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Selective induction of apoptosis in murine skin carcinoma cells (CH72) by an ethanol extract of *Lentinula edodes*

Yu-Huan Gu[a], Martha A. Belury[b,*]

[a]*Bastyr University, 14500 Juanita Drive NE, WA 98028, USA*
[b]*Department of Human Nutrition, The Ohio State University, 1787 Neil Avenue, 325 Campbell Hall, Columbus, OH 43210, USA*

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Abstract

The effects of ethanol extracts from four species of mushroom fruiting bodies, mushroom spores and mushroom cultured broth, were assessed for modulation of cell proliferation and apoptosis in murine skin carcinoma cells (CH72) and non-tumorigenic epidermal cells (C50). While extracts from mycelia of *Grifola frondosa*, *Ganoderma lucidum*, *Hericium erinaceus*, or from spores of *G. lucidum* exerted little, if any, effect on proliferation, the ethanol-soluble extract of *Lentinula edodes* (*L. edodes*) significantly decreased cell proliferation of CH72 cells. There were no changes in the proliferative response of the non-tumorigenic keratinocyte cell line, C50, to any of the mushroom extracts tested. To analyze cell proliferation and apoptosis, fluorescent DNA-microscopy with ethidium bromide and acridine orange staining of cells revealed *L. edodes* reduced cell proliferation and induced apoptosis in time- and dose-dependent manners in carcinoma cells but had no effect in non-tumorigenic cells (C50). Cell cycle analysis demonstrated that *L. edodes* extract induced a transient G_1 arrest, with no changes observed in the non-tumorigenic cells (C50).

Keywords: Cell proliferation; Apoptosis; *Lentinula edodes*; Cell cycle arrest; Skin cancer; Shiitake mushroom

1. Introduction

For centuries, medicinal properties of edible mushrooms have been used for treating various health conditions including cancer, immunity, viral infections, and bacterial infections [1]. Since Ikekawa's group first noted that a water extract of shiitake fruiting bodies could inhibit transplanted tumors in mice in 1969 [2], the anti-tumor property of mushrooms has been reported in many edible species of mushrooms, such as shiitake (*Lentinula edodes* or *L. edodes*), maitake (*Grifola frondosa*), Turkey tail (*Coriolus versicolor*), reishi (*Ganoderma lucidum*), and lion's mane (*Hericium erinaceus*). Polysaccharides derived from mushrooms are considered one of the potent anti-tumor constituents in vitro and in animal studies [3]. In recent years, clinical trials using medicinal mushrooms or mushroom polysaccharides for the treatment of various types of cancers have achieved some success in

* Corresponding author. Tel.: +1-614-292-1680; fax: +1-614-292-8880.
E-mail address: belury.1@osu.edu (M.A. Belury).

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extending survival of cancer patients and/or improving the quality of life in patients with advanced cancer [4,5]. The possible role of mushrooms to prevent cancer has been summarized in several recent review studies [1,6]. Cancer inhibition by mushrooms or mushroom polysaccharides was generally achieved through activating immune responses of the host rather than directly altering tumor cell growth. However, information is lacking of: (i) the identity of bioactive compounds; and (ii) putative mechanisms of action to influence growth properties of tumor cells. Before effective therapeutic treatments are available to cancer patients, research is needed to address the mechanisms of action of mushrooms as anti-cancer therapeutic agents.

Currently, most drugs intended for cancer therapy are not specific to target cancer cells and therefore may be highly toxic to normal, surrounding tissues. Toxicity due to chemotherapy leads to the many serious adverse effects resulting in morbidity and mortality not just due to the cancer but to the chemotherapy. 'Designer drugs' selectively act on cancer cells the development of which represents a major effort of the pharmaceutical industry. Our study compares an ethanol-soluble extract from *L. edodes* to extracts from other edible mushrooms (e.g. maitake (*G. frondosa*), reishi (*G. lucidum*) and lion's mane (*H. erinaceus*)) for their effects in either a tumorigenic or non-tumorigenic keratinocyte cell line for modulating cellular (i) growth; (ii) apoptosis; and (iii) cell cycle progression. We found that *L. edodes* selectively induced apoptosis and cell cycle arrest in the G_1 phase in the mouse skin cancer cell line, CH72, with no deleterious effects on non-cancerous mouse epidermal cells, C50.

2. Materials and methods

2.1. Cell culture and mushroom extracts

Ethanol-based extracts of four species of mushrooms, *G. frondosa*, *G. lucidum*, *H. erinaceus*, and *L. edodes* were obtained from Fungi Perfecti, LLC (Olympia, WA). The same amount of fresh cultured mycelia from each species of mushrooms were soaked in water/ethanol (2:1) and then combined with hot water extracts obtained from boiling the same species of mushrooms for several hours (Paul Stamets, personal communication). Spore extract of *G. lucidum* was extracted by boiling 1 g of spore powders in 5 ml of 30% ethanol for 1 h and then centrifuging at 750*g* for 5 min. A cultured broth was collected by liquid culturing *G. lucidum* mycelia in 2% of corn meal broth for 72 h at 28 °C under shaking conditions. All extracts were sterile filtered and stored at −20 °C.

Multistage mouse skin carcinogenesis induced by chemicals is a well-characterized model for studying epithelial cancers [7]. The mouse skin carcinoma cell line, CH72, and the non-tumor cell line, C50, were generous gifts from Dr Susan Fischer (University of Texas, M.D. Anderson Cancer Center, Smithville, TX). The CH72 cell line was derived from a squamous cell carcinoma that resulted from 7,12-dimethylbenz-[a]-anthracene initiation and TPA promotion of SENCAR mice [7]. Cell lines were cultured in modified Eagle's Minimal Essential Medium [8] supplemented with 1% fetal bovine serum at 37 °C in a humidified atmosphere containing 5% CO_2. Mushroom extracts were mixed with fresh conditioned medium (serum-free) and introduced to cell culture plates after cells reached ~70% confluence.

2.2. Cell proliferation

Cell density of CH72 was determined following exposure to mushroom extracts for evaluating the potential of mushroom extracts to modulate cell proliferation. About 1×10^6 CH72 cells were seeded onto plates (60 mm diameter) and the conditioned medium mixed with mushroom extracts in 30% ethanol (4 μl per ml of conditioned medium) was then added to the cells. The same volume (4 μl) of 30% ethanol was premixed with per ml of conditional medium to equal a final dilution of ethanol in media of 0.12% (v/v). All plates were continuously incubated at 37 °C, 5% CO_2 for 15 h or time points shown.

Cell growth of both CH72 and C50 cells was determined using two methods, trypan blue exclusion and CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI). For trypan blue exclusion, a cell suspension of (1×10^5/ml; 0.5 ml) was mixed with 0.4% trypan blue stain (0.1 ml, Sigma). Two samples were counted for each suspension and three replicates were performed for each treatment. Viable cells were measured over time using