Abstract. Xanthohumol may prevent and cure diabetes and atherosis, have oxidation resistance and antiviral function as well as anticancer effect preventing cancer cell metastasis. We investigate whether the anticancer effect of xanthohumol induces growth inhibition and apoptosis of human liver cancer through NF-κB/p53-apoptosis signaling pathway. Human liver cancer HepG2 cell were treated with 10, 20, 30 and 40 µM xanthohumol for 48 h. The present study showed that the anticancer effect of xanthohumol was effective in inhibiting proliferation and inducing apoptosis of human liver cancer HepG2 cells. Furthermore, the caspase-3 activity of human liver cancer HepG2 cells was increased by xanthohumol. In addition, 48-h treatment with xanthohumol suppressed NF-κB expression and promoted p53, cleaved PARP, AIF and cytochrome c expression and downregulated XIAP and Bcl-2/Bax expression in human liver cancer HepG2 cells. Therefore, the anticancer effect of xanthohumol induces growth inhibition and apoptosis of human liver cancer through the NF-κB/ p53-apoptosis signaling pathway.

Introduction

Liver cancer is one of the most common malignant cancers (1). It is ranked 3rd in deaths from various malignant cancers (1). Liver cancer mostly occurs in the conditions of hepatic disease and liver cirrhosis, and most patients have weak liver function (2). Therefore, patients often cannot tolerate an operation or endure traditional chemotherapeutics in strong enough doses. Also, liver cancer is insensitive to traditional chemotherapeutics (3). Hence, chemotherapy often has no satisfactory results for the improvement of prognosis for patients. Therefore, identification of natural chemotherapeutics without harmful effects has become one of the strategies to improve therapeutic effect of liver cancer (4).

The combination between NF-κB and sequence κB on DNA may adjust the transcriptional activation of multiple genes, which are closely related with the inhibition of transcriptional activation, vasculogenesis, tumor metastasis and apoptosis, which are the key link of promoting tumor growth and resistance (5). The inhibition of NF-κB activity may increase the sensitivity of cancer cells for chemotherapeutics and radiotherapy (6). The expression and mutation of cancer suppressor gene p53 is closely related with tumorigenesis, development and apoptosis of multiple tumors (7). However, the expression of both p53 and NF-κB is adjusted by Akt (8).

As an isoprene flavonoid existing in hops, xanthohumol has multiple biological activities. It plays a significant role in prevention and cure of diabetes and atherosis. Also, it has antioxidant, and antiviral functions, inhibiting cancer cell growth in breast, colon, ovarian and prostatic cancer (9,10). However, the molecular mechanism of such function is not yet clear. In the present study, we elucidated anticancer effect of xanthohumol inducing growth inhibition and apoptosis of human liver cancer through the NF-κB/p53-apoptosis signaling pathway.

Materials and methods

Chemicals and materials. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were acquired from Gibco Technologies (Carlsbad, CA, USA). Penicillin and streptomycin were acquired from Life Technologies. Xanthohumol and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) were acquired from Sigma-Aldrich (St. Louis, MO, USA). The chemical structure of xanthohumol is shown in Fig. 1. FITC Annexin V apoptosis detection kit was acquired from BD Biosciences (San Jose, CA, USA). Caspase-3 activity kit was acquired from GeneTex, Inc. (Irvine, CA, USA).

Cell culture. Human liver cancer HepG2 cells were maintained in DMEM medium (Gibco) supplemented with 10% (v/v) FBS and 2% penicillin/streptomycin (Life Technologies) in 5% CO₂ incubator at 37°C in a humidified atmosphere.
Cell viability measurement. The effect of xanthohumol on cell viability was measured using the MTT assay. HepG2 cells were seeded in 96-well culture plates (5,000 cells/well) and treated with concentrations of (0-200 µM) xanthohumol for 1-3 days. After treatment, cells were incubated with 200 µl MTT (1 mg/ml) for 4 h, followed by removal of the supernatant and dissolution of 20 µl DMSO. The absorbance of the resulting solution was recorded using a microplate reader (Perkin Elmer Inc., Waltham, MA, USA) at 570 nm.

Apoptosis detection by Annexin V-FITC/PI staining. HepG2 cells were seeded in 6-well culture plates (2.5x10^5 cells/well) and treated with 10, 20, 30 and 40 µM of xanthohumol for 1 day (24 h). The cells were resuspended in 1X binding buffer according to the manufacturer's instruction. Then, HepG2 cells were stained with 5 µl V-FITC and 5 µl propidium iodide (PI) for 15 min on ice. At the end of the staining process, 10,000 cells were acquired for each replicate using Accuri C6 flow cytometer.

Caspase-3 activity. HepG2 cells were seeded at a density of 1x10^6 cells/culture dish. At the end of the incubation period, the cells were centrifuged at 3,000 rpm for 5 min and the supernatant was removed. Then, the cells were resuspended in 0.5 ml wash buffer and centrifuged at 2,000 rpm for 10 min. An equal amount of total protein was incubated with Ac-IETD-pNA for caspase-3 assay for 4-6 h. Fluorescence intensity was measured at 485 nm (excitation wavelength) and 535 nm (mission wavelength).

Western blot analysis. HepG2 cells were seeded at a density of 1x10^6 cells/culture dish. After xanthohumol treatment, cells were washed with cold PBS and lysed with cold RIPA buffer containing protease inhibitors. Protein concentrations were measured using BSA (Bio-Rad Laboratories, Hercules, CA, USA). Next, 40 µg protein was separated with 10-12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to 0.2 µm nitrocellulose membrane blocking with 1% BSA in PBS-T. Each membrane was incubated with a specific primary antibody NF-κB (1:1,000), p53, PARP, XIAP, AIF, Bax, cytochrome c and β-actin at 4°C overnight. After three washes in 1% BSA in PBS-T, each membrane was incubated with the appropriate secondary antibody at room temperature for 2 h and visualized using an ECL Advanced Western blot detection kit (Thermo Fisher Scientific, Waltham, MA, USA).

Results

Anticancer effect of xanthohumol induces growth inhibition of human liver cancer. HepG2 cells were subjected to the MTT assay to evaluate the anticancer effect of xanthohumol treatment by measuring cell proliferation. Xanthohumol reduced cell proliferation of HepG2 cells in a concentration- and time-dependent manner (Fig. 2). As shown in Fig. 2, this change was markedly observed after exposure to 200 µM of xanthohumol for 1 day, 100-200 µM of xanthohumol markedly reduced cell proliferation of HepG2 cells in 2 or 3 day treatments (Fig. 2). At 50 µM xanthohumol significantly inhibited cell proliferation of HepG2 cells up to 3 days (Fig. 2).

Anticancer effect of xanthohumol induces apoptosis of human liver cancer. The cell apoptosis was determined by analysis using PI staining. As shown in Fig. 2, apoptosis of HepG2 cells was treated with 0-150 µM of xanthohumol. The group treated with 0-150 µM of xanthohumol showed a significant increase in apoptosis rate in comparison with the 0 µM xanthohumol group (Fig. 3).

Figure 1. The chemical structure of xanthohumol.

Figure 2. Anticancer effect of xanthohumol induces growth inhibition of human liver cancer. *P<0.01 compared with 0 µM xanthohumol group.

Figure 3. Anticancer effect of xanthohumol induces apoptosis of human liver cancer. *P<0.01 compared with 0 µM xanthohumol group.

Figure 4. Anticancer effect of xanthohumol induces caspase-3 activity of human liver cancer. *P<0.01 compared with 0 µM xanthohumol group.
Anticancer effect of xanthohumol induces caspase-3 activity of human liver cancer. To determine whether xanthohumol induced apoptosis in HepG2 cells, caspase-3 activity was conducted. Fig. 4 shows caspase-3 activity was significantly increased by treatment with xanthohumol.

Anticancer effect of xanthohumol inhibits NF-κB signaling of human liver cancer. Furthermore, we explored whether xanthohumol inhibits NF-κB signaling in HepG2 cells, NF-κB protein expression was executed using western blot analysis. Dose response curves shown in Fig. 5 indicate that NF-κB protein expression was significantly inhibited with 100 or 150 µM of xanthohumol.

Anticancer effect of xanthohumol induces p53 signaling of human liver cancer. The anticancer effect of xanthohumol induces p53 signaling of human liver cancer, western blot analysis was executed for p53 protein expression. As shown in Fig. 6, after treatment with xanthohumol, promotion of p53 protein expression was significantly observed in HepG2 cells exposed with 100 or 150 µM of xanthohumol.

Anticancer effect of xanthohumol induces PARP signaling of human liver cancer. We identified the anticancer effect of xanthohumol on PARP signaling of human liver cancer. When HepG2 cells treated with xanthohumol, PARP protein expression was significantly increased in HepG2 cells (Fig. 7).

Anticancer effect of xanthohumol inhibits XIAP signaling of human liver cancer. Furthermore, the anticancer effect of xanthohumol inhibiting XIAP signaling of human liver cancer was determined. As shown in Fig. 8, following xanthohumol treatment, the XIAP proteins expression decreased in HepG2 cells.
Anticancer effect of xanthohumol inhibits AIF signaling of human liver cancer. Increased AIF signaling is an important characteristic of cell apoptosis. In addition, a remarkable increase in the abundance of AIF protein expression was also detected after xanthohumol treatment (Fig. 9).

Anticancer effect of xanthohumol induces Bax signaling of human liver cancer. To investigate whether Bax signaling is involved in xanthohumol-induced apoptosis, we measured Bax protein expression in HepG2 cells by western blot analysis. As shown in Fig. 10, HepG2 cells treated with 20 μM
of xanthohumol showed a time-dependent promotion of Bax signaling.

**Anticancer effect of xanthohumol induces cytochrome c signaling of human liver cancer.** Activation of cytochrome c signaling is involved in the anticancer effect of xanthohumol in HepG2 cells. As expected, the xanthohumol treatment (100-150 µM) resulted in a significant decrease in cytochrome c signaling of HepG2 cells (Fig. 11).

**Discussion**

Apoptosis, which is an intrinsic function of cells, is the reverse of cell proliferation (3). Cell death that occurs via triggering the suicide program of cells is called apoptosis. The essence of the so-called apoptosis program is a set of gene programs in cells responsible for perception, adjustment and execution of the cell death signal (11). Apoptosis is an initiative death, which is very common and has important physiological and pathological significance. Under physiological status, the death of body cells is a very common phenomenon, which happens continually. Nevertheless, the essence of such physiologic death is apoptosis, which is a significant method to adjust the balance between growth and death by the body. It keeps the homeostasis of body cell population together with cell proliferation. The normal physiological process of body cannot do without apoptosis. In hodiernal study, the anticancer effect of xanthohumol induces growth inhibition, enhance apoptosis and advance caspase-3 activity of HepG2 cells. Yong et al (12) indicated that xanthohumol induces growth inhibition and apoptosis of human cervical cancer cells, breast (10) and prostate cancer (9). These results confirmed that xanthohumol can suppress cell growth and induce apoptosis of human liver cancer.

NF-κB may adjust the transcriptional activation of multiple genes, which is closely related with the inhibiting effect of cell proliferation, vasculogenesis, tumor metastasis and apoptosis and also is a key link of promoting tumor growth and resistance (13). The inhibition of NF-κB activity may increase the sensitivity of cancer cells against chemotherapeutics and radiotherapy (14). Research has shown that by the inhibition of NF-κB excitation, the expression of p53 significantly increases by downstream factors of upregulated p53 (15). The loss of gene structural stability is a key factor of multiple tumorigenesis, in which the cancer suppressor gene p53 is the stress reaction gene of cells, responding to DNA damage arising from various factors (16). About half of human tumors show loss of p53 function. The structure and function of p53 as well as the gene activity network focusing on p53 have been summarized (16). Also, the correlation between p53 and HBV and their effect in primary hepatocarcinogenesis have been emphasized (17). In the present study, the anticancer effect of xanthohumol inhibited the NF-κB signaling and induced p53 signaling of human liver cancer as demonstrated in Fig. 12. Dell’Eva et al (18) reported xanthohumol, an AKT/NF-κB inhibitor, treatment in leukemia and endothelial cells.

PARP can keep the structural integrity of chromosome and participate in DNA replication and transcription (19). It plays a role in maintaining a stable genome and apoptosis course. PARP is activated when DNA is broken and damaged as a molecular receptor of DNA damage (20). It distinguishes and combines with the DNA breakages, activating and catalyzing the poly ADP ribosylation of receptor protein and participating in DNA repair (21). PARP combines with histone H1, which influences the normal structure of nucleosome, allowing the chromosome to form an open and loose structure, which assists in DNA repair. PARP is activated after DNA damage, identifying and combining the DNA breakages, thereby protecting bare DNA terminal from the catabolic reaction by nuclease (20). In the present study, anticancer effect of xanthohumol induced PARP signaling of human liver cancer (Fig. 12). Drenzek et al (22) suggested that xanthohumol decreases cell growth and induces epithelial ovarian cancer via cleaved caspase-3 and cleaved PARP.

The XIAP regulation and control of cell proliferation and migration are reported in the literature. The conclusion has proved that such a course is achieved by a signal path, which may allow the activation of NF-κB transcription factor, thereby promoting the expression of genes for cell proliferation and migration (23). Research has shown that XIAP is the critical regulatory protein between the apoptosis pathway and cell cycle pathway of XIAP; XIAP may promote the hepatoma carcinoma cell to enter the G1 cycle by adjusting the expression of cdk4, cdk6 and cyclin D1 of G1 phase protein of hepatoma carcinoma cells, thus reducing cells entering apoptosis pathway and promoting cell proliferation (24,25). In the present study, the anticancer effect of xanthohumol inhibits XIAP signaling of human liver cancer (Fig. 12). Taken together, Yong et al (12) indicated that xanthohumol induces growth inhibition and apoptosis through increasing of cleaved PARP, p53 and AIF, and decreasing of Bcl-2 and XIAP pathways in CaSki human cervical cancer cells.

Tumorigenesis and tumor progression are related with an imbalance between cell proliferation and apoptosis. NCTD not only inhibits the tumor cell proliferation, but also induces cell apoptosis (26). AIF is a flavoprotein with relative molecular weight of 57 kD encoded by nuclear gene, located
in the intermembrane zone of mitochondria with double-layer coating. AIF may enter the karyon from mitochondria by transposition, independently splitting DNA into DNA fragments with ~60 kb, directly causing the chromatin condensation and DNA breakage (27). Bel family may adjust and control the release of AIF by opening and closing the PT pore, but it cannot influence its activity (28). Apoptosis of AIF is independent of the activity of caspase, self-oxidase and reductase. Furthermore, research shows that AIF antibody may inhibit the release of cytochrome c and the grade chain reaction of caspase, but such inhibition cannot influence the release of AIF. Thus, it can be seen that AIF has an apoptosis-promoting effect in the upstream of the apoptotic pathway of cytochrome c or caspase (29). In the present study, the anticancer effect of xanthohumol inhibited AIF signaling, induced Bax and cytochrome c signaling of human liver cancer. Festa et al. (30) suggested that the anticancer effect of xanthohumol induces apoptosis through activation of caspase-3, caspase-9, PARP cleavage, Bcl-2 and cytochrome c in human malignant glioblastoma. Yong et al. (12) indicated that xanthohumol induces growth inhibition and apoptosis through increasing cleaved PARP, p53 and AIF, and decreasing of Bcl-2 and XIAP pathways in CaSki human cervical cancer cells.

The present study showed that xanthohumol induced growth inhibition, apoptosis and caspase-dependent cell death, by the NF-κB/p53-apoptosis signaling pathway, as well as upregulation of Bax/Bcl-2-cytochrome c-caspase-3-PARP and AIF; suppression of XIAP signaling pathway in HepG2 cells. Our findings provide confirmation of the anticancer effect of xanthohumol on human liver cancer.

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