5′-GGGGC GTAGC ATCAT AGTAG T-3′</td>
<td>5′-TTTGG CTGCG TTTG TCCAG-3′</td>
<td>35</td>
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<tr>
<td>CCNE</td>
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<td>5′-TTTGC TCGGG CTTTG TCCAG-3′</td>
<td>35</td>
</tr>
<tr>
<td>p16Ink4a</td>
<td>5′-GCTCT GAGAAACCTC GGGAAAC-3′</td>
<td>5′-CTCGC AAGAA ATGCC CACAT-3′</td>
<td>35</td>
</tr>
<tr>
<td>p27Kip1</td>
<td>5′-CGCTC GCCAG TCCAT TTGAT-3′</td>
<td>5′-TTCTC CACCT CTTGC CACTC-3′</td>
<td>35</td>
</tr>
<tr>
<td>β-Actin</td>
<td>5′-ATCATGTTTGAGAGACCTTCAAC-3′</td>
<td>5′-CATCTCTTGCTC GAAGTCCA-3′</td>
<td>33</td>
</tr>
</tbody>
</table>

concentration of CF, CF + RA, and RA of serum-free medium for the indicated time. The treated cells were collected to trypsinize, then were washed once with cold PBS, and fixed in cold 75% ethanol at 4°C. Cells were then washed once again with cold PBS and re-suspended with PBS, then stained with 50 mg/ml propidium iodide (Sigma, St. Louis, MO, USA) and 100 mg/ml RNase A solution (Genview, Carlsbad, CA) for 20 min at 37°C in the dark. Stained cells were subjected to analysis immediately by flow cytometry. The proportion of cells in each phase of cell cycle was determined by a BD FACScan for Quantitative Cell Analysis, three independent measurements were made and averaged. Statistical significance was determined using a Student’s t-test.

2.6. Total RNA isolation and semi-quantitative RT-PCR

HepG2 cells were treated with the indicated concentration of CF, CF + RA, and RA of serum-free medium for 3 days. Total RNA of seedlings was isolated using TRIZOL Reagent (Gibco BRL, Rockville, MD, USA) according to the manufacturer’s instruction and stored at −80°C. The amounts of the proteins were estimated from calibration curve of bovine serum albumin as measured by spectrophotometry analysis. Thirty micrograms of the whole protein in each lysate was loaded in the gel. Cell lysate was blotted onto a nitrocellulose membrane (Hybond-C, Amersham, Vienna, Austria) after separation by SDS-PAGE [19]. The blots were blocked for 2 h on a shaker at room temperature in TBST containing 5% (w/v) dry milk and then incubated with a primary antibody overnight on a shaker at 4°C. After incubation, all membranes were washed with TBST for 3 × 10 min and then with TBS 10 min. Fluorescent bands were developed with 1 ml of substrate containing same volume of each Super Signal West Pico Luminol Enhancer solution and Super Signal West Pico-stable Peroxidase solution (Pierce, Rockford, IL, USA) at room temperature for 5 min. The X-ray films were exposed to the membrane and then developed. The autoradiographies were scanned and semi-quantitatively analyzed.

3. Results

3.1. CF mediated growth inhibition and differentiation induction in HepG2 cells

The effect of CF on HepG2 cells proliferation and viability was evaluated using the MTT assay, the results showed...
Fig. 1. Effect of CF on HepG2 cells proliferation. MTT assay for HepG2 cells which were pretreated at the indicated time points with CF (0.1 mM), CF + RA (CF:RA = 1:1, v/v), RA (0.01 mM), cells treated with serum-free medium were considered as controls. Control-FBS means cells treated with medium supplemented with 10% heat-inactivated fetal bovine serum. Data are mean ± S.D. (n = 3).

The cell viability of CF-, CF + RA-, and RA-treated HepG2 cells was decreased compared control (P < 0.01) (Fig. 1). This result also suggested that CF and RA have the similar anti-tumor effect on hepatoma HepG2 cell line. Further, to investigate the effects of CF on HepG2 cells differentiation, we detected the mRNA expression of AFP and enzyme activity of γ-GT and TAT after 3 day treatment, results showed CF could considerably down-regulate the expression of AFP mRNA (P < 0.01) (Fig. 2a and b) and the activity of γ-GT (after CF, CF + RA, and RA 3 days of treatment, the γ-GT activity of cells decreased to 75.2%, 51.5%, and 38.5% of that of control cells, respectively) (Fig. 2d), up-regulate the activity of TAT (after CF, CF + RA, and RA 3 days of treatment, the TAT activity of cells was 2.5-fold, 2.1-fold, and 2.1-fold of that of control cells, respectively) (Fig. 2c).

3.2. CF induced G1 arrest in HepG2 cells

Growth inhibition and differentiation induction by CF in the studied HepG2 cells were accompanied by cell cycle G1 arrest, which was indicated by a decrease in DNA synthesis and retardation of cell growth. CF could inhibit DNA synthesis and increased the percentage of cells in G1/G0 phase (after 3 day treatment, the percentage of G0/G1 phase cells was 73.1% in CF compared with 64.7% in control) in a time-dependent manner (from 63.1% after 1 day treatment to 73.1% after 3 day treatment in CF induced cells). This increase was coupled with the decreased percentage of cells in S phase (after 3 day treatment, the percentage of S phase cells was 18.0% in CF compared with 24.2% in control) in a time-dependent manner (from 24.0% after 1 day treatment to 18.0% after 3 day treatment in CF induced cells). RA and FA have the similar retardant effect on cell cycle progression (Fig. 3).

3.3. Up-regulation of p27Kip1 down-regulation of cyclin D1 by CF treatment of HepG2 cells correlating to G1 arrest

To investigate mechanisms involved in the regulation of HepG2 cell G1 arrest and differentiation by CF, expres-
Fig. 3. HepG2 cells blocks cell cycle progression from G0/G1 to S phase. The graph indicates the percentage of HepG2 cells in the G0/G1 and S phases during CF (0.1 mM), CF + RA (CF:RA = 1:1, v/v), RA (0.01 mM) treatment at the indicated times, cells treated with serum-free medium were considered as controls. Data are mean ± S.D. (n = 3) * P < 0.01 with respective control.

Fig. 4. Expression of cell cycle G1 phase related Genes. HepG2 cells were treated 3 days with CF (0.1 mM), CF + RA (CF:RA = 1:1, v/v), RA (0.01 mM) as stated above, cells treated with serum-free medium were considered as controls: (a) expression of CCND1, CCNE, p16Ink4a, and p27Kip1 at the mRNA level was detected by RT-PCR, the results were scanned and quantified by densitometric analysis (b). Data are mean ± S.D. (n = 3). (c) Expression of cyclin D1, p16Ink4a, p27Kip1, cdk2, and cdk4 was detected by Western Blot, blots were scanned and quantified by densitometric analysis (d). Data are mean ± S.D. (n = 3) * P < 0.01 with respective control.

Fig. 5. Western Blot analysis of cyclin D1, p27Kip1 protein expression in HepG2 cells after induction of CF at the indicated times, cells treated with serum-free medium were considered as controls. One representative experiment of three is shown.

Expression of cyclins and other regulators of G1 related cell cycle genes at the mRNA level was studied by comparative RT-PCR with β-actin gene as the control one. The results of comparative RT-PCR of CCND1, CCNE, p16Ink4a, and p27Kip1 showed a considerable down-regulation of CCND1, CCNE and up-regulation of p27Kip1 after 3 days induction with CF (Fig. 4a and b). This up-regulation of cyclin D1 and down-regulation of p27Kip1 found by the comparative RT-PCR was confirmed by Western Blot (Fig. 4c and d).

Results also showed RA did not take effect on expression of cyclin D1 at both mRNA and protein levels, but the up-regulation of p27Kip1 by RA at mRNA level was not confirmed by Western Blot at protein level. Both CF and RA have no influence on p16Ink4a expression. To detect the influences on two G1 phase related cdks, expression of cdk2 and cdk4 was measured using Western Blot, the results showed, at the progression of G1-S transition, CF had no effect on their expression level, so do the CF + RA and RA.
The time course study (Fig. 5) continued to investigate the effect of CF on p27Kip1 and cyclin D1 protein expression. Results suggested that induction of p27Kip1 and inhibition of cyclin D1 protein expressions were regulated by CF in a time-dependent manner.

4. Discussion

Continual division and constant proliferation are important characteristics of tumor cells. In this study, we first examined the effects of CF on HepG2 cells proliferation and differentiation, the results obtained by MTT assay demonstrated that CF could repress the proliferation, especially the viability of HepG2 cells, RA showed the similar effect as positive control. It suggested that CF could suppress tumor cell growth as the differentiation inducer RA. This anti-proliferative effect was coupled with the reversion of malignant HepG2 cell differentiation.

The proliferation activity of cells is inversely correlated with the degree of differentiation. Therefore, inhibiting the proliferation of tumor cells is a significant index in induction of differentiation [20]. We chose three major differentiation markers for hepatic cell to investigate the effect of CF on cell differentiation. AFP is one of the earliest markers for hepatic cell differentiation, the expression of AFP transcripts or the products have been used as a marker of hepatic cell [21,22]. γ-GT is another differentiation marker for hepatocyte which is normally expressed at low levels in the liver in the adult state but has been shown to be elevated in premalignant and malignant liver lesions [23,24]. TAT represents an excellent enzymatic marker for postnatal hepatocyte specific differentiation, which is not synthesized in significant quantities prior to birth but are rapidly activated early in the neonatal developmental period [25]. Therefore, evaluating the changes of these three characteristics in hepatoma cells is important in determining the effects of exotic substances on hepatoma cells. Our results showed CF could down-regulate the AFP mRNA expression and activity of γ-GT, up-regulate the activity of TAT, and this effect is more significant than that of RA (P < 0.01).

Generally, cell growth and differentiation are tightly controlled processes that coordinate maintain normal tissue homeostasis. The decision of cells to differentiate is commonly made in the G1 phase of the cell cycle, and induction of differentiation is believed to require cell cycle arrest [7]. Inhibition of cell growth and induction of differentiation in HepG2 cells by CF were accompanied by retardation of G1/S transition, and this G1 arrest was induced by CF in the time-dependent manner. It has been proposed that cdk activities control the G1/S transition in mammalian cells [26]. Both D- and E-type cyclins are known to be important regulators in G1/S control, even though some reports raised the possibility that they have distinct roles [27]. cdk4 is a main catalytic partner of cyclin D, and cdk2 is known to form complexes with cyclin E and cyclin A. In the present study in HepG2 cells, cyclin D1, and cyclin E mRNA levels and Cyclin D1 protein expression are both significantly decreased after CF 3 day stimulation. But two primary G1 phase cdks, cdk2 and cdk4, protein expressions were not modified by either CF or RA.

A family of cdk inhibitors plays a major role in the cell cycle machinery [28]. The activities of these cyclin D/cdk complexes are negatively regulated by the cdk inhibitors \( p16^{INK4a} \), \( p21^{CIP1} \), and \( p27^{KIP1} \) through directly inhibiting cdk activities and preventing their phosphorylation [29]. \( p16^{INK4a} \) accumulates progressively as cells age, possibly being induced by a senescence timer [30]. \( p27^{KIP1} \) has been shown to mediate cell cycle arrest in response to various factors, including transforming growth factor-β [31], zaspamycin [32], and cAMP [33]. In our report, \( p27^{KIP1} \) mRNA and protein expression both up-regulated by CF compared no changes by RA at protein level, but \( p16^{INK4a} \) expressions were not influenced by both CF and RA.

Our unpublished work demonstrated that after CF treatment, one of the early events was cAMP accumulation. Some effects or function of CF may be contributed to this accumulation. Many mechanisms have been proposed to explain the anti-proliferative effects of cAMP, increasing cell-cycle inhibitor proteins \( p21^{CIP1} \) [34] or \( p27^{KIP1} \) [35], as well as decreasing the levels of cyclin D1 [36] and cyclin D3 [37]. cAMP-mediated cell differentiation has also been characterized by the induction of specific genes through phosphorylation of the transcription factor CREB [38]. Consequently, we tentatively put forward that up-regulation of \( p27^{KIP1} \) and down-regulation of cyclin D1 by CF may be mediated by cAMP, leading to a G1-phase arrest. The present results also indicated CF has the different mechanism with RA to regulate the cell cycle. The ability of RA to regulate cell growth and promote cell differentiation has been established in numerous cell culture and animal models [39,40]. In our study RA arrested HepG1 cells G1 phase but did not affect the expression of \( p27^{KIP1} \), \( p16^{INK4a} \), cyclin D1, cdk2, cdk4 after 3 day treatment. This incongruous result can be accounted for the decrease in activity of the cyclin-cdk complexes [41]. We hypothesize this RA-induced G1 arrest may be associated with decreased activity of cyclin E/A-cdk2 complex by \( p21^{CIP1} \) [42,43]. Though RA can cooperate with cAMP to induce several human leukemia cell lines and other cell lines maturation or differentiation [44–47], in this study CF had not significant synergistic effect with RA on HepG2 treatment.

In conclusion, our study showed CF could inhibit HepG2 proliferation and reverse its transformed phenotype. This anti-tumor effect is due to G1 arrest in cell cycle which is associated with decreased expression of cyclin D1 and increased expression of \( p27^{KIP1} \). CF has not significant synergistic effect with RA on HepG2 treatment. Our further study will focus on effects of CF on the activity of related cyclin-cdk complexes, the status of G1 phase related cdk inhibitors and relationship with cAMP or other mediators.
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References


