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Release of cytochrome c in MCF-7 cells treated with 7, 3', 5'-trihydroxyflavanone of *Hydnophytum formicarium*

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ABSTRACT

Cytochrome c is a central protein that plays a role in triggering apoptosis in cell induced by any agent either drug or plant extracts. Here, we present data of an active antiproliferative agent 7,3',5'-trihydroxyflavanone (3HFD) that is elucidated from the plant *Hydnophytum formicarium* showed to trigger cytochrome c release in treated MCF-7 cell. By performing Western Blot, cytochrome c was seen to be elevated throughout the experiments. The increase level of cytochrome c was confirmed by ELISA and remarked as the beginning of the caspase cascade without altering caspase-8 level. In conclusion, the 3HFD seems to significantly induced apoptosis via mitochondria pathway as reflected by up regulation of cytochrome c.

Key words: Cytochrome c, MCF-7 cels, *Hydnophytum formicarium*.

INTRODUCTION

Cytochrome c is an ancient protein, developed early in the evolution of life. The familiar function of cytochrome c, is its role as a carrier of electrons (Reed, 1997). It is a small, mobile molecule that shuttles electrons through the last step of aerobic energy production. These electrons are obtained from the dATP, which are shuttled through a series of proton pumping proteins (Yang *et al.,* 1997). Cytochrome c shuttles these electrons in the narrow space between the two mitochondrial membranes. It diffuses from protein to protein, picking up electrons from one huge membrane-bound complex and placing them at their final destination on another (Liu *et al.,* 1996).

Cytochrome c and the mitochondria play a central role in apoptosis, signaling the cell to begin the process of programmed cell death (Goldstein *et al.,* 2005a; Goa *et al.,* 2001). Apoptosis is triggered when something is amiss with the cell: DNA damage, detachment from neighbors, growth factor deprivations, infection, or a host of other signs. The cell then initiates one or more cascades of signaling proteins that spread the message through the cell and ultimately orchestrate a controlled self-destruction. Apoptosis is essential in many natural processes, such as the coordinated growth and selective pruning that shapes a growing embryo. If the system is corrupted, however, the consequences are dire, leading to degenerative diseases if overactive and allowing the growth of cancers if blocked (Goldstein *et al.,* 2005b). Although studies of plant-derived anti-cancer agents are fast-progressing, but the precise mechanism of plant-derived agents on the inhibition of cancer cell growth is still not completely understood. Previous studies reported that cytochrome c play as central role in apoptosis regulation, and contribute significantly to the pathogenesis of cancer.

In this study, we tested the 7, 3', 5'-trihydroxyflavanone (3HFD) that is elucidated from the plant *Hydnophytum formicarium* (Hasmah et al. 2008) of the rubiaceae family which is native to Malaysia and Indonesia (Huxley, 1992) for cytochrome c signaling on human breast cancer cell line, MCF-7. This compound was reported to exert potent antiproliferative activity towards MCF-7 cell without affecting normal cell line, MDBK (Hasmah et al. 2008). Previously chloroform extract of this plant was reported to have anti multidrug resistant towards mouse lymphoma cell lines transfected with human *mdr1* gene with moderate effect (Hasmah et al. 2004). Here in this study, we treated MCF-7 cells with the concentration of 3HFD as reported by Hasmah et al. (2008) to observe the expression of cytochrome c with regard to the involvement of procaspase-8. We also performed the ELISA analysis to figure out the concentration of cytochrome c release in treated and untreated control cells.

MATERIALS AND METHOD

Cell culture

MCF-7 human mammary carcinoma cells were obtained from the American Type Culture Collection (ATCC) and maintained in DMEM supplemented with 10% fetal bovine serum and 2 mM glutamine (Lee *et al.,* 2003).

Isolation of cytosolic fractions

Cytosolic extracts were prepared as previously described by Yang et al. (1997). Briefly, treated cells were harvested by centrifugation and washed with ice-cold phosphate-buffered saline and re-suspended in 5 volumes of extraction buffer containing 250 mM sucrose. Cells were homogenized and the homogenates were centrifuged twice at 750 × g for 10 min at 4°C. The supernatant was then centrifuged at 10,000 × g for 15 min at 4°C, and the resulting mitochondrial pellets were discarded. The supernatant was then dissolved in electrophoresis sample buffer and used for Western blotting.

Western Blotting

After electrophoresis, proteins were blotted onto polivynyl-difluoride membranes (PolyScreen, Nen Life Science). Membranes were dried, pre-blocked with 5% non-fat milk in phosphate-buffered saline and 0.1% Tween-20, then incubated with a primary antibody for caspase-8 and cytochrome *c* (Clone 7H8.2C12) (all from Pharmingen), and detected with horseradish peroxidase-labeled antibodies to rabbit or mouse IgG. Following exposure on a Kodak BIOMAX x-ray film, densitometry analysis was done with a GS 670 Imaging Densitometer with the software Molecular Analyst (Bio Rad). Blots were stripped with Re-Blot Plus (Chemicon) before reprobing with β-actin antibody to determine equal loading.

ELISA analysis of cytochrome c

Quantification of cytochrome c concentration was assayed by means of ELISA analysis according to User Protocol QIA74 provided in the test kit by Qiagen. The assay was done according to the manufacturer's instructions. Briefly, MCF-7 cells were treated with 3HFD at 9 µg/ml 3HFD for 0, 3, 6, 12 and 24 in 5% CO_2 at 37 °C. Control were treated with 1% DMSO. After the treatment period, cells were counted and then pelleted at 1,500 rpm for 10 min. Cells were then re-suspended in chilled Cell Lysis Buffer and incubated at room temperature before centrifugation at 1000 × g for 15 min. The supernatant (cytosolic extract) was then diluted for 5 times and assayed immediately. Alternatively, supernatant could be aliquot and stored at -80 °C. The protein concentration for each sample set was then assayed using standard protocols. Assay mixture was prepared in a 96-well plate and mixed with calibrator diluents, sample and standard and shielded with provided plastic cover and left for 2 hours at room temperature. After 2 hours, 96 well plates were washed with wash buffer and dried. Lastly, cytochrome c conjugate solution were added and incubated for 2 hours, washed and mixed with substrate solution for 30 minutes followed by adding stop solution and mixed well. The mixture was read with a Dynex MRX microtiter plate reader at 450 nm and 540 nm. The concentrations were determined from regression equation of standard curve times dilution factor.

RESULTS

During apoptosis, initiator caspases are activated in response to proapoptotic signals (Diaz

et al., 2003). By SDS-PAGE and subsequent Western Blot analysis with a caspase-8 specific antibody, it was found that 3HFD treatment did not lead to the activation of the initiator caspase-8. Procaspase-8, expressed in two functionally active isoforms, caspase-8a and caspase-8b (Diaz *et al.,* 2003) was not processed. From immunoblotting, the two bands observed were the 55/50-kDa procaspase-8 isoforms (Figure 1) similarly as reported by Sun *et al.* (1999) and Dirsch *et al.* (2001) and the active p18 subunit could not be detected. As processing of this caspase did not occur, it is possible that the other initiator caspase, caspase-9 may be involved in 3HFD-induced apoptosis.

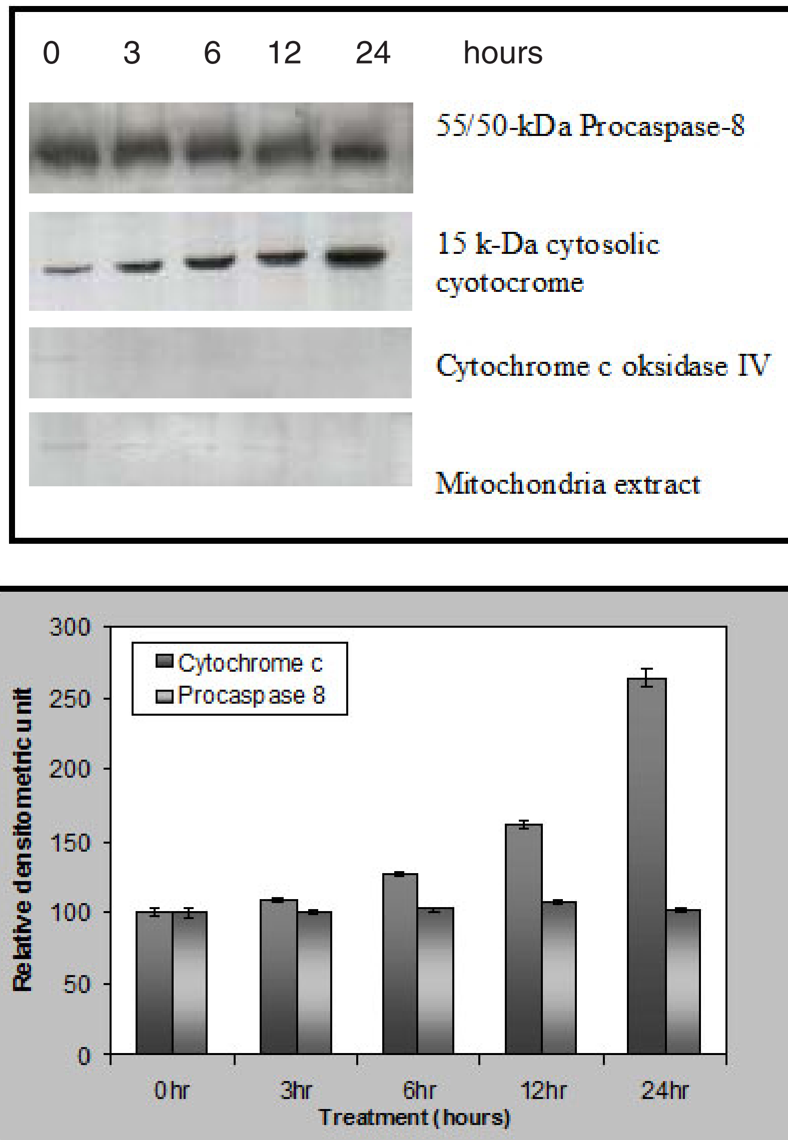

Fig. 1: Proteins from MCF-7 cells treated with 9μg/ml for the indicated times were resolved on 12% SDS-PAGE and submitted to Western Blotting with an anti-procaspase-8 antibody. Two bands were observed, corresponding to the uncleaved 55/50-kDa procaspase-8 isoforms. The active p18 subunit was not detected. The cytosolic fractions of MCF-7 cells treated with 9μg/ml were also resolved on 15% SDS-PAGE and submitted to immunoblotting with the cytochrome *c* antibody (Clone 7H8.2C12). Increasing amounts of cytochrome *c* were detected in the cytosol in a time-dependent manner. All blots were then washed and re-probed with â-actin to confirm equal loading. The concentration value represented as means ± SEM, $P<0.05$ of three independence experiments.

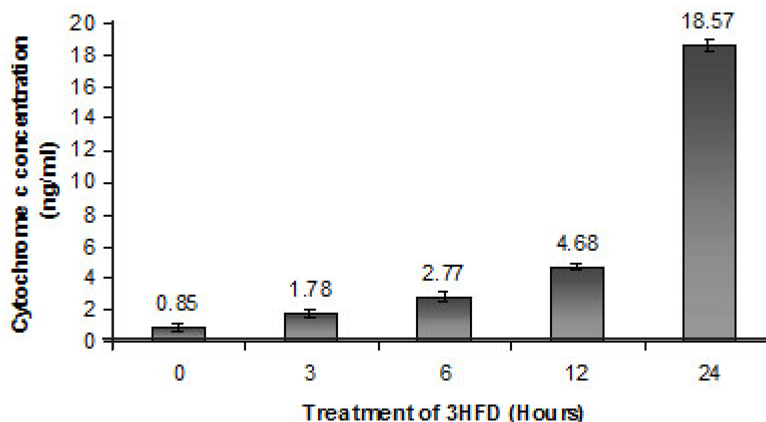

Fig. 2: MCF-7 treated with 9µg/ml 3HFD was assayed for cytochrome c concentration. Level of cytochrome c increased as measured with ELISA method using ELISA Cytochrome c kit (QIAGEN) in time dependence manner. The concentration value represented as means ± SEM, $P<0.05$ of three independence experiments.

Cytochrome *c* seems to be a major trigger for the assembly of this complex, and various studies have found that cytochrome *c* is released from the mitochondria into the cytosol during cell death (Li et al. 1997). When cytochrome *c* levels in the cytosol were examined, we detected increasing levels in the 3HFD-treated MCF-7 cells (Figure 1). Untreated control cells did not exhibit similar high levels of cytochrome *c*, indicating that the release of cytochrome *c* from the mitochondria into the cytosol was an effect of 3HFD treatment.

We further examined the concentration of cytochrome c by means of enzymatic assay through the same experimental period. The level of cytochrome c concentration increased in time dependence manner. This result supported the Western Blot analysis and high peaks were observed at 24 hours treatments (Figure 2).

DISCUSSION

Western Blott and ELISA analysis has evidenced the importance of cytochrome c in MCF-7 treated 3HFD apoptosis. These data provided basis for 3HFD mechanism of action through mitochondria pathway. Release of cytochrome c will triggered a formation of activation of caspase cascade to produce apoptotic characteristic such as nuclear condensation, substrate degradation and fragmentation DNA as suggested by Slee et al. (1999). Slee et al. (1999) also reported that cytochrome c involves in activation of downstream caspases (kaspase-2, -8 -9 dan -10), and upstream caspases (kaspase-3, -6 dan -7). Liu et al. (1996) concluded that cytochrome c will trigger the processing of casopase 3. Reed (1997) suggested that cytochrome c is a central role of apoptosis that released from mitochondria. and function as central controller of apoptosis. The exact mechanism involved in cytochrome c release is still undiscovered (Renz *et al.,* 2001).

Determination of cytochrome c placement is also important to support this apoptosis precursor through Western blot analysis. In this study, we evaluated the concentration of cytochrome c in treated cells using ELISA assay to support the elevated expression in Western Blot analysis. Han et al. (2003) reported the evidenced of cytochrome c in cell cytosol through immunoflourescene libeling with FITC cytochrome c specific antibody. Luetjens et al. (2001) reported the multiple kinetic release of cytochrome c in MCF-7 with caspase-3 drug induced apoptosis (staurosporin and valiomysin) and tumour necrosis factor TNF-alfa. Bax or Bid from Bcl-2 family was evidenced to influence the cytochrome c release from mitochondria through the phosphorilation process (Jürgensmeier *et al.,* 1998; Li *et al.,* 1998; Luo *et al.,* 1998). Smac/Diablo also reported to influence the cytochrome c release (Carson *et al.,* 2002; Kandasamy *et al.,* 2003) in prostate cancer cell (Ln CaP). This is due to the needs of Smac to combat epidermal growth inhibitor.

Caspase-8 was reported to influence cytochrome c release through Bid processing and triggered the activation of caspase 9 (Granville *et al.,* 1999). However, in this study, caspase-8 was not processed. This finding supported study by Scaffidi et al. (1999) which stated that caspase-8 was not activated in early stage of caspase activation in CD95 signaling. This is because the existing of two types of different cell which were: type I involve caspase-8 activation at early stage and type II occurred at late stage of aggregation involved mitochondria. Dirsch et al. (2001) and Tang et al. (2000) also reported the inactivation of procaspase 8 in MCF-7 treated with helenalin and staurosporin respectively.

Previous reports have found that MCF-7 cells are relatively insensitive to many chemotherapeutic agents due to the absence of caspase-3 (Yang *et al.,* 2001). Our studies here have shown that the mechanism for apoptosis is functional in MCF-7 and 3HFD is able to trigger cytochrome c release through mitochondria pathway. Therefore, finding new therapeutic agents that induce tumor cells apoptosis in a manner independent of caspase-3 with promoting cytochrome c release may have important clinical implications. By releasing cytochrome c without requiring caspase-3, 3HFD may evoke an apoptotic pathway different from clinical oncology drugs such as doxorubucin and etoposide (Yang *et al.,* 2001) thus making it a promising agent for combination chemotherapy that merits further study.

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