Inhibition of HepG2 cell proliferation by ursolic acid and polysaccharides via the downregulation of cyclooxygenase-2

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Abstract. Cyclooxygenase (COX)-2, a multi-functional molecule, is overexpressed in hepatocellular carcinomas. In order to understand cell proliferation and its association with COX-2 in HepG2 cells in the presence of ursolic acid (UA), viili exopolysaccharides (VEPS) and Astragalus polysaccharides (APS), the cell proliferation, superoxide dismutase (SOD) and metabolic malondialdehyde (MDA) of fatty acids, COX-2, prostaglandin E2 (PGE2), as well as apoptotic morphology and rate were investigated. The results revealed that the activities of SOD, COX-2 and PGE2 were reduced, MDA was markedly decreased, apoptotic blebs were induced, and HepG2 cells were accumulated in the G1 and sub G1/apoptotic phases in test groups. The results indicated that UA, VEPS, APS and any combination of these possess anticancer properties, particularly by downregulating COX-2 expression, which may have increased internal oxidation and triggered apoptosis together with a change in internal antioxidant response elements, leading to a reduction in cell proliferation.

Introduction

Hepatocellular carcinoma (HCC) is estimated to be the fifth most common cause of cancer-related mortality worldwide (1). Although ~80% of cases are reported in developing countries, where the prevalence of hepatitis is high, HCC is one of the few types of cancer whose incidence is on the increase in developed countries (2,3). Although chemotherapy has provided significant survival benefits for HCC patients, such drugs are associated with marked tissue toxicity, and drugs or alternative therapies that target tumor cells without compromising normal tissue function are required (4). Increased concentrations of cytotoxic drugs and higher doses of radiation often fail to improve the health of liver cancer patients, and may cause resistance to apoptosis. An anticancer agent with lower toxicity that preferentially induces apoptosis in human cancer cells while creating an internal oxidative environment would be useful.

Ursolic acid (UA), a pentacyclic triterpenoid, has been identified in various natural products, such as vegetables and medicinal herbs (5). UA may inhibit cell growth and induce apoptosis in certain tumors (6,7) through multiple pathways, including inhibiting DNA replication, activating caspases and downregulating anti-apoptotic genes (8,9). UA specifically inhibits tumorigenesis (10), tumor progression (11), angiogenesis and tumor invasion (12).

Viili, a Nordic traditional fermented dairy product containing lactobacillus, yeast and filamentous fungi, generates large quantities of extracellular polysaccharide (EPS) (13). Viili exopolysaccharides (VEPS) reportedly have antioxidant properties (14), regulate immunity function and lower cholesterol (15). Astragalus, particularly A. membranuse, is a common traditional Chinese medicine; its polysaccharides [or Astragalus polysaccharides (APS)] reportedly improve immune function (16), modulate the immune system and promote tumor cell apoptosis (17).

Cyclo-oxidase (COX)-2 is a key enzyme that catalyzes arachidonic acid into prostaglandins (18,19). COX-2 is not expressed in the majority of organs under normal physiological conditions, but it is expressed in the majority of cancer cells (20). COX-2 is believed to inhibit cancer cell apoptosis (21), thus causing resistance to chemotherapy as COX-2 selective inhibitors suppress tumor cell proliferation and induce apoptosis (22). For these reasons, naturally derived COX-2 inhibitors have been used to study chemotherapy and chemoprevention. In this study we analyzed the synergistic effect of UA in combination with VEPS and APS, on cell proliferation, morphologic change, anti-oxidation and COX-2 expression.

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Materials and methods

Chemicals. Ursolic acid (>99.8%) was purchased from Sigma (St. Louis, MO, USA). VEPS (>78%) and APS (>80%) were extracted in our laboratory. DMEM and the RevertAid First Strand cDNA Synthesis kit were purchased from Thermo Fisher Scientific Inc. (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Gibco (Milan, Italy). Penicillin streptomycin solution, trypsin, phosphate-buffered saline (PBS), DMSO, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and cell lysis solution were purchased from Solarbio (Beijing, China). Anti-COX-2 and anti-β-actin antibodies were purchased from Bioworld (Minneapolis, MN, USA). Anti-rabbit IgG antibody (H+L) was purchased from Thermo Fisher Scientific Inc. (Rockford, IL, USA). Superoxide dismutase (SOD) and malondialdehyde (MDA) test kits were purchased from Nanjing Biological Engineering (Nanjing, China), human COX-2 and human prostaglandin E2 (PGE2) enzyme-linked immunosorbent assay (ELISA) kits were purchased from Biovision (山景城, CA, USA), the RNeasy Mini kit was purchased from Qiagen (Hilden, Germany), and the West Pico Mouse IgG Detection kit was purchased from Thermo Fisher Scientific.

Cell culture and reagents. The HepG2 human HCC cell line was a gift from the Academy of Military Science (Beijing, China). Cells were maintained in DMEM medium supplemented with 10% FBS, 100 U/ml of penicillin and 100 µg/ml of streptomycin, and incubated in a humidified 5% CO2 incubator at 37°C. The culture medium was changed every two days and the cells were subcultured every fifth day. Cells in the mid-log phase were used for experiments.

Stock solutions of UA, VEPS and APS were prepared in DMSO and diluted with medium. The final concentration of DMSO was <0.1%, which demonstrated no effect on cell viability and DNA fragmentation. DMSO was also used in the controls. For all assays, the HepG2 cells (5x10^4/ml) were treated with UA at 0.1, 1, 2.5, 5, or 10 µg/ml; or VEPS or APS at 10, 20, 40, 50 or 100 µg/ml, respectively. After treatment, the cells were washed twice with PBS, and incubated for 48 h. The cells were collected and resuspended in 0.5 ml of PBS, and the absorbance value was measured at 570 nm using a multiwell spectrophotometer (Bio-Rad, Hercules, CA, USA). Percentage of cell inhibition was calculated using the formula: inhibitory rate(%) = 1 - (absorbance_experiment well/absorbance_control well) x 100.

Flow cytometric analysis. HepG2 cells (1x10^5 cells/ml) were treated with varying concentrations of UA (5 µg/ml), VEPS (50 µg/ml), APS (50 µg/ml), UA/VEPS 1:1 in combination, or UA and APS 1:1 in combination. 48 h later, the cells were collected and washed twice with PBS, and incubated for 2 h at 4°C with 2.5% 3,3’-diaminobenzidine (DAB) to detect COX-2 expression. The cells were then fixed in 4% paraformaldehyde and 2% glutaraldehyde for 60 min at 4°C. After washing, the cells were incubated with the primary antibody (anti-COX-2) overnight in the dark at 4°C. After three washes with PBS, the cells were incubated with the secondary antibody (FITC-Rat IgG) for 30 min at RT. Finally, the cells were washed, resuspended in PBS, and analyzed by flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA).

Determination of intracellular SOD activity. HepG2 cells were plated at 5x10^4 cells/ml in 96-well plates, and incubated with varying concentrations of UA, VEPS, APS, combined 1:1 UA and VEPS, or combined 1:1 UA and APS for 48 h. The cells were washed twice with ice-cold PBS, and incubated in 0.5 ml of PBS for 10 min in lysis buffer, and centrifuged at 5000 rpm for 5 min at 4°C. The cells were then resuspended in 1 ml of SOD assay buffer, and the absorbance value was measured at 570 nm using a spectrophotometer (Bio-Rad, Hercules, CA, USA). Percentage of cell inhibition was calculated using the formula: inhibitory rate(%) = 1 - (absorbance_experiment well/absorbance_control well) x 100.

Determination of intracellular MDA content. HepG2 cells were plated at 5x10^4 cells/ml in 96-well plates, and incubated with varying concentrations of UA, VEPS, APS, combined 1:1 UA and VEPS, or combined 1:1 UA and APS for 48 h. The cells were washed twice with ice-cold PBS, and incubated in 0.5 ml of PBS for 10 min in lysis buffer, and centrifuged at 5000 rpm for 5 min at 4°C. The intracellular MDA content was determined using the MDA test kit (Nanjing Biological Engineering, Nanjing, China). Percentage of cell inhibition was calculated using the formula: inhibitory rate(%) = 1 - (absorbance_experiment well/absorbance_control well) x 100.

Table I. Primer sequences used for PCR.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primers (5'-3')</th>
<th>Primers (5'-3')</th>
<th>Size (bp)</th>
<th>Accession</th>
</tr>
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<tr>
<td>COX-2</td>
<td>TGAAACCCACTCCAAACACAG</td>
<td>TCATCAGGCACAGGGAAG</td>
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<td>NM_000963</td>
</tr>
<tr>
<td>β-actin</td>
<td>AAATCTGGGACACACACCTT</td>
<td>AGCACTGTGTTGGCGTAGAG</td>
<td>646</td>
<td>NG_007992</td>
</tr>
</tbody>
</table>

PCR, polymerase chain reaction; COX-2, cyclooxygenase-2.
MDA content was measured in non-protein cell lysates using a commercially available MDA assay kit according to the manufacturer's instructions.

Reverse-transcription polymerase chain reaction (RT-PCR). After HepG2 cells were exposed for 48 h to 5 µg/ml of UA, 50 µg/ml of VEPS, 50 µg/ml APS, combined 1:1 UA and VEPS, or combined 1:1 UA and APS, total RNA was extracted from the cells using RNeasy Mini kit. First strand cDNA was generated via reverse transcription of 2 µg of the total RNA using a RevertAid First Strand cDNA Synthesis kit (Fermentas, USA). The standard PCR conditions for COX-2 were: 94˚C for 4 min, then 30 cycles at 94˚C for 45 sec, 56˚C for 45 sec and 72˚C for 1 min, followed by 10 min at 72˚C. β-actin, a housekeeping gene, was selected as an internal standard to account for variability in amplification due to differences in the starting mRNA concentrations. The PCR conditions were as follows: 94˚C for 3 min, then 35 cycles at 94˚C for 30 sec, 57˚C for 45 sec and 72˚C for 30 sec, followed by 10 min at 72˚C. The correct fragment of PCR was confirmed by a commercial sequencing service company (BGI, Beijing, China). Primer sequences for COX-2 and β-actin are listed in Table I.

Measurement of intracellular COX-2 by ELISA. HepG2 cells were plated at 5x10^3 cells/well in 96-well plates, and incubated with varying concentrations of UA, VEPS, APS, combined 1:1 UA and VEPS, or combined 1:1 UA and APS for 48 h. Cells were washed twice with ice-cold PBS. Lysates were incubated for 10 min on ice, sonicated and centrifuged for 15 min at 12,000 x g. After protein concentrations were determined using the Bradford assay, the samples were boiled for 10 min, equal amounts of protein (20 µl/lane) were separated by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with a 1:1000 dilution of primary antibody against COX-2 and 1:4000 dilution of primary antibody against β-actin at 4˚C overnight. The secondary antibody was goat anti-rabbit IgG antibody (H+L) diluted 1:5,000 in blocking solution for 1 h at room temperature. Immunoreactivity was detected using West Pico Mouse IgG Detection kit and visualized by autoradiography.

Measurement of PGE2 levels by ELISA. HepG2 cells were plated at 5x10^3 cells/well in 96-well plates, and incubated with varying concentrations of UA, VEPS, APS, combined 1:1 UA and VEPS, or combined 1:1 UA and APS for 48 h. Cells were washed twice with ice-cold PBS and lysed for 10 min in lysis buffer. COX-2 concentration was measured in plates coated with purified human COX-2 antibody, using a commercially available human COX-2 ELISA kit, according to the manufacturer's instructions.

Results

UA, VEPS, APS and combined treatments inhibited HepG2 cell proliferation. UA, VEPS, APS and combination treatments induced HepG2 cell deaths in a time- and dose-dependent manner (Fig. 1). Incubation with varying doses of UA, VEPS, APS and combined treatments for different time periods (24, 48, or 72 h) resulted in the significant inhibition of cell proliferation (P<0.05). However, inhibition at 48 h was stronger than that at 24 h or 72 h (P<0.05). Furthermore, there was greater inhibition by the combined treatments than with individual treatments (P<0.05).

UA, VEPS, APS and combined treatments induced apoptotic blebs in HepG2 cells. Morphological changes to cells

Statistical analysis. Data are reported as the means ± SD. Statistical analyses used analysis of variance (ANOVA) tests. Differences among means were determined by the least significance difference test. P<0.05 was considered to indicate a statistically significant difference.
following exposure to UA (5 µg/ml), VEPS (50 µg/ml), APS (50 µg/ml), and combined treatments for 48 h were visualized under a SEM (Fig. 2). Following treatment with UA, VEPS, or APS, HepG2 cells demonstrated characteristic apoptotic features, with shrinkage, nuclear condensation and DNA fragmentation.

**UA, VEPS, APS and combined treatments increased cell arrest in the HepG2 cell cycle.** Following the exposure of HepG2 cells to UA (5 µg/ml), VEPS (50 µg/ml), APS (50 µg/ml), and combined treatments for 48 h, the cell cycle and apoptosis were monitored by flow cytometry (Fig. 3). The percentage of cells increased in the G1- and S-phases. A sub-G1 peak (apoptosis peak) was also observed. Cell apoptosis increased when HepG2 cells were treated with UA and combined compounds. The results indicate that UA, VEPS and APS are capable of inducing cell cycle arrest.

**UA, VEPS, APS and combined treatments reduced intracellular ROS activity.** SOD is a critical antioxidant enzyme that cleans up cytosolic ROS efficiently. As the drug concentrations increased, SOD activity was significantly downregulated in HepG2 cells (Fig. 4; P<0.05). SOD activity was markedly affected at concentrations of 5 µg/ml UA, 50 µg/ml VEPS, 50 µg/ml APS and by the combined treatments (P<0.01). Similarly, MDA is an indicator for intracellular ROS, as the accumulation of MDA is normally increased in cancer cells. Results of our assay showed that the MDA content increased at a low concentration, but decreased at a high concentration of VEPS and APS in HepG2 cells (Fig. 5, P<0.05). However, MDA content was significantly decreased by UA from 0.1 to 10 µg/ml, and when combined with 40, 50 and 100 µg/ml VEPS and APS (P<0.01), respectively.

**UA, VEPS, APS and combined treatments reduced COX-2 expression in HepG2 cells.** RT-PCR analysis results (Fig. 6A,
P<0.05) revealed that COX-2 mRNA expression in HepG2 cells was higher in the control group, and downregulated by various compounds (Fig. 6B, P<0.05). For HepG2 cells treated with 5 µg/ml of UA, COX-2 mRNA expression was markedly downregulated compared with the controls (P<0.05), and COX-2 mRNA expression decreased further when treated with combinations of UA and VEPS or APS compared with 50 µg/ml of VEPS or APS alone, compared with the controls (P<0.05). Western blotting results (Fig. 6C) revealed that COX-2 protein expression was higher in the control compared with the experimental groups (Fig. 6D). Additionally, COX-2 protein expression following treatment with 5 µg/ml UA or either combination of UA and VEPS or APS was markedly
Table III. Inhibitory effects of different compounds on PGE2 concentration in HepG2 cells at 48 h.

<table>
<thead>
<tr>
<th>Samples</th>
<th>PGE2 concentration</th>
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<tbody>
<tr>
<td></td>
<td>0 (µg/ml)</td>
</tr>
<tr>
<td>UA</td>
<td>120.67±7.31</td>
</tr>
<tr>
<td>VEPS</td>
<td>120.67±7.31</td>
</tr>
<tr>
<td>APS</td>
<td>120.67±7.31</td>
</tr>
<tr>
<td>UA+VEPS</td>
<td>120.67±7.31</td>
</tr>
<tr>
<td>UA+APS</td>
<td>120.67±7.31</td>
</tr>
</tbody>
</table>

A standard curve with absorbance value on the vertical (y) axis and concentration on the horizontal (x) axis: y=0.0013x + 0.0848; R²=0.9984. The corresponding concentration of PGE2 in the table was from the standard curve. *P<0.05; ^P<0.01, compared with control group. PGE2, prostaglandin E2; UA, ursolic acid; VEPS, viili exopolysaccharides; APS, Astragalus polysaccharide.

reduced, compared with the controls (P<0.01). However, no clear change was observed in the VEPS group, and reduction with 50 µg/ml of APS was less significant when compared with the controls (P<0.05).

**Discussion**

Although the occurrence and development of tumors and malignancies are complex, cancer events are not unusual processes. Potentially cancerous cells are constantly produced, but are usually eliminated in a healthy environment. However, carcinogenesis may occur if the body's internal antioxidant or anti-inflammatory environment changes entirely, or the mechanisms that inhibit abnormal cell proliferation disappear. Previous studies have revealed that antioxidant and anti-inflammation may increase the risk of cancer in certain environments (23,24), where antioxidants are over-enriched, due to the internal oxidative system, or ARE genes, may be triggered, which eventually increase cell proliferation (25,26). Thus an antioxidant environment may not curb cancer, particularly in cancerous organisms, where COX-2 is overexpressed. COX-2, a double-edged molecule, participates in inflammatory and anti-inflammatory processes. As a downstream product of COX-2, PGE2 frequently affects inflammation, and may be further derived into several so-called electrophilic oxo-derivative (EFOX) molecules with short life-cycles that strongly regulate cell proliferation through the Nrf2/keap1/ARE pathways (27).

In conclusion, this study has demonstrated that UA, VEPS, APS and their combined treatments markedly reduced COX-2 expression, and reduced the concentration of PGE2 in HepG2 cells, as shown by RT-PCR, western blot and ELISA analyses. The inhibition of COX-2 in cancer cells may increase oxidative stress due to decreased levels of EFOXs molecules that mediate the gene expression of SOD and other AREs (28). This is due to the fact that the ARE family, including SOD, create an antioxidative and anti-inflammatory protective environment in order to increase cell proliferation (29). Increased MDA levels, metabolic products of fatty acids, also indicate an oxidative environment. UA is a potent antioxidant with multiple functions, which reduced MDA significantly, while the inhibition of MDA and cell proliferation occurred only with a high concentration of VEPS and APS. Complications in in vivo metabolism occur when the metabolites of fatty acids accumulate. However, it is possible to ignore MDA as it minimizes the metabolism of fatty acids in vitro. Thus it is possible that the inhibition of HepG2 cell proliferation by UA, VEPS, APS and the combined treatments may be attributed to the inhibition of COX-2 and the associated decreases in SOD activity, which increase the oxidative environment and induce apoptosis, as shown by the higher rate of apoptotic blebs which were observed under the SEM, and the cell-cycle arrest detected by flow cytometry.

These compounds, particularly VEPS, may also have cancer-preventive roles in vivo through the innate immune system (30-32), as VEPS are capable of activating macrophages and lymphocytes without causing severe inflammation or other diseases, and a correlation between its dietary use and cancer epidemiology has been suggested (33).

In conclusion, this study has demonstrated that UA, VEPS, APS, and their combined treatments inhibit HepG2 cell growth. The mechanism for this inhibition may be through the inhibition of COX-2, which in turn reduces EFOXs that activate the internal anti-oxidative elements, including SOD, thus leading HepG2 cancer cells into an over-oxidative environment, causing apoptosis and retarding cell proliferation.
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