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Induction of apoptosis in human hepatoma cells by mycelia of *Antrodia camphorata* in submerged culture

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Abstract

The effect of methanolic extracts of mycelia (MEM) from *Antrodia camphorata* (Polyporaceae, Aphyllophorales) of submerged culture (ACSC) on the inhibition of cell viability and the mechanism of MEM-induced cytotoxic in hepatoma cells were investigated. The IC_{50} of MEM on the cytotoxicity of HepG2 (wild type p53) and Hep3B (delete p53) were 49.5 and 62.7 μg/ml, respectively, on 48 h incubation. There is no observable cytotoxicity of MEM in Chang liver cells and rat primary hepatocytes at the concentration of 100 μg/ml. Cell cycle analysis revealed that MEM induced apoptosis on HepG2 via G0/G1 cell cycle arrest. MEM (100 μg/ml) treated HepG2 and Hep3B for 72 h, the apoptotic cells were 98.3 and 39.5%, respectively. The activities of caspase-3, -8 and -9 in HepG2 induced by MEM (50 μg/ml) were increased 5.3, 6.7 and 2.2-fold, respectively. MEM-induced apoptotic cell death was accompanied by up-regulation of caspase-3 and -8 in HepG2 cells. Combined treatment with MEM and caspase-3, -8 and -9 inhibitors, the caspase-3 and -8 inhibitors were accounting for 63 and 47% inhibition in MEM-induced apoptosis, respectively; however, caspase-9 inhibitor exhibited no obvious inhibition effect on the apoptosis percentage ($p > 0.05$). The results indicated that MEM induced HepG2 apoptosis through activation of caspase-3 and -8 cascades and regulation of the cell cycle progression to inhibit hepatoma cells proliferation.

Keywords: *Antrodia camphorata* in submerged culture; Apoptosis; Hepatoma cells; Cell cycle; Caspases

1. Introduction

Cell death may occur by two different death mechanisms, necrosis and apoptosis. Necrosis is accompanied by inflammation. Apoptosis is usually induced by receptor-regulatory signal such as hormone, cytokines, and growth (Hayashi, 1998). The characteristic features of apoptosis have been studied extensively.

In the last few decades, mushrooms have increasingly been used as a source of therapeutic agents for several types of cancer in human or health food supplements (Hong and Sporn, 1997; Agarwal et al., 2002). Mushrooms have been proved to express promising antitumor, immune modulating, cardiovascular and hypercholesterolemia, antivival, and antiparasitic effects (Wasser and Weis, 1999). It has been reported that mushrooms showed the cytotoxicity effects in cancer cells by inducing cells apoptosis which regulated of cell cycle progression (Fujimiya et al., 1998; Hsieh et al., 2002). Han et al. (1999) have reported that *Coprinus disseminatus* (Per.: Fr.) S.F. Gray mycelial culture broth extract could induce apoptosis by activation caspase-3 activity in human cervical carcinoma cells. Furthermore, a microbial secondary metabolite, Tetrocarcin A, inhibited mitochondrial functions regulated by Bcl-2, resulting in Fas-triggered mitochondrial transmembrane potential loss and cytochrome c release to regulate the activation of caspase-3. Experiments in animal models have provided direct evidence of the protective effects of mushrooms on tumor development (Mizuno et al., 1990; Mizuno, 1999). Caspases were down stream executioner of

Abbreviations: MEM, methanolic extracts of mycelia; ACSC, *antrodia camphorata* of submerged culture

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apoptosis, which may occur by cell cycle arresting, the death receptor (DR) and mitochondrial pathways. Thus, caspases activation was regard as an important indicator by inducing apoptosis.

Antrodia camphorata (Polyporaceac, Aphyllophorales), a parasitical microorganism on the inner cavity wall of local evergreen *Cinnamomm kanchirai* Hay (Lauraceae), is a species known to be available only in Taiwan. It was initially identified by Zang and Su as a new *Ganoderma* species in 1990 (Zang and Su, 1990), but was recently identified as a new basidomycete *Antrodia camphorata* in the Ployporaceae (Wu et al., 1997). This species is well known in Taiwan under the name of "niu-chang-chih" or "niu-chang-ku", and is also popular there. However, because its host wood is a local species that is getting scarce, *Antrodia camphorata* is difficult to find in the forest and is also very expensive. Because of the similarity of *Antrodia camphorata* and *Ganoderma* species in several characteristics, *Antrodia camphorata* is said to have many medicinal uses, such as remedy for food, alcohol and drug intoxication, diarrhea, abdominal pain, hypertension, skin itches and liver cancer among Chinese (Tsai and Liaw, 1985). Since the growth rate of the natural *Antrodia camphorata* in the wild is very slow, and it is difficult to cultivate in green house, thus, it is expensive to obtain fruiting bodies. Therefore, using a submerged cultured method to obtain useful cellular materials or to produce effective substances from cultured mycelia, might be a possible way to overcome the disadvantage of the retardant growth of fruiting bodies (Sone et al., 1985).

In our previous studies (Song and Yen, 2002, 2003), *Antrodia camphorata* of submerged culture (ACSC) had antioxidant activity in vitro and in vivo. Hseu et al. (Hseu et al., 2002) reported that mycelial aqueous extract from ACSC could decrease cell viability in HL-60 but not the human umbilical vein endothelial cells. However, the anti-tumor ability of MEM has not yet been studied on apoptosis mechanism and comparing with the fruiting bodies of *A. camphorata*. The objectives of this study were to investigate the cytotoxicity effect of MEM in hepatoma cells (HepG2 and Hep3B) and to elucidate its possible regulation pathway of apoptosis.

2. Materials and methods

2.1. Materials and chemicals

Antrodia camphorata An5 in submerged culture (BCRC 930032), human hepatocyte (Chang liver cell, BCRC 60024) and human hepatoma cell line (HepG2, BCRC 60025; Hep3B, BCRC 68001) were purchased from Food Industry Research & Development Institute, Hsin Chu, Taiwan). Caspase activity assay kits, including the substrates of caspase-3 (DEVD), caspase-8 (LETD) and caspase-9 (LEHD) were purchased from Intergen Co. (Burlington, MA). Anti-caspase-3 and anti-caspase-8 antibodies were purchased from Pharmingen (San Diego, CA). Caspase-3/CPP32 inhibitor (z-DEVD-FMK), caspase-8/FLICE inhibitor and caspase-9 inhibitor (z-LEHD-FMK) were purchased from KAMIYA BIOMEDICAL Co. (Seattle, WA). 4′,6′-Diamidino-2-phenylindole (DAPI) was obtained from Sigma Chemical Co. (St. Louis, MO). Male Sprague-Dawley rats (180 ± 20 g) were obtained from National Laboratory Animal Breeding and Research Center (Taipei, Taiwan).

2.2. Culture conditions of Antrodia camphorata

Antrodia camphorata hyphae were separated from the fruiting bodies and was inoculated into a culture medium which was composed of 2.5% corn starch, 2% sucrose, 0.5% yeast extract, 0.1% KH_2PO_4, 0.3% $MgSO_4$, 0.3% $(NH_4)_2SO_4$ and 0.05% citric acid in distilled water, and adjusted to initial pH range of 5.3–5.5. Each shaking flask culture was carried out in a 2 L Erlenmeyer flask containing 1 L of medium and incubated at 27–30 °C for 7 days. Thereafter, 3.5 L of shaking flask cultures was inoculated into a 500 L fermented tank containing 350 L of culture medium, and then cultured at 27–30 °C for 7 days with a 0.5 vvm aeration rate (aeration volume/medium volume (L)/min) by shaking at 50 rpm with a rotary shaker to obtain a mucilaginous medium containing mycelia. Residual sugar concentration detected by using the phenol-H_2SO_4 method was about 0.1 g/L after cultivation for 7 days. The mycelia were collected by means of centrifugation (4 °C, 8000 rpm for 15 min) and then washed with distilled water. Finally, the mycelia were freeze-dried to a powder form. The yield of mycelia in submerged culture was 1.1 g of dry weight of mycelia/100 g of ACSC.

2.3. Preparation of mycelial extracts from ACSC

The methanol extracts of mycelia (MEM) were obtained by extracting freezing-dry mycelia (10 g) with methanol (2 L) three times under 30 °C. The extracts were filtered through Whatman No. 2 filter paper, and then concentrated to dryness. The yield of MEM was 3.0 g.

2.4. Cell culture and cell viability assay

Chang liver cells and human hepatoma HepG2 and Hep3B cells, were maintained in Dulbecco's modified Eagle's medium (Flow Laboratories), supplemented with 10% fetal bovine serum, 2 mM glutamine and antibiotics (100 U/ml pencillin and 100 μg/ml streptomycin), at 37 °C in a humidified atmosphere of 5% CO_2. The medium was changed every 2 days. Cells were seeded at a density of 1×10^5 cells/well onto a 12 well-plate (FALCON, Becton Dickinson, NJ, USA) 24 h prior to drug treatment. MEM was added to medium, at various times and concentrations. After incubation, cells were washed with phosphate buffered saline (PBS). Morphological changes were observed after the addition of MEM using a phase-contrast inverse microscope (IMT-2, Olympus Co.