Chemopreventive effect of saikosaponin-d on diethylinitrosamine-induced hepatocarcinogenesis: Involvement of CCAAT/enhancer binding protein β and cyclooxygenase-2

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Abstract. Cyclooxygenase-2 (COX-2) and CCAAT/enhancer binding protein β (C/EBPβ) have been shown to be involved in inflammation and carcinogenesis, and our previous study revealed that they were co-overexpressed in human hepatocellular carcinoma (HCC) tissue and a positive correlation was found. Saikosaponin-d (SSD), a triterpene saponin extracted from Bupleurum falcatum L. (Umbelliferae), is known to exert inhibitory effects on COX-2 expression, together with inflammation and hepatic fibrosis. These findings prompted us to investigate the chemopreventive potential of SSD against hepatocarcinogenesis and its possible molecular mechanism in vivo. An experimental model with diethylinitrosamine (DEN)-treated Sprague Dawley rats was used in the present study. DEN (50 mg/kg body weight) and SSD (2 mg/kg body weight) were intraperitoneally injected weekly and daily, respectively. Administration of SSD alone had no side effects. The liver nodule formation, tumorous invasion to surrounding organs and increased cellular atypia induced by DEN were markedly reduced by SSD in the SSD + DEN group compared with the DEN group. On the other hand, immunohistochemical staining demonstrated that the expression of COX-2 and C/EBPβ proteins was significantly increased in tumor cells and macrophages of liver tissue from DEN-treated rats, whereas the expression of the two proteins was markedly lowered in the SSD + DEN group. Overall, our results suggest that SSD prevents DEN-induced hepatocarcinogenesis in rats through inhibition of C/EBPβ and COX-2, providing indispensable experimental evidence for the clinical application of SSD as a novel chemopreventive agent against HCC in the future.

Introduction

Hepatocellular carcinoma (HCC) is a common human malignant disease, accounting for 748,300 new cases and 695,900 deaths worldwide in 2008 (1). Although approaches such as surgery and radiochemotherapy have been established for treatment, the high incidence, quick progression and poor prognosis of HCC still remain as critical issues leading to high cost and mortality (2). Thus, the exact molecular mechanisms underlying hepatocarcinogenesis and potent preventive agents require urgent investigation.

A variety of risk factors such as chronic hepatitis virus infection, chemical carcinogen exposure and obesity have been thought to foster hepatocarcinogenesis (3). Additionally, HCC is known to be an inflammation-related malignancy, which is attributed to the above-mentioned risk factors. Cyclooxygenase-2 (COX-2), a rate-limiting enzyme in the production of prostaglandins, is usually induced by stimuli involved in inflammatory responses and has been shown to be associated with carcinogenesis and tumor progression (4,5). Various HCC-related molecules such as hepatitis B virus X protein (6), hepatitis C virus (7) and diethylinitrosamine (DEN) (8), have been reported to induce the expression of COX-2, which finally facilitates hepatocarcinogenesis and cancer progression. Moreover, studies have revealed that COX-2 overexpression is present in well-differentiated HCC and is an early event in the process of hepatocarcinogenesis (9,10). Deregulation of the COX-2 signaling pathway serves as a basis for designing novel-targeted therapeutic strategies for cancer therapy. Consequently, a variety of anti-inflammatory drugs targeting COX-2 have been demonstrated to exert strong chemopreventive capabilities in various types of cancers including tongue carcinoma (11), mammary carcinoma (12) and HCC (13).

In recent years, the importance of the transcription factor CCAAT/enhancer binding protein β (C/EBPβ) in promoting tumorigenesis and regulating COX-2 expression has been
recognized. C/EBPβ belongs to a basic-leucine zipper transcription factor family and plays multiple roles in the control of inflammation, cellular proliferation, survival and differentiation, and tumorigenesis (14-17). Recent evidence indicates that C/EBPβ is critical for carcinogen- and oncogene-mediated cell transformation (18,19). Furthermore, C/EBPβ activation is indispensable to liver proliferation and fibrosis (20-22).

C/EBPβ is one of the regulators implicated in COX-2 expression (23), and the two proteins are co-overexpressed in gastric carcinomas and play a crucial role in gastric tumorigenesis (24). HBV has been also reported to induce COX-2 expression by recruitment of C/EBPβ to the promoter (25). Furthermore, anti-inflammatory drugs such as salicylate suppress COX-2 expression via inhibition of C/EBPβ binding to the COX-2 promoter (26,27). Overall, these findings suggest a vital role of C/EBPβ/COX-2 in carcinogenesis, and render the two molecules as potential targets for the intervention of cancer by chemopreventive or chemotherapeutic agents.

Saikosaponin-d (SSD) is a triterpene saponin extracted from Bupleurum falcatum L. (Umbelliferae), a herb used to cure chronic liver diseases in traditional Chinese medicine (28). SSD exhibits multiple pharmacological activities including anti-inflammatory and anti-cancer effects (29-31). It has been shown that SSD reduces phorbol 12-myristate 13-acetate (PMA)-induced inflammation in vivo and COX-2 and lipoxygenase production in vitro (31). Moreover, SSD has been reported to attenuate toxin-induced hepatocyte injury and hepatic fibrosis in animal models though the inhibition of several types of inflammatory mediators (32,33). Our previous studies revealed that C/EBPβ and COX-2 were overexpressed in human HCC tissues and a positive correlation was found (34). We also found that SSD inhibited lipopolysaccharide-induced COX-2 expression in the HCC SMMC-7721 cell line in vitro (35).

However, to date, the effects of SSD on hepatocarcinogenesis and C/EBPβ and COX-2 expression in vivo, have not been clearly analyzed. To evaluate these, a DEN-induced model in rats was used, since weekly DEN injections efficiently promote hepatocarcinogenesis, which mimics the genetic process of human HCC (36). In this study, we determined that SSD chemoprevented hepatocarcinogenesis though inhibition of C/EBPβ and COX-2 expression.

Materials and methods

Reagents and chemicals. SSD (≥98% pure) was obtained from Jiangxi Herbfine Hi-Tech Co., Ltd. (China). DEN was purchased from Sigma Chemical Co. (St. Louis, MO). Rabbit polyclonal antibody against C/EBPβ and goat polyclonal antibody against COX-2 were both purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals were of the highest quality available and were obtained from authentic sources.

Experimental animals. Eight-week-old male Sprague Dawley rats weighing 180-200 g were obtained from the Laboratory Animal Centre of Xian Jiaotong University. All housing and animal procedures were carried out in accordance with the NIH Guidelines on the Use of Laboratory Animals. Animal care and protocols were approved by the Institutional Animal Care and Use Committee of Xi’an Jiaotong University.

Histology and immunohistochemistry. Paraformaldehyde-fixed tissues were embedded in paraffin blocks and cut into 4-µm sections. The sections were used for haematoxylin and eosin (H&E) staining according to conventional procedures.

Immunohistochemistry was conducted using a Dako Autostainer Plus system (Dako Corporation, Carpinteria, CA) as described previously (38). Briefly, sections were dewaxed, rehydrated and subjected to 5 min of antigen retrieval by pressure cooking, followed by blocking of endogenous enzyme and nonspecific antigens for 15 min. The sections were incubated with specific primary antibodies to C/EBPβ and COX-2 for 1 hour, washed in PBS, and then incubated with biotinylated conjugated secondary antibody for 30 min, followed by streptavidin-HRP reagent for 30 min and then incubation with diaminobenzidine for 30 min. The sections were counterstained with haematoxylin and mounted with cover slips. The specificity of the antibody was determined in tissue sections from rats injected with saline only and from rats injected with saline only and then with 50 mg/kg body weight of DEN weekly. Immunoreactivity was scored in a blinded manner by an investigator that did not have knowledge of the experimental conditions.

Experimental design. Fifty rats were randomly assigned into 4 experimental groups: normal group (n=10), rats were used as controls without any additional treatment; DEN group (n=15), rats received weekly intraperitoneal (i.p.) injections of DEN at a dose of 50 mg/kg body weight for 16 weeks; SSD + DEN group (n=15), rats received daily i.p. injections of SSD at a dose of 2 mg/kg body weight daily. The number of tumors (whitish nodules, >3 mm in diameter) at the liver surface was estimated (37). The livers were removed immediately and fixed in 4% paraformaldehyde.
tion for goat antibody. This was followed by incubation with dianinobenzidine (DAB) in a dark room. The sections were then counterstained with hematoxylin followed by dehydration. Sections incubated with PBS without the primary antibody were used as negative controls. All of the stained sections were reviewed in a blinded manner by two pathologists using light microscopy. The results were assessed using the average percentage of positive cells (No. of positive cells x 100/total no. of cells) in 5 different random microscopic fields (x400) in each slice.

Statistical analysis. Data are presented as the means ± SD. Statistical significance was determined using one-way ANOVA or Student’s t-test (SPSS 16.0 for windows). Significance was accepted at the level of P<0.05.

Results

Effects of SSD on hepatocarcinogenesis and tumor development in DEN-treated rats. To determine the chemopreventive effects of SSD against hepatocarcinogenesis in rats, SSD was administered starting 1 week before induction of DEN and lasting until the end of the experiments (Fig. 1).

As shown in Table I, administration of DEN markedly caused hepatomegaly, while SSD treatment led to a significant reduction in liver weight and the liver to body weight ratio in the SSD + DEN-treated rats (P<0.05). No mortality or adverse effects suggestive of toxicity of SSD was observed in the SSD group.

Macroscopically, no nodules or additional abnormalities were found in the livers of the normal (Fig. 2A) and SSD groups. As shown in Fig. 2C and D, in the DEN group, irregular appearing livers with cirrhosis and multiple whitish nodules distributed on the surface (15 of 15), were noted, and invasion to surrounding organs including colon and blood vessel (3 of 15; 20%) were visibly observed. In the SSD + DEN group, 2 of 15 animals (13.3%) were observed to have no nodules, while the other rats in this group were found to have less tumors and reduced tumor size (Fig. 2E). The number of nodules (>3 mm in diameter) over the liver surface in the SSD + DEN group was significantly less than that in the DEN group (Fig. 2F). No invasion to surrounding tissues was observed in the SSD + DEN group.

Hepatic histopathology from the various groups of rats was examined by H&E staining. Microscopically, the hepatic sections from the normal and SSD-treated animals

Table I. Effect of DEN and SSD treatment on body and liver weight.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of rats</th>
<th>Body weight (g)</th>
<th>Liver weight (g)</th>
<th>Ratio (%) of liver to body weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>10</td>
<td>315±6.57</td>
<td>11.9±0.975</td>
<td>3.8±0.38</td>
</tr>
<tr>
<td>SSD</td>
<td>10</td>
<td>311±11.95</td>
<td>11.8±1.26</td>
<td>3.8±0.29</td>
</tr>
<tr>
<td>DEN</td>
<td>15</td>
<td>346±5.51</td>
<td>17.6±1.49</td>
<td>5.1±0.43</td>
</tr>
<tr>
<td>SSD + DEN</td>
<td>15</td>
<td>321±11.10</td>
<td>12.7±0.87</td>
<td>4.0±0.28</td>
</tr>
</tbody>
</table>

Data are expressed as the means ± SD; P<0.05 compared with normal group or DEN-treated group.
revealed normal liver parenchyma characterized by typical hepatic lobules and small uniform nuclei (Fig. 3A and B). However, as shown in Fig. 3C, liver tissue samples from the DEN group exhibited disordered architecture with a large number of pseudolobules, collagen deposition, infiltration of inflammatory cells, and abnormal cells with irregular-shaped cytoplasm and enlarged hyperchromatic nuclei, and these HCC cells generally tended to be moderately differentiated. In the SSD + DEN group, the hepatic pathological lesions with pseudolobules, fibrosis, inflammatory cell infiltration and cellular atypia were markedly improved compared with the DEN group, and the tumors in the SSD + DEN group were histologically well-differentiated (Fig. 3D and E).

**Effects of SSD on DEN-induced intracellular expression of COX-2 and C/EBPβ proteins.** Since chemopreventive effects of SSD against hepatocarcinogenesis in rats was observed, the possible molecular mechanisms were then explored. The expression levels of COX-2 and C/EBPβ proteins in tissue specimens were examined by immunohistochemical staining.

COX-2 expression was merely detectable in minor hepatocytes in normal hepatic tissue from the normal and SSD groups (Fig. 4A and B). In contrast, an increased number of elevated positive cells (HCC cells and macrophages) in the DEN-induced tumor tissue were clearly observed (Fig. 4C), whereas SSD markedly suppressed COX-2 expression in the tumorous and non-tumorous tissues from the SSD + DEN group (Fig. 4D and E). As shown in Fig. 4F, a decreased number of COX-2 positive cells was observed in the SSD + DEN group.

Immunohistochemistry revealed the absence of C/EBPβ expression in the normal liver tissues from the normal and SSD groups (Fig. 5A and B), while hepatic tissue from the DEN-treated group showed markedly strong staining of C/EBPβ in tumor cells and macrophages (Fig. 5C). Both the tumorous and non-tumorous tissues from the SSD + DEN-treated group showed significantly lower expression of C/EBPβ protein compared with the DEN group (Fig. 5D and E). Additionally, a decreased number of C/EBPβ-positive cells was observed in the SSD + DEN group (Fig. 5F).

**Discussion**

The goals of the present study were to show the in vivo chemopreventive effects of SSD on hepatocarcinogenesis and to
elucidate the novel underlying mechanisms of the action. We demonstrated that SSD suppressed hepatocarcinogenesis and the overexpression of C/EBPβ and COX-2 induced by DEN. To the best of our knowledge, this is the first report on the effects of SSD on hepatocarcinogenesis involving regulation of C/EBPβ and COX-2.

The molecular mechanisms of HCC formation and effective approaches to prevent HCC still remain poorly defined. In recent years, studies have emphasized the potential role of COX-2 in linking chronic inflammation with carcinogenesis and progression in various cancer types (4). It has been shown that COX-2 plays crucial roles in fibrogenesis and hepatocarcinogenesis (9,39). In the present study, the increase in COX-2 expression was observed in DEN-induced HCC tissues in rats, consistent with a recent report (8). These results suggest that, under some conditions, COX-2 overexpression promotes hepatocarcinogenesis.

As an upstream regulator of the COX-2 gene, the transcription factor C/EBPβ is significantly elevated in colorectal tumors and is associated with human ovarian epithelial tumor progression, suggesting that C/EBPβ may be involved in tumorigenesis and cancer development (40,41). Bundy and Sealy identified the role of C/EBPβ in carcinogenesis by evidence that the human breast epithelial cell line MCF10A overexpressing C/EBPβ acquired malignant phenotypes including anchorage-independent growth, colony formation in soft agar and invasion (42). Zhu et al demonstrated that C/EBPβ-null mice are completely refractory to skin tumor formation induced by a variety of carcinogens which produce tumors containing oncogenic Ras mutations, and that C/EBPβ participated in Ras-induced transformation of NIH 3T3 fibroblasts (16). In Ras-transformed MCF10A cells, C/EBPβ suppressed expression of tumor suppressor Sigleminded-2, which has been found to inhibit malignant transformation of mammary ductal cells (19). Thus, C/EBPβ is downstream of the Ras signaling pathway and is required for Ras-induced malignant transformation. A recent study found elevated Ras activity in DEN-induced hepatocarcinogenesis (37), which may explain our finding that C/EBPβ expression was significantly increased in hepatic tumor tissue from DEN-treated rats. Moreover, C/EBPβ has been shown to be essential for TGFα-stimulated proliferation.
of murine hepatocytes and CCl₄-induced liver fibrosis and regeneration (precursors to HCC) (22), which supports our hypothesis that C/EBPβ is involved in hepatocarcinogenesis.

Activation of C/EBPβ is crucial for the initial induction of COX-2 by growth factors, tumor promoters, cytokines and other inflammatory mediators in various types of cells (43,44). Further study revealed that C/EBPβ and COX-2 showed overlapping overexpression in gastric carcinomas and that C/EBPβ has the potential to mediate gastric carcinogenesis via the regulation of COX-2 expression (24). In human prostate tissues, the expression of C/EBPβ and COX-2 was highly correlated and was involved in chronic inflammation and prostate cancer development (45). Our previous study demonstrated that C/EBPβ overexpression was correlated with COX-2 overexpression in human HCC tissue (34). All of these investigations provide evidences for our present finding that C/EBPβ and COX-2 are relatedly overexpressed in rat liver tumors induced by DEN, although double immunostaining to define the precise co-expression was not carried out. Moreover, our in vitro study showed that lysophosphatidic acid (LPA), a growth factor-like phospholipid, potently stimulated the cell proliferation and cell cycle progression of human hepatocarcinoma SMMC-7721 cells through induction of C/EBPβ and COX-2 expression (data not shown). Given the drastic and invariable roles of C/EBPβ and COX-2 in liver fibrogenesis and carcinogenesis, potential drugs targeting these molecules may have preventive effects against liver fibrogenesis and tumorigenesis (22,46).

Previous in vivo studies have reported that SSD exerted hepatoprotective effects and attenuated toxin-induced liver fibrosis, due to the downregulation of pro-inflammatory cytokines such as TNF-α, IL-6 and TGF-β1 (32,33). In other words, the therapeutic effects of SSD on liver fibrosis and cirrhosis may be attributed to its anti-inflammatory pharmacological activity. At the cellular and molecular level, it has been reported that SSD suppresses T cell activity through inhibition of the NF-κB signaling pathway and COX-2 expression (31,47). Since SSD inhibits COX-2 activity, chronic inflammation, liver fibrosis and cirrhosis, which are relevant to HCC, it is reasonable to characterize SSD as a potential chemopreventive or chemotherapeutic agent against hepatocarcinogenesis. In this study, we found that SSD administration significantly inhibited liver inflammation, fibrosis and
tumor formation and invasion in rats in comparison to those administered DEN only, suggesting that SSD plays roles in inhibiting HCC in both the early and late stages. Since C/EBPβ and COX-2 play key roles in inflammation, hepatic fibrosis and tumorigenesis, we determined the effect of SSD on expression of these two proteins. As expected, SSD markedly reduced DEN-induced activity of C/EBPβ and COX-2. Additionally, we found that SSD inhibited LPA-stimulated proliferation of hepatocarcinoma cells by suppression of C/EBPβ and COX-2 expression (data not shown). Thus, it can be inferred from our finding that SSD-mediated downregulation of C/EBPβ and COX-2 may be one of the mechanisms by which SSD chemoprevents DEN-induced hepatocarcinogenesis in rats. However, further studies are needed to define the molecular mechanisms.

In conclusion, using a DEN-induced rat HCC model, we reported that SSD prevents hepatocarcinogenesis through inhibition of C/EBPβ and COX-2. Our findings may provide important insights into the mechanism of HCC and indispensable experimental evidence for the clinical application of SSD in the future.

Acknowledgements

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References