Available online at www.sciencedirect.com
SCIENCE DIRECT
Toxicology and Applied Pharmacology 203 (2005) 124–131
Toxicology and Applied Pharmacology
www.elsevier.com/locate/ytaap

Induction apoptosis of luteolin in human hepatoma HepG2 cells involving mitochondria translocation of Bax/Bak and activation of JNK

Herng-Jiun Lee[a], Chau-Jong Wang[a], Hsing-Chun Kuo[a], Fen-Pi Chou[a], Lian-Fwu Jean[a], Tsui-Hwa Tseng[b,*]

[a]*Institute of Biochemistry, Chung Shan Medical University, Taichung, Taiwan*
[b]*School of Applied Chemistry, Chung Shan Medical University, Taichung, Taiwan*

Received 8 June 2004; accepted 10 August 2004
Available online 28 September 2004

Abstract

Since hepatocellular carcinoma remains a major challenging clinical problem in many parts of the world including Eastern Asia and Southern Africa, it is imperative to develop more effective chemopreventive and chemotherapy agents. Herein, we present an investigation regarding the anticancer potential of luteolin, a natural flavonoid, and the mechanism of its action in human hepatoma HepG2 cells. Using DNA fragmentation assay and nuclear staining assay, it showed that luteolin induced apoptosis of HepG2 cells. Luteolin induced the cytosolic release of cytochrome *c* and activated CPP32. We found that Bax and Bak translocated to mitochondria apparently, whereas Fas ligand (FasL) was unchanged after a treatment with luteolin for 3 h. In addition, it showed that c-Jun NH_2-terminal kinase (JNK) was activated after the treatment of luteolin for 3–12 h. Further investigation showed that a specific JNK inhibitor, SP600125, reduced the activation of CPP 32, the mitochondrial translocation of Bax, as well as the cytosolic release of cytochrome *c* that induced by luteolin. Finally, the apoptosis induced by luteolin was suppressed by a pretreatment with SP600125 via evaluating annexin V-FITC binding assay. These data suggest that luteolin induced apoptosis via mechanisms involving mitochondria translocation of Bax/Bak and activation of JNK.

Keywords: Apoptosis; Bax; Bak; JNK; Luteolin

Introduction

Apoptosis, or programmed cell death, has an essential role in controlling cell number in many developmental and physiological settings. Apoptosis is impaired in many human tumors, suggesting that disruption of apoptotic function contributes substantially to the transformation of a normal cell into a tumor cell. Apoptosis is an important phenomenon in chemotherapy-induced tumor-cell killing. Components of the apoptosis signaling cascade including caspases (Nicholson et al., 1995; Talanian et al., 1997) along with several other triggers and regulators such as Fas ligand (FasL) (Sharma et al., 2000) and Bcl-2 family members, are among the most promising targets for pharmacological modulation of cell death (Gross et al., 1999; Kluck et al., 1997).

Accumulating evidence suggests that protein kinases, such as the mitogen-activated protein kinase (MAPK) superfamily (including ERK, JNK, and p38 MAPK) are important regulators of apoptosis (Xia et al., 1995; Tournier et al., 2000). Furthermore, several studies have revealed that activation of c-*jun* NH_2-terminal kinase (JNK) is involved in apoptosis induced by various cellular stresses and anti-cancer reagents (Amato et al., 1998; Mizukami et al., 2001; Zanke et al., 1996). However, the mechanism that accounts for the proapoptotic actions of JNK has not yet been elucidated. Recent studies have focused on two different possible mechanisms. First, JNK may cause cell death by regulating the expression of death receptor ligands (Faris et al., 1998).

Abbreviations: CPP32, cysteine protease 32 kDa proenzyme; DMSO, dimethyl sulfoxide; JNK, c-Jun NH_2-terminal kinase; p38 MAPK, p38 mitogen-activated protein kinase; MTT, (3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyl tetrazolium bromide; PARP, poly (ADP-ribose) polymerase.

* Corresponding author. School of Applied Chemistry, Chung Shan Medical University, No. 110, Section 1, Chien Kuo N. Road, Taichung, Taiwan. Fax: +886 4 23248189.
E-mail address: tht@csmu.edu.tw (T.-H. Tseng).

0041-008X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.taap.2004.08.004

The second possible mechanism is that JNK may contribute to apoptotic response by regulating the intrinsic cell death pathway involving the mitochondria (Tournier et al., 2000).

Many studies have demonstrated that flavonoids are present in medical and edible plants, and epidemiological studies suggest that flavonoids play an important role in the prevention of carcinogenesis (Birt et al., 2001; Middleton and Kandaswami, 1994). Flavonoids are known to suppress tumor cell growth that is mediated by different type of cell cycle arrest and the induction of apoptosis in several tumor cell lines (Casagrande and Darbon, 2001; Iwashita et al., 2000; Kawaii et al., 1999; Kobayashi et al., 2002; Wang et al., 1999). Luteolin (3′,4′,5,7-tetrahydroxyflavone), an important member of the flavonoid family, is present in various fruits and vegetable. It exhibits a wide spectrum of pharmacological properties including anti-inflammatory and anti-allergic properties (Kimata et al., 2000). Recently, much attention has been paid to its antioxidant properties and to its antiproliferative effects (Lee et al., 2002; Perez-Garcia et al., 2000). It was reported that luteolin has potential for anticancer therapy by inhibiting DNA topoisomerase I and II (Chowdhury et al., 2002; Mittra et al., 2000). In addition, Ko et al. (2002) reported that luteolin demonstrated an effect on the inhibition of proliferation and induction of apoptosis in human myeloid leukemia cells. However, little is known about its biochemical targets.

In recent years, it has become clear that many anticancer drugs induce apoptosis in target cells (Debatin, 2000; Kaufmann and Earnshaw, 2000). Although the participation of apoptosis pathways in drug-induced apoptosis appears to be complex, perturbation of mitochondrial function with opening of permeability transition pores and release of apoptogenic molecules has been observed in most cases of drug-induced apoptosis (Green and Reed, 1998). Consistent with our results, it has been demonstrated that luteolin induces apoptosis of human hepatoma HepG2 cells through increasing cytochrome *c* release from mitochondria. In addition, it has been shown that translocation of Bax and Bak to mitochondria and activation of JNK involved in the action (Wei et al., 2001; Wolter et al., 1997). Thus, the evidence suggests that luteolin possess anticancer potential.

Materials and methods

Cell culture. Human hepatoma HepG2 cells were grown in Dulbecco's modified Eagle's medium (GIBCO BRL, Grand Island, NY) supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. The cells were plated at a density of $2 \times 10^4/cm^2$ into tissue-culture dishes and grown at 37 °C in a humidified, 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium.

Assessment of cell viability. The cells were seeded at 2×10^4 cells/ml density and incubated with luteolin (0–100 μM) for 24 h. Thereafter the medium was changed and incubated with MTT (0.5 mg/ml) for 4 h. The viable cell number was directly proportional to the production of formazan, which was then solubilized with isopropanol, and measured spectrophotometrically at 563 nm.

Determination of DNA fragmentation. Both detached and attached cells were harvested by scraping and centrifugation. After been washed with PBS (with 1 mM $ZnCl_2$), the cells were resuspended in 0.5 ml lysis buffer (0.5% Triton X-100, 20 mM EDTA, and 5 mM Tris; pH = 8.0) for 45 min. Fragmented DNA in the supernatant fraction after centrifugation at 14000 rpm was extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v) and once with chloroform; and then it was precipitated with ethanol and 5 M NaCl overnight at −20 °C. The DNA pellet was washed once with 70% ethanol, resuspended in Tris-EDTA buffer (pH 8.0), and treated with 100 μg/ml RNase A for 2 h at 56 °C. After quantitative analysis of DNA content by spectrophotometry (260 nm), an equal amount of DNA was electrophoresed in horizontal agarose gel (1.8%) performing at 1.5 V/cm for 3 h. DNA in gel was visualized under UV light after staining with ethidium bromide (0.5 mg/ml).

DAPI staining. Changes in cell morphology characteristic of apoptosis were examined by fluorescence microscopy of DAPI-stained cells. The monolayer of cells was washed in PBS and fixed with 4% paraformaldehyde for 30 min at room temperature. The fixed cells were permeabilized with 0.2% Triton X-100 in PBS for three times and incubated with 1μg/ml of 4,6-diamidino-2-phenylindole (DAPI) for 30 min, and, then, washed with PBS for three times. The apoptotic nuclei (intensely stained, fragmented nuclei and condensed chromatin) were examined under 400× magnification using a fluorescent microscope with a 340/380 nm excitation filter and scored according to the percentage from 200 to 300 cells/sample by at least two investigators.

Release of cytochrome c and translocation of Bax, Bak. The basic methodology for the preparation of mitochondria and cytosol fractions was as described as Tang et al. (1998). Cells (3×10^6) were harvested and washed with ice-cold PBS at the end of treatment. The cell pellet was resuspended in 500 μl of buffer A (20 mM HEPES–KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM leupeptin, 1 μg/ml pepstatin A, and 1 μg/ml chymostatin), and homogenized in the same buffer with a Pyrex glass homogenizer using a type B pestle (40 strokes). The homogenate was then centrifuged at 1000 × *g* for 10 min at 4 °C. The resulting supernatant was subjected to another centrifugation at 10000 × *g* for 20 min at 4 °C. The resulting supernatant contained the cytosolic fraction, and the pellets contained the enriched mitochondria fraction. Pellets containing mitochondria were treated with lysis buffer (1x PBS, 1% NP40, 0.5% sodium deoxycho-