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Genistein induces apoptosis in human hepatocellular carcinomas via interaction of endoplasmic reticulum stress and mitochondrial insult

Ting-Chun Yeh [a], *Po-Cheng Chiang* [a], *Tsai-Kun Li* [b], *Jui-Ling Hsu* [a], *Chun-Jung Lin* [a], *Shih-Wei Wang* [c], *Chieh-Yu Peng* [c], *Jih-Hwa Guh* [a,*]

[a] *School of Pharmacy, National Taiwan University, No. 1, Section 1, Jen-Ai Road, Taipei, Taiwan*
[b] *Department of Microbiology, College of Medicine, National Taiwan University, No. 1, Section 1, Jen-Ai Road, Taipei, Taiwan*
[c] *Pharmacological Institute, College of Medicine, National Taiwan University, No. 1, Section 1, Jen-Ai Road, Taipei, Taiwan*

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ABSTRACT

Hepatocellular carcinoma is a very common malignancy and is chemoresistant to currently available chemotherapeutic agents. Endoplasmic reticulum (ER) stress-induced apoptotic pathway is suggested to be less affected by the resistance mechanisms, becoming a potential target of chemotherapeutic strategy. The anticancer effects and expression of GADD153, a transcription factor induced by ER stress, were examined in hepatocellular carcinoma Hep3B cells. The correlation between these two parameters was constructed under flavonoid stimulation with a correlation coefficient (r) of 0.8. The data also showed that genistein (isoflavone) was the most effective one. Genistein induced the activation of several ER stress-relevant regulators, including m-calpain, GADD153, GRP78 and caspase-12. Furthermore, genistein-induced effect was inhibited in cells transfected with antisense GADD153 cDNA, indicating a functional role of GADD153. Notably, genistein induced the activation of caspase-2, whereas did not cause the DNA damage. It also triggered the production of ROS. The antioxidant trolox significantly reduced ROS accumulation, but did not modify genistein-induced apoptotic cell death. The long-term exposure (48 h) of cells to genistein caused Mcl-1 down-regulation and Bad cleavage; furthermore, cyclosporin A (an inhibitor of mitochondrial permeability transition pore) almost completely abolished genistein-induced loss of mitochondrial membrane potential, and induced a 30% reverse of apoptosis caused by long-term treatment (48 h) of genistein, suggesting the involvement of mitochondrial stress in the late phase of genistein-induced effect. Taken together, it is suggested that genistein induces the anticancer effect through a mechanism initiated by ER stress and facilitated by mitochondrial insult in Hep3B cells.

1. Introduction

Flavonoids, chemical structures of a common phenylchromanone skeleton with one or more hydroxyl substituents, are part of a family of naturally occurring compounds and represent one of the most widespread classes of component in fruits, vegetables and medical herbs [1]. The flavonoids exert a wide spectrum of pharmacological activities, with one of the most elucidated effects being the anticancer activities. The flavonoids are well known to inhibit cell growth and

* *Corresponding author*. Tel.: +886 2 2391 9098; fax: +886 2 2391 9098.
E-mail address: jhguh@ha.mc.ntu.edu.tw (J.-H. Guh).
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induce apoptosis in numerous types of cancer cells [2,3]. Increasing evidence suggests that the anticancer effects of flavonoids result from various mechanisms, including the regulation of cell cycle progression [3], inhibition of kinase and protease activities [4,5], suppression of the secretion of matrix metalloproteinases [6] and inhibition of the induction of activator protein-1 activity [7].

Human hepatocellular carcinoma (HCC) is a very common malignancy and is highly chemoresistant to currently available chemotherapeutic agents [8]. In a large screening test, we found that numerous flavonoids could prevail over the resistant capacity and display effective anti-proliferative activities in HCC. In this study, 16 flavonoids of several classes, including flavones, flavonols, flavanones, flavanols, and isoflavones, were used to examine their anticancer effects in HCC. Moreover, the anticancer mechanisms were investigated based on three predominant apoptosis pathways, such as mitochondria-mediated intrinsic pathway, death receptor-induced extrinsic pathway and the apoptotic signaling evoked by endoplasmic reticulum (ER) stress [9–11]. Caspases are intracellular cysteine protease responsible for and associated with these three apoptosis pathways. The intrinsic apoptotic pathway is a mitochondria-involved signaling cascade in which caspase-9 is the predominant initiator caspase. In the presence of ATP, the association of procaspase-9 with cytochrome c and the adaptor molecule apoptotic protease-activating factor 1 (Apaf-1) and oligomerization of this complex cause the activation of caspase-9 [9,10]. With contrast, the extrinsic apoptotic pathway is mediated by death receptors, such as the receptors for Fas ligand (FasL) and tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), and caspase-8 is a major initiator caspase in this pathway [9,10,12]. Furthermore, it has been suggested that there is cross-talk between the intrinsic and extrinsic apoptosis pathways [12,13]. Recent studies identify the ER as a third subcellular compartment implicated in apoptotic execution [11]. The ER, which is the site for folding and assembly of proteins, lipid biosynthesis, vesicular traffic, and cellular calcium storage, is sensitive to alterations in homeostasis. Several stimuli, such as the expression of misfolded proteins, glucose deprivation, misrepresented glycosylation, and perturbation in calcium homeostasis, can disrupt ER homeostasis and, subsequently, induce ER stress [11,14]. Notably, it has been suggested that ER stress and the associated activation of NF-κB, ATF-6 and mitogen-activated protein kinases (MAPKs) may contribute to hepatocarcinogenesis [15,16]. However, recently the trigger of ER stress as an anticancer strategy has been suggested in a variety of tumor types including HCC [17,18]. Furthermore, increasing evidence suggests that the ER stress precedes the mitochondria event in HCC responsive to apoptotic stimuli [18,19]. To date, there are few studies delineating flavonoid-mediated anticancer mechanisms in HCC based on the aforementioned three apoptosis pathways. In this study, several biological assays and techniques were used and the apoptosis pathways were examined to identify the anticancer mechanisms of flavonoids. To our knowledge, this is the first report that the intrinsic, extrinsic and ER stress-mediated apoptosis pathways are investigated in flavonoid-induced apoptosis in human hepatocellular carcinoma cells.

2. Materials and methods

2.1. Materials

FBS and penicillin/streptomycin were obtained from GIBCO/BRL Life Technologies (Grand Island, NY, USA). Antibodies to Bid, caspase 7, 8 and 9 were from Cell Signaling (Beverly, MA, USA). Anti-Bcl-xL antibody was from Upstate. Antibodies to other Bcl-2 family member proteins and horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies were from Transduction Lab (Lexington, KY, USA). Antibodies to caspase 3 and GADD153 were from IMGENEX (San Diego, CA, USA) and Affinity BioReagents (Golden, CO, USA), respectively. Antibody to caspase-12 was from Abcam Limited (Cambridgeshire, UK). 2′,7′-Dichlorofluorescin diacetate (DCF-DA) was from Molecular Probes (Eugene, Oregon, USA). RPMI-1640 medium, phenylmethylsulphonylfluoride, leupeptin, aprotinin, glycerophosphate, NaF, sodium orthovanadate, sulforhodamine B (SRB), Hoechst 33342, etoposide (VP-16) and all of the flavonoids were obtained from Sigma Chemical (St. Louis, MO, USA). The purities of the flavonoids are also obtained: quercetin, ≥98%; (−)-epigallocatechin-3-gallate (EGCG), ≥95%; diosmin, ≥95%; genistin, ≥99%; genistein, ≥98%; epicatechin, ≥98%; catechin, ≥98%; hesperidin, 80%; hesperetin, ≥95%; naringin, ≥90%; naringenin, 95%; rutin, ≥95%; myricetin, ≥85%; gossypin, ≥90%; phloridzin, ≥99%; daidzein, ≥98%.

2.2. Cell cultures

Hep3B and HepG2 cells (American Type Culture Collection) were cultured in RPMI-1640 medium supplemented with 10% FBS (v/v) and penicillin (100 U/ml)/streptomycin (100 μg/ml). Cultures were maintained in a humidified incubator at 37 °C in 5% CO_2/95% air.

2.3. SRB assay method

Cells were seeded in 96-well plates in medium with 5% FBS. After 24 h, cells were fixed with 10% trichloroacetic acid (TCA) to represent cell population at the time of flavonoid addition (T_0). After additional incubation of vehicle (0.1% DMSO) or flavonoid for 48 h, cells were fixed with 10% TCA and SRB at 0.4% (w/v) in 1% acetic acid was added to stain cells. Unbound SRB was washed out by 1% acetic acid and SRB bound cells were solubilized with 10 mM Trizma base. The absorbance was read at a wavelength of 515 nm. Using the following absorbance measurements, such as time zero (T_0), control growth (C), and cell growth in the presence of flavonoid (T_x), the percentage growth was calculated at each of the compound concentrations levels. Percentage growth inhibition was calculated as: $[(T_x - T_0)/(C - T_0)] \times 100$ for concentrations for which $T_x \geq T_0$. Growth inhibition of 50% (IC_{50}) is determined at the drug concentration which results in 50% reduction of total protein increase in control cells during the compound incubation.

2.4. Immunoprecipitation assay

After treatment with vehicle (0.1% DMSO) or compound, cells were washed twice with ice-cold PBS, lysed in 700 μl ml of lysis