Growth-inhibitory effects of a ß-glucan from the mycelium of *Poria cocos* on human breast carcinoma MCF-7 cells: Cell-cycle arrest and apoptosis induction

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Abstract. Because of the reported immune-enhancing and anti-tumor activities of some mushroom polysaccharides, their applications as biological response modifiers have attracted significant attention. We have purified a water-soluble ß-glucan PCM3-II, comprising mainly 1→3 and 1→4 linkages, from the mycelia of *Poria cocos* (Schw.) Wolf (Fu-ling). In this study, the growth-inhibitory effect of PCM3-II was further explored on the human breast carcinoma MCF-7 cells *in vitro*. The dose effect of PCM3-II was studied by incubating the breast cancer cells with 12.5-400 μg/ml of the glucan for 72 h. The MTT study showed that PCM3-II reduced proliferation and viability of the MCF-7 cells dose-dependently, so that the cancer-cell growth was decreased by 50% of the control level at 400 μg/ml of the glucan. The time effect of PCM3-II was then investigated by treating the breast cancer cells with 400 μg/ml of the glucan for 24, 48 and 72 h, respectively. Results from the flow cytometry study demonstrated that PCM3-II induced cell-cycle G_1 arrest time-dependently and about 90% of the cells in cell cycle were accumulated at G_1 phase after 72 h of treatment. The G_1 arrest was associated with downregulations of the unscheduled cyclin D1 and cyclin E expressions in the breast cancer cells. Apoptosis was also induced by PCM3-II in the MCF-7 cells, so that the $subG_1$ cells in DNA histogram of the flow cytometry were elevated by 5-fold of the control level at 48 h and by 24-fold at 72 h of treatment. The immunoblot study also showed that the glucan induced depletion of the antiapoptotic Bcl-2 protein, but not the proapoptotic Bax protein, so that the Bax/Bcl-2 ratio was elevated in the breast cancer cells at the time when the most prominent apoptosis was also observed. In conclusion, although the detailed mechanism for the anti-tumor activity of the *P. cocos* ß-glucan still needs further investigation, this study provides preliminary insights into its mode of action and perspectives of its development as a water-soluble anti-tumor agent.

Introduction

Poria cocos (Schw.) Wolf (Fu-ling) has been used as an important medicinal herb in China and some Asian countries, particularly Japan and Korea, for its diuretic and anti-depressant activities. Results from the recent experimental studies further supported application of the chemical constituents from *P. cocos*, especially triterpenes and poly-saccharides, as novel anticancer agents. The triterpenes, namely poricoic acids, tumulosic acids, polyporenic acid, and dehydroabietic acid inhibit the tumor-promoting activity of 12-O-tetradecanoylphorbol-13-acetate (TPA); poricoic acid and tumulosic acid, on the other hand, exhibit cytotoxicity on a number of human cancer cells *in vitro* (1). Dehydroebriconic acid, a selective topoisomerase II inhibitor, prevents the growth of human gastric cancer cells by inducing the cell-cycle G_1 arrest (2). Pachymic acid reduces the proliferation of and induces apoptosis in both the androgen-insensitive DU145 and androgen-responsive LNCaP prostate cancer cells, with decrease in expression and activation of the proteins in the AKT cell survival pathway (3). Although the knowledge on the *P. cocos* polysaccharides is relatively limited comparing to that on its triterpenes, application of the polysaccharides as antitumor agent has attracted significant attention in recent years (4). A neutral polysaccharide fraction from *P. cocos* was found to exhibit growth-inhibitory and differentiation-inducing activities on the human leukemic U937 and HL-60 cells, mainly due to the elevated IFN-γ and TNF-α expressions (5). We also purified two alkali-soluble polysaccharides, namely PCM3-I and PCM3-II, from the *P. cocos* mycelia; structural elucidation with infrared (IR), gas chromatography (GC), and carbon-13 nuclear magnetic resonance (NMR) spectroscopy revealed that PCM3-I is a water-insoluble α-heteropolysaccharide, consisting of xylose, mannose, and glucose, and PCM3-II, on the other hand, is a ß-type polysaccharide, comprising mainly glucose (98.9% in the total carbohydrate content) and has a very good water solubility (6). These polysaccharides inhibited the proliferation of and exhibited cytotoxic effect on human leukemic HL-60 cells *in vitro* and retarded growth of the mouse sarcoma S-180

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in BALB/c mice *in vivo* (6). However, their growth-inhibitory effects have not yet been evaluated on cancers of other histotypes and the antitumor mechanisms are not known.

Breast cancer is a complex disease with a significant number of genetic and environmental risk factors associated with an increased occurrence (7) and is the most malignant disease in women (8). The growth-inhibitory effect of PCM3-II on the human breast carcinoma MCF-7 cells was explored in the present study. MCF-7, which is one of the cell lines commonly used for breast cancer research, co-expresses both the insulin-like growth factor 1 receptor (IGF-1R) and the estrogen receptor (ER), so that the two signaling systems are engaged in a crosstalk that leads to synergistic growth (9). The present results showed that PCM3-II retarded growth of the breast cancer cells, mediated by both cell-cycle arrest and apoptosis induction. The arrest of cell-cycle progression was associated with depletions of cyclin D1 and cyclin E expressions, while apoptosis was associated with elevation of the proapoptotic Bax/Bcl-2 ratio. This investigation focused on the mechanisms for the anti-proliferative and cytotoxic effects of PCM3-II, which would have great significance in its development as a novel and water-soluble antitumor agent.

Materials and methods

PCM3-II, antibodies, and chemicals. PCM3-II, which is a water-soluble ß-glucan, was purified from the mycelia of *P. cocos*, according to the procedures described in our previous study (6). Mouse anti-human cyclin D1 (clone G124-326) and cyclin E (clone HE12) antibodies, and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse Ig specific polyclonal antibody (multiple adsorption) were purchased from BD Pharmingen, San Jose, CA. Mouse anti-human bcl-2 (clone Bcl-2/100) and bax (clone 6A7) antibodies were provided by BD Transduction Laboratories, San Jose, CA. Alkaline phosphatase (AP)-conjugated goat anti-mouse Ig specific polyclonal antibody was obtained from Bio-Rad, Hercules, CA. Unless otherwise stated, all the other chemicals were obtained from Sigma, St. Louis, MO.

Cell culture and in vitro proliferation and cytotoxicity assay. Human breast carcinoma MCF-7 cells were obtained from the American Type Culture Collection (ATCC), Manassas, VA. The cells were grown in RPMI-1640 medium, supplemented with 10% FBS, and incubated at fully humidified atmosphere of 95% room air and 5% CO$_2$ at 37˚C.

The breast cancer cells were plated in 96-well microtiter plates at 2.5x10^3/well, and then incubated with 12.5, 25, 50, 100, 200 and 400 μg/ml of PCM3-II respectively for 72 h. Metabolic activity of the living cells, indicating their proliferation and viability, was determined by a previously described colorimetric method based on [4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT) with some modifications (10). In brief, 10-μl MTT solution (5 mg/ml) was added to each well, and the plate was then incubated for 4 h at 37˚C. The precipitated formazan was solubilized with 150-μl 0.04 N HCl-isopropanol, and the absorbance was measured by a microplate reader at 570 nm. Inhibition ratio of the cellular metabolic activity was calculated as $[(a - b) / a]$,

where a and b are the microplate readings of the control and treatment cells, respectively.

Flow cytometric analyses of cell cycle, apoptosis, and cyclin D1 and E expressions. The breast cancer cells were incubated with 400 μg/ml of PCM3-II for 24, 48 and 72 h, respectively. After trypsinization, the cell pellet was collected by centrifugation, followed by washing twice with PBS and fixing in 70% ethanol at 4˚C overnight. After the fixation, the ethanol was removed and the cells were washed with 1% bovine-serum albumin (BSA) solution. The cyclin D1 or cyclin E antibody, at 10 μg/ml, was added to the cells and incubated for 1 h, with vortexing every 30 min. Isotype control was also prepared with the same steps except IgG$_1$, instead of the cyclin antibody, was used. The excess antibody was removed by washing with the BSA solution. The cells were then incubated with the FITC-conjugated secondary antibody in dark for 1 h, with vortexing every 30 min. The excess antibody was removed, and the cells were resuspended in 1 ml of 500-μg/ml propidium iodide (PI) solution, containing 1-mg/ml RNase A. The stained cells were then analyzed by the EPICS-XL flow cytometer from Beckman Coulter, Miami, FL. The cyclin-positive cells were determined using the control software of the flow cytometer; a cell was considered as cyclin-positive if its FITC level was above the mean plus three times the standard deviation of its isotype control at the same cell-cycle phase. Apoptotic cells, because of their hypochromic subdiploid staining profile, were determined from the DNA histogram as subG$_1$ cells, using the control software of the flow cytometer. Cells in the different cell-cycle phases were measured from the DNA histogram using the Multicycle software from Phoenix Flow Systems, San Diego, CA.

Immunoblot analysis. The breast cancer cells were incubated with 400 μg/ml of PCM3-II for 24, 48 and 72 h, respectively. After the treatment, lysate was prepared from 2x10^6 cells. The proteins in the lysate were resolved by 13% SDS-poly-acrylamide gel. The resolved proteins were then transferred to a nitrocellulose membrane (Amersham Life Science, Buckinghamshire, UK). The membrane was first blocked with 0.2% Aurora® blocking reagent (ICN Biomedicals, OH), followed by overnight incubation with the bcl-2 or bax anti-body at 4˚C. ß-actin was also measured as the loading control. The membrane was washed, and then incubated with the AP-conjugated secondary antibody for 1 h, with shaking at room temperature. Color development was performed using the Immun-Blot Assay Kit (Bio-Rad). A densitometer was used to measure the relative intensity of the protein bands.

Statistical analysis. Difference in means between the control and experimental groups was compared by Student's t-test.

Results

PCM3-II reduces the proliferation and viability of MCF-7 cells. MTT assay, depending on the cellular mitochondrial dehydrogenase activity, offers a rapid and economical method for measuring cell proliferation and viability *in vitro* (10). In this study, the dose effect of PCM3-II on proliferation and viability
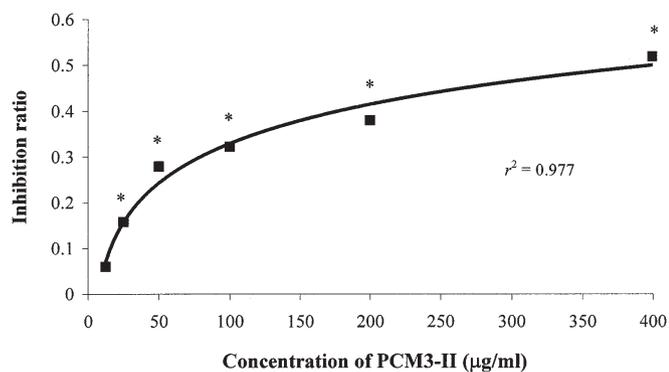
Figure 1. Effect of PCM3-II on the proliferation and viability of MCF-7 cells. The breast cancer cells were incubated with 12.5, 25, 50, 100, 200 and 400 μg/ml of PCM3-II respectively for 72 h. Inhibition ratio of the cellular metabolic activity was calculated as $[(a - b) / a]$, where a and b are the MTT readings of the control and treatment cells, respectively. The glucan reduced the proliferation and viability of the cancer cells dose-dependently. Results are expressed as mean of three independent replicates. *p<0.05, Student's t-test.

of the human breast carcinoma MCF-7 cells were investigated using the MTT method. At 72 h of treatment, PCM3-II reduced the proliferation and viability of the cancer cells dose-dependently, so that they were decreased by about 50% of the control level at 400 μg/ml of the glucan (Fig. 1).

PCM3-II induces G_1 arrest and apoptosis in the breast cancer cells. Cell cycle comprises a series of cellular events that an eukaryotic cell has to proceed orderly before it can divide

into daughter cells; the rate of cell proliferation is therefore determined by the rate that the cell proceeds through the different cell-cycle phases and checkpoints (11). Results from the DNA-flow cytometry showed that 400-μg/ml PCM3-II did not affect cell cycle of the breast cancer cells at 24 h of incubation [Fig. 2(a) and (b)]. However, the glucan induced cell-cycle arrest so that the G_1 cells increased from the control 45% of the cells in cell cycle to 73% at 48 h [Fig. 2(c) and (d)] and from 47% to 90% at 72 h of treatment [Fig. 2(e) and (f)]. Accompanying the elevations of G_1 cells, the G_2/M cells decreased from 38% to 12% at 48 h and from 37% to 2% at 72 h of incubation, while the S cells decreased from 17% to 13% and from 16% to 8%, respectively.

Apoptosis is a mode of programmed cell death, which plays an important role in cancer chemotherapy (12). DNA fragmentation is one of the characteristic features of apoptotic cells, so that they are shown as subG$_1$ cells in the DNA histogram of flow cytometry (13). PCM3-II was found to induce apoptosis time-dependently in the breast cancer cells. No apoptosis was induced by the glucan at 24 h of incubation; however, the subG$_1$ cells elevated by 5-fold of the control level at 48 h [Fig. 2(c) and (d)] and by 24-fold at 72 h of incubation [Fig. 2(e) and (f)].

PCM3-II downregulates cyclin D1 and cyclin E expressions in the breast cancer cells. Transitions through the different cell-cycle phases and checkpoints are at least regulated by different cyclins and their dependent kinases (Cdks) (11). Since PCM3-II arrested cell cycle at G_1 phase, effects of the glucan
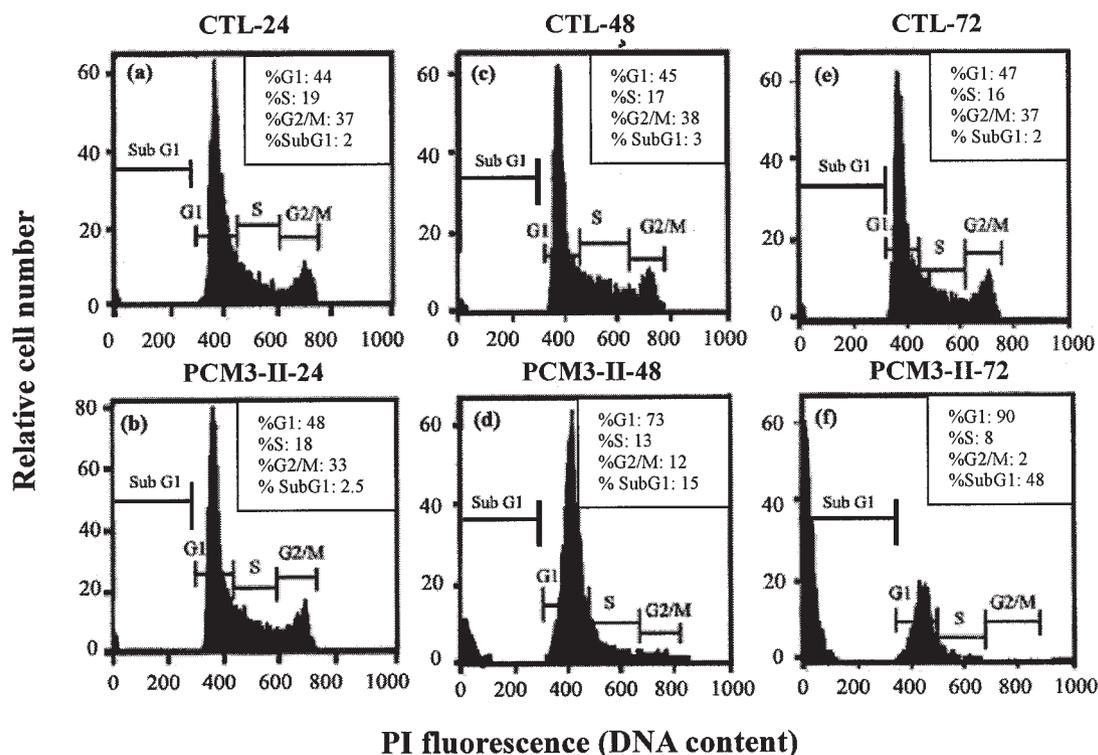

Figure 2. Representative DNA histograms showing the effects of PCM3-II on cell cycle and apoptosis in MCF-7 cells. The breast cancer cells were incubated with 400-μg/ml PCM3-II for (b) 24 h, (d) 48 h, and (f) 72 h respectively, comparing to their corresponding time controls (CTL) (a, c, and e). The glucan induced G_1 arrest after 48 h and apoptosis at 72 h of incubation. Numeric data showing the subG$_1$ cells, i.e., apoptotic cells, and the cells in different cell-cycle phases, i.e., G_1, S, and G_2/M, are also listed.
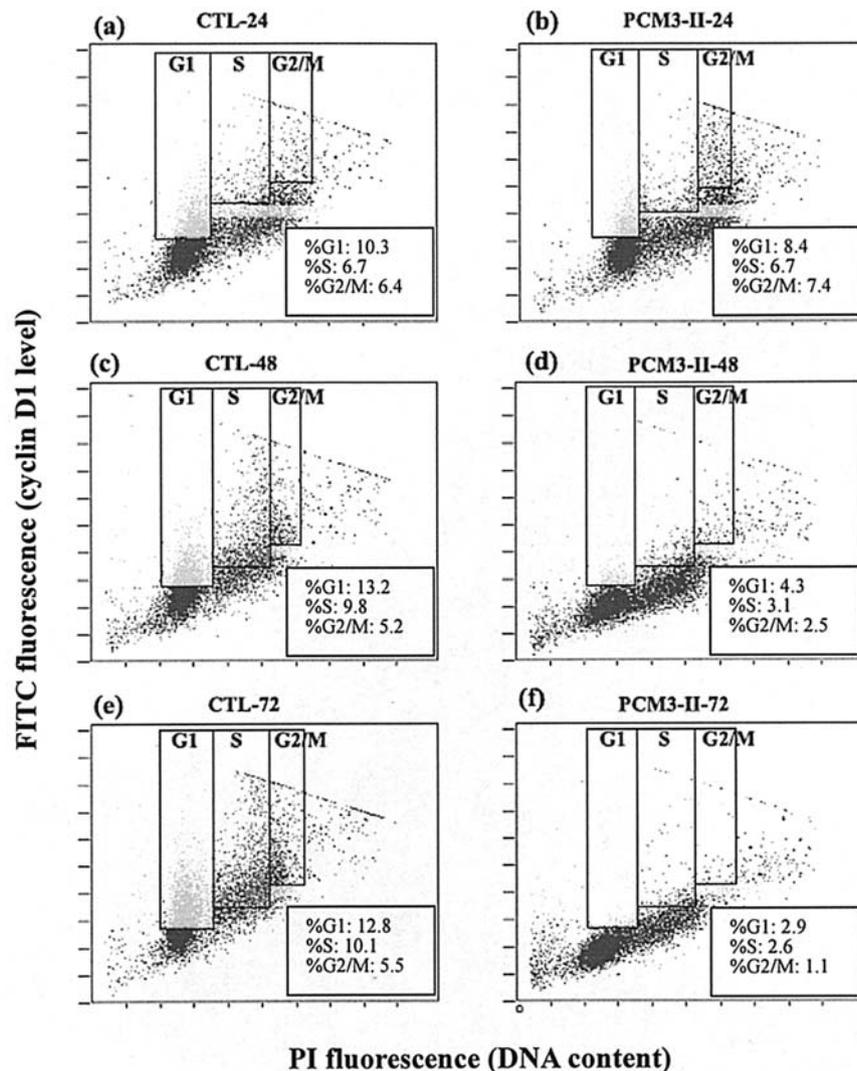
Figure 3. Effect of PCM3-II on the cyclin D1 expression in MCF-7 cells. The breast cancer cells were incubated with 400-μg/ml PCM3-II for (b) 24 h, (d) 48 h, and (f) 72 h respectively, comparing to their corresponding time controls (CTL) (a, c and e). Cyclin D1 expression was found to be deregulated in the MCF-7 cells, so that it was found not only in G$_1$ but also in other cell-cycle phases. PCM3-II induced downregulation of cyclin D1 expressions in all the cell-cycle phases after 48 h of treatment. Numeric data showing the proportion of cyclin D1-positive cells in different cell-cycle phases are also listed.

on cyclin D1, i.e., a G$_1$ cyclin, and cyclin E, i.e., a G$_1$/S cyclin, were investigated. Results from the bivariate cyclin/DNA flow cytometry showed that both cyclin D1 and cyclin E expressions were deregulated in the MCF-7 cells so that they could be found in all the cell-cycle phases, instead of only G$_1$ and S phases (Figs. 3 and 4). PCM3-II affected neither the cyclin D1 [Fig. 3(a) and (b)] nor cyclin E [Fig. 4(a) and (b)] expression in the breast cancer cells at 24 h of treatment. Interestingly, the cyclin D1-positive cells were decreased from 13.2% of G$_1$ cells in the control group to 4.3% in the treatment group at 48 h [Fig. 3(c) and (d)] and from 12.8% to 2.9% at 72 h of incubation, with 400 μg/ml of the glucan [Fig. 3(e) and (f)]. Similarly, the cyclin E-positive cells were also decreased from 11.6% of G$_1$ cells in the control group to 4.6% in the treatment group at 48 h [Fig. 4(c) and (d)] and from 9.4% to 1.4% at 72 h of treatment [Fig. 4(e) and (f)]. Both cyclin D1 and cyclin E expressions were also prominently depleted in other cell-cycle phases after 48 h of the PCM3-II treatment.

PCM3-II downregulates Bcl-2 expression in the breast cancer cells. Triggering and progression of the apoptosis pathways are tightly regulated by a number of gene products. Bcl-2 family proteins play a central role in the regulation of apoptosis (14). In this study, the time effects of PCM3-II on the pro-apoptotic Bax and antiapoptotic Bcl-2 expressions in the breast cancer cells were investigated. The glucan, at 400 μg/ml, induced downregulation of the Bcl-2 expression after 72 h of treatment, while there was no significant change of the Bax expression with the different periods of incubation [Fig. 5(a)]. Elevated proapoptotic bax/bcl-2 ratio was observed at 72 h of treatment, when the most prominent increase of the apoptotic cells occurred [Fig. 5(b)].

Discussion

Because of their reported immune-enhancing and antitumor activities, applications of mushroom polysaccharides as biological response modifiers have attracted recent attention (4).
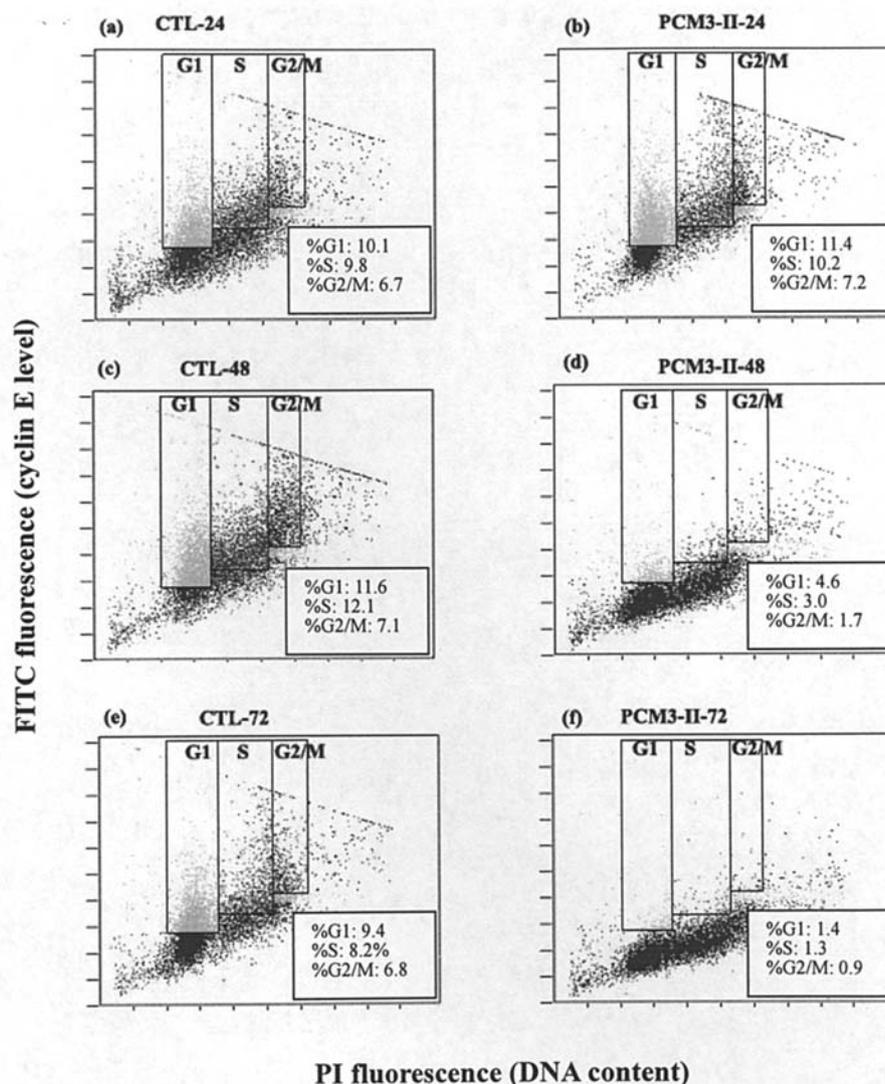
Figure 4. Effect of PCM3-II on the cyclin E expression in MCF-7 cells. The breast cancer cells were incubated with 400-μg/ml PCM3-II for (b) 24 h, (d) 48 h and (f) 72 h respectively, comparing to their corresponding time controls (CTL) (a, c and e). Cyclin E expression was found to be deregulated in the MCF-7 cells, so that it was found not only in the late G_1 and early S but also in other cell-cycle phases. The glucan induced downregulation of the cyclin E expression after 48 h of treatment. Numeric data showing the proportion of cyclin E-positive cells in different cell-cycle phases are also listed.

In our previous study, we had purified a ß-type glucan, PCM3-II, from the mycelia of *Poria cocos* and its antitumor activity was partially elucidated on human leukemic HL-60 and mouse sarcoma S-180 cells (6). However, mechanisms for the antitumor effect of PCM3-II have not yet been investigated. The water-soluble PCM3-II was further characterized as being composed of 54.2% 1→3 linked and 45.8% 1→4 linked glucose residues (data not shown). To further increase our knowledge on the effects of PCM3-II on cancers of other histotypes, growth-inhibitory effects of PCM3-II were further investigated on the human breast carcinoma MCF-7 cells, which were found to be mediated at least via cell-cycle arrest and apoptosis induction.

In general, cell-cycle transitions are controlled by cyclin-dependent kinases (Cdks), which contain both regulatory (cyclin) and catalytic (Cdk) subunits. In mammalian cells, cyclin D-Cdk4/6 and cyclin E-Cdk2 are important for G_1 progression and G_1/S transition, respectively (11). Both cyclin D-Cdk4/6 and cyclin E-Cdk2 complexes have been shown to

phosphorylate the retinoblastoma protein (pRb); successive phosphorylations of pRb by both of these complexes may be required to inactivate the protein completely (15). The ability of pRb to bind to E2F-1 and hence repress the E2F-1-dependent gene expression for S phase entry is well documented (16). Results from the bivariate cyclin/DNA flow cytometry illustrated that both cyclin D1 and cyclin E expressions were deregulated in the MCF-7 cells, so that the proteins were found not only in G_1 and S but also in other cell-cycle phases. This unscheduled pattern of cyclin expressions has also been observed in other cancers and has been suggested as a potential cause of tumor transformation (17,18). Cyclin D1 is over-expressed in 30-40% of human breast cancers and is associated with poor prognosis in ERα-positive cases (19). We determined in this study that both cyclin D1 and cyclin E expressions were depleted in the breast cancer cells after the PCM3-II treatment, suggesting that the pRb might remain bound to E2F-1 and the entry into S phase was thus prohibited. However, the signaling cascades
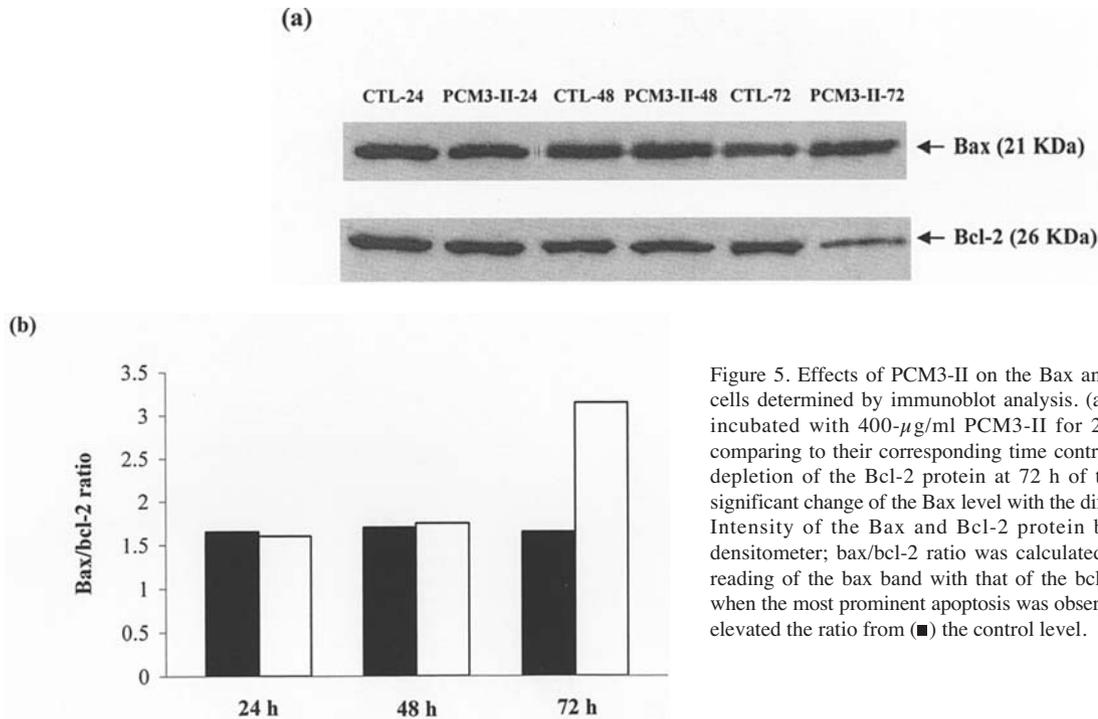
(a)

(b)

Figure 5. Effects of PCM3-II on the Bax and Bcl-2 expressions in MCF-7 cells determined by immunoblot analysis. (a) The breast cancer cells were incubated with 400-μg/ml PCM3-II for 24, 48 and 72 h respectively, comparing to their corresponding time controls (CTL). The glucan induced depletion of the Bcl-2 protein at 72 h of treatment, while there was no significant change of the Bax level with the different periods of incubation. (b) Intensity of the Bax and Bcl-2 protein bands were measured with a densitometer; bax/bcl-2 ratio was calculated by dividing the densitometry reading of the bax band with that of the bcl-2 band. At 72 h of treatment, when the most prominent apoptosis was observed, (□) the glucan significantly elevated the ratio from (■) the control level.

leading to the downregulation of cyclin expressions need further investigations.

Apoptosis is a form of cell death triggered during a variety of physiological conditions and is tightly regulated by a number of gene products that promote or block cell death at different stages. The PCM3-II-induced G$_1$ arrest was followed by apoptosis after a longer period of treatment. The most extensively studied and perhaps most important are Bcl-2 family proteins (14). In mammals, Bcl-2 has at least 20 relatives, including 4 other antiapoptotic proteins: Bcl-xL, Bcl-w, A1 and Mcl1; and 2 groups of proteins that promote cell death: Bax and BH3-only families. Bax family, on the other hand, consists of Bax, Bak and Bok; and BH3-only family comprises Bid, Bim, Bik, Bad, Bmf, Hrk, Noxa and Puma (14,20). These molecules either homodimerize or heterodimerize with molecule of opposing function. Bax may homodimerize so that apoptosis is promoted, or it may heterodimerize with Bcl-2 or Bcl-xL so that apoptosis is inhibited (21). The PCM3-II-induced apoptosis in the breast cancer cells was associated with downregulation of the anti-apoptotic Bcl-2 expression; however, no significant change of the proapoptotic Bax expression was observed. The bax/bcl-2 ratio was found to be elevated at the time when most prominent apoptosis occurred. It has been shown previously that the Bax/Bcl-2 ratio determines the susceptibility of cells to the induced apoptosis (22). Bax associates *in vivo* with Bcl-2 protein and down-regulation of Bcl-2 favors the formation of Bax homodimer that increases the rate of apoptosis and sensitivity of cells to apoptotic stimuli (23). Bax and a constitute mitochondrial protein, adenine nucleotide trans-locator, cooperate within the permeability transition pore complex to increase mitochondrial membrane permeability (24). The permeabilization results in the release of cytochrome *c*, apoptosis-inducing factor (AIF), and other pro-apoptotic molecules from mitochondria into the cytoplasm, which in turn

activate executioner caspases to orchestrate apoptosis (25). In conclusion, although the detailed mechanism for the anti-tumor activity of the *P. cocos* ß-glucan still needs further investigation, results from this study provide preliminary insights into its mode of action and perspectives of its being developed as a novel and water-soluble antitumor agent.

Acknowledgements

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