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Hericium erinaceus enhances doxorubicin-induced apoptosis in human hepatocellular carcinoma cells

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ABSTRACT

It has been demonstrated that the *Hericium erinaceus* (HE) mushroom, which primarily consists of polysaccharides, possesses anti-tumor activities. However, the mechanisms by which HE inhibits human hepatocellular carcinoma growth remain unknown. Our study demonstrates that HE acts as an enhancer to sensitize doxorubicin (Dox)-mediated apoptotic signaling, and this sensitization can be achieved by reducing c-FLIP expression via JNK activation and enhancing intracellular Dox accumulation via the inhibition of NF-κB activity. These findings suggest that HE in combination with Dox serves as an effective tool for treating drug-resistant human hepatocellular carcinoma.

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the fourth leading cause of cancer-related mortality worldwide, with the highest incidence in Asia [1]. Despite surgical management and application of non-surgical therapeutic modalities, the incidence of HCC continues to rise [2]. Doxorubicin (Dox) is an effective chemotherapeutic drug for the treatment of patients with HCCs [3]. However, Dox has been shown to cause high systemic toxicity to normal tissues, as well as immunosuppression, secondary cardiomyopathy, and development of drug resistance during cancer therapy, thereby limiting successful outcomes of cancer chemotherapy [4,5]. Accordingly, several new therapeutic strategies are being developed to treat cancer. Recently, combination (rather than single-agent) chemotherapy has been found to be a superior treatment strategy that offers the potential for lowering the dose of chemotherapeutic drugs to reduce side effects. A related strategy is to evaluate the mechanisms of natural medicines [6,7]. Many studies have demonstrated that polysaccharides from basidiomycetes mushrooms had highly beneficial therapeutic effects including: (1) preventing oncogenesis after administration of peroral medications developed from these mushrooms or their extracts, (2) direct anti-tumor activity against various tumors, (3) synergistic anti-tumor activity in combination with chemotherapy, and (4) preventive effects on tumor metastasis [8–11]. In addition, unlike existing cancer chemotherapeutic drugs, mushroom-derived polysaccharides are known to have no toxic side effects. Most of the related clinical evidence was determined for the commercial polysaccharides lentinan (*Lentinus edodes*), krestin (*Coriolus versicolor*), and schizophyllan (*Schizophyllum commune*) [12]. Data on polysaccharides isolated from *Hericium erinaceus* (HE) is highly impressive, and these polysaccharides show promise for use as cancer therapeutics. Studies have shown that HE extracts strongly suppress the growth of various tumors *in vitro* and *in vivo* [13,14]. However, the molecular signaling involved in HE-mediated anti-tumor activity has never been investigated in HepG2 human hepatocellular carcinoma cells.

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Apoptosis, or programmed cell death, is an important physiological process of cell death and occurs during tissue remodeling, immune regulation, and tumor regression. Most of the chemotherapeutic agents kill cancer cells by inducing apoptotic death pathways [15]. Cells undergoing apoptosis show a sequence of cardinal morphological features, including membrane blebbing, cellular shrinkage, and condensation of chromatin [16]. The two primary apoptotic pathways are the extrinsic death receptor-mediated pathway and the intrinsic mitochondria-mediated pathway. These two pathways, when stimulated, lead to the release of cytochrome c from the mitochondria and to the activation of the death signal [17]. Apoptotic signaling and execution through these two pathways depends on caspases, or aspartate-specific cysteine proteases, which are the key effector molecules in the apoptotic process [18]. It has been suggested that Dox, an anticancer agent, elicits a caspase cascade, leading to apoptosis [19,20]. A member of the mitogen-activated protein kinase (MAPK) family, c-Jun NH_2-terminal kinase (JNK), is rapidly phosphorylated and subsequently activated by a diverse spectrum of different cell stimuli. The JNK pathway has been shown to be closely linked to apoptosis by activation of the mitochondria-mediated apoptosis pathway and to regulate the degradation of cellular Fas-associated death domain (FADD) interleukin-1 β-converting enzyme (FLICE)-like inhibitory protein (c-FLIP), which is an important caspase cascade inhibitor [21–23].

To improve chemotherapeutic treatment, much effort has been made to identify chemosensitisers; that is, agents that are able to overcome multi-drug resistance (MDR) [24]. Interestingly, apart from MDR, nuclear factor κB (NF-κB) has been shown to be involved in chemoresistance in different cell types, suggesting a possible role of this transcription factor in the chemosensitising effects of agents [25].

Although there are many therapeutic strategies to treat cancer, including chemotherapy, high systemic cytotoxic toxicity and drug resistance have limited the successful outcomes in most cases. Recently, TNF-related apoptosis-inducing ligand (TRAIL) and non-steroidal anti-inflammatory drugs (NSAIDs) have been found to sensitize Dox-induced apoptosis in various types of tumors [26–28]. In addition, the combination treatment of Dox and natural products dramatically augments the therapeutic effects against breast cancer, prostate cancer, and hepatocellular carcinoma [29,30].

In this study, we investigated the ability of HE to synergize the inhibitory action of Dox in human hepatocellular carcinoma HepG2 cell lines. We also evaluated the molecular mechanisms underlying this synergistic effect and sought evidence of synergy between Dox and HE.

2. Materials and methods

2.1. Materials

The human hepatocellular carcinoma cell line, HepG2 (ATCC HB-8065), was obtained from Korean Cell Line Bank (Seoul, Korea). Fetal bovine serum, penicillin G, streptomycin, and RPMI 1640 were obtained from GIBCO (Grand Island, NY, USA). Dox, acridine orange, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Cell-permeable inhibitor peptide (NF-κB SN50), Bay 11-7082, and SP600125 were obtained from Calbiochem (La Jolla, CA, USA). General caspase inhibitor z-VAD-fmk was purchased from BD Bioscience (San Diego, CA, USA). All other chemicals were of Sigma grade. Antibodies to caspase 3, p53, phospho-p53, JNK, phospho-JNK, NF-κB p105/p50, NF-κB p65, and horseradish peroxidase (HRP)-linked anti-rabbit IgG were obtained from Cell Signaling (Beverly, MA, USA). Antibodies to cytochrome c, Bax, Bcl-2, c-FLIP, β-actin, and HRP-linked goat anti-mouse IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Mushroom extracts and the chemotherapeutic drug

The crude water-soluble polysaccharide, which was obtained from the fruiting body of HE by hot water extraction and ethanol precipitation, was fractionated by DEAE-cellulose and Sepharose CL-6B column chromatography, as previously reported [31]. The purified components of HE primarily consisted of polysaccharide, which was a low-molecular-mass (13 kDa) 1,3-branched-β-1,6-glucan with a triple helix conformation. HE contained a level of endotoxin below the detection limits (0.0015 EU/ml) as assessed by an E-TOXATE kit (Sigma, St. Louis, MO, USA) [32].

Dox was dissolved in 1× phosphate–buffered saline (PBS) for injection at a concentration of 5 mg/ml and stored in aliquots at 4 °C. Immediately before each experiment, serial dilutions of this drug were performed in culture media to obtain the required final concentrations.

2.3. Cell culture

HepG2 cells were cultured in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained at 37 °C in a 5% CO_2 incubator, and the media were changed twice weekly.

2.4. In vitro cytotoxic assay; colorimetric MTT assay

The cytotoxicity of HE and Dox on the viability of HepG2 cells was measured using the MTT assay, which relies on the ability of viable cells to metabolically reduce the tetrazolium salt MTT to a purple formazan product, which can be quantified colorimetrically [33]. Briefly, cell suspensions (2×10^5 cells/ml) were seeded in 96-well plates and incubated at 37 °C to allow for cell attachment. After 48 h, the cells were treated with serum-free medium containing the indicated additives. The plates were incubated for 1 h (d 0) and 72 h (d 3) at 37 °C. At the end of each exposure time, 50 μl of the MTT stock solution (5 mg/ml) in serum-free medium were added to each well to reach a total reaction volume of 200 μl. After incubation for 2 h at 37 °C, the supernatants were aspirated. The formazan crystals in each well were dissolved in 150 μl dimethylsulfoxide, and the A_{540} was read on a scanning multiwell spectrophotom-