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Cell-cycle arrest and apoptosis induction in human breast carcinoma MCF-7 cells by carboxymethylated β-glucan from the mushroom sclerotia of *Pleurotus tuber-regium*

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Abstract

The mechanism for the anti-tumor activity of a water-soluble carboxymethylated β-glucan (CMPTR), partially synthesized from an insoluble native glucan isolated from the sclerotia of *Pleurotus tuber-regium*, was studied using human breast carcinoma MCF-7 breast cancer cells *in vitro*. CMPTR-induced anti-proliferative activity dose-dependently, with an IC_{50} of 204 μg/ml. CMPTR inhibited the cell proliferation of MCF-7 by arresting the G_1 phase of its cell cycle after 48 h of incubation as shown by flow cytometry. Such G_1 phase arrest was associated with the down-regulation of cyclin D1 and cyclin E expressions in the breast cancer cells. In addition, the CMPTR-treated MCF-7 cancer cells were associated with decreased expression of anti-apoptotic Bcl-2 protein and increased expression of Bax/Bcl-2 ratio. This study shows that CMPTR can inhibit the proliferation of MCF-7 by cell-cycle arrest and apoptosis induction. The potential development of this mushroom polysaccharide as a water-soluble anti-tumor agent requires further investigation.

Keywords: *Pleurotus tuber-regium*; Sclerotia; Carboxymethylation; β-Glucan; Cell-cycle arrest; Apoptosis; Mushroom sclerotia; MCF-7 cells

1. Introduction

It has been shown that mushroom polysaccharides exhibited direct inhibitory effects on cancer cell growth by modulating cell-cycle progression and inducing apoptosis (Wang et al., 2002). A polysaccharide-peptide complex (PSP) extracted from *Trametes versicolor* significantly reduced proliferation of MAD-MB-231 breast cancer cells by increasing p21 expression and decreasing cell-cycle protein cyclin D1 expression (Chow, Lo, Loo, Hu, & Sham, 2003). A protein bound polysaccharide (PBP) isolated from *Phellinus linteus* had an anti-proliferative effect on SW480 human colon cancer cells mediated by inducing apoptosis and G_2/M cell-cycle arrest with a decrease of Bcl-2 expression, an increase of cytochrome *c* release and reduced cyclin B1 expression (Li, Kim, Kim, & Park, 2004). In addition, a water-soluble β-glucan isolated from *Poria cocos* was shown to have growth-inhibitory effects on human breast carcinoma MCF-7 cells mediated by cell-cycle arrest and apoptosis induction (Zhang, Chiu, Cheung, & Ooi, 2006). Therefore, anti-tumor activities of mushroom polysaccharides are not only mediated by the immunopotentiation (Zaidman, Yassin, Mahajna, & Wasser, 2005), but can also be resulted from a direct inhibition on the tumor cells. However, structure of mushroom polysaccharides are very diverse in terms of their monosaccharide composition, linkages in the main chain, degree of branching, percentage of non-carbohydrate components such as protein or peptide percentage as well as functional group modification. Thus, the correlation of the chemical structure of mushroom polysaccharides and the mechanisms of their anti-proliferation activities is still unclear.

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The sclerotia of *Pleurotus tuber-regium* were firstly discovered in Africa and are used as functional food to promote health and longevity (Zoberi, 1973). In China, these sclerotia are mainly distributed in Yunnan province (Southwestern China) and are used by local people as folk medicine to treat asthma, stroke, and breast cancer as well as to promote the development of fetus (Huang, Guo, & Huang, 1996). The sclerotia of *P. tuber-regium* are edible and extremely rich in non-starch polysaccharides that are mainly composed of β-glucan (Cheung & Lee, 1998). A native water-insoluble β-glucan (PTR) isolated from the sclerotia of *P. tuber-regium* was characterized by our laboratories to be a $1 \rightarrow 3$ linked β-glucan, with one $1 \rightarrow 6$ branches at every third glycosidic residues (Zhang, Zhang, & Cheung, 2001). PTR was demonstrated to have both immunomodulatory and direct cytocidal anti-tumor activities (Zhang, Cheung, & Zhang, 2001). We have recently introduced carboxymethylated groups to the native glucan, producing a novel water-soluble CMPTR with enhanced anti-tumor activity (Zhang, Cheung, & Zhang, 2004). Our previous study showed that the administration of CMPTR to the BALB/c mice bearing Sarcoma S-180 cells enhanced the production of TNF-α and might thereby inhibit the tumor growth *in vivo*. Compared to PTR, CMPTR had generally higher *in vitro* antiproliferation of cancer cell lines including HL-60 and HepG2. Therefore, CMPTR has very good potential in the development as an anti-tumor agent because of its higher water solubility and enhanced immunomodulatory and anti-tumor activities. However, the anti-proliferative effects of CMPTR on other cell lines and their mechanisms are still not clear. In this project, a mechanistic study of the anti-proliferative activity of CMPTR on the human breast cancinoma MCF-7 cells *in vitro* was investigated.

2. Materials and methods

2.1. Chemicals and antibodies

Samples of carboxymethylated β-glucan (CMPTR) were obtained from the sclerotia of *P. tuber-regium* by methods described previously (Zhang et al., 2004). Mouse anti-human cyclin D1 monoclonal antibody (Cat. No. 554181), cyclin E monoclonal antibody (Cat. No. 551160), and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse Ig specific polyclonal antibody (multiple adsorption) (Cat. No. 554001) were purchased from BD Pharmingen. Mouse anti-human Bcl-2 monoclonal antibody (Cat. No. 610538) and Bax monoclonal antibody (Cat. No. 610982) were provided by BD Transduction Laboratories. Goat anti-mouse IgG antibodies conjugated with alkaline phosphatase were obtained from Bio-Rad (Cat. No. 1705010); Unless otherwise stated, all the other chemicals were obtained from Sigma Chemical Co., St. Louis, MO, USA.

2.2. Cell lines and cell culture

Human breast carcinoma MCF-7 cells were obtained from American Type Culture Collection (ATCC). The cells were grown in RPMI-1640 medium supplemented with 10% FBS. The cell cultures were incubated at fully humidified atmosphere of 95% room air and 5% CO_2 at 37 °C, and were passed three times a week.

2.3. In vitro proliferation and cytotoxicity assay

The breast cancer cells were incubated with CMPTR at the concentrations of 12.5, 25, 50, 100, 200, 400 μg/ml. The metabolic activity of the living cells, indicating their proliferation and viability at the end of incubation period was determined by colorimetric method based on a tetrazolium salt (MTT) (Mosmann, 1983). The treatment groups were compared with control groups in the absence of CMPTR; results were expressed as an inhibition ratio of the control cell metabolic activity calculated as $[(A - B)/A]$, where A and B are metabolic activities of the control and the treatment cells, respectively. The value of IC_{50}, which is the concentration of CMPTR required to inhibit the breast cancer cells by 50% of the control level, was estimated from the plot.

2.4. Cell-cycle analysis

The breast cancer cells were incubated with the IC_{50} of CMPTR for 24, 48, and 72 h, respectively. After treatment, the cells were washed with PBS twice and fixed by 70% ice ethanol at −20 °C for at least 2 h. Prior to the analysis, the fixed cells were again washed with PBS and stained with 50 μg/ml of propidium iodide (PI) solution. The stained cells were then transferred to flow tubes by passing through nylon mesh with pore size of 40 μm. Flow cytometric analysis was performed on a flow cytometer (Beckman Coulter, Epics XL MCL). Apoptotic cells were determined by their hypochromic sub-diploid staining profiles. The distribution of cells in the different cell-cycle phases was analyzed from the DNA histogram using Multicycle software (Phoenix Flow Systems, San Diego, CA).

2.5. Western blot analysis

Western blot was conducted to analyse the effects of CMPTR on the expressions of cell-cycle regulators cyclin D1 and cyclin E as well as apoptosis regulators Bcl-2 and Bax in the breast cancer cells. Equal amounts of proteins (30 μg/lane) were diluted by loading dye and subjected to 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). The gel was then transferred to a nitrocellulose membrane (Amersham). The membrane was then blocked in the blocking solution containing 0.2% Aurora@ blocking reagent (ICN Biochemical) and 0.1% Tween in PBS solution at room temperature (r.t.) for at least 2 h, which was subsequently incubated with