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Apoptosis Study on the Effect of PMF on Different Cancer Cells

[1]F.A. Khorshid, [2]S.A. Rahimaldeen and [1]J.S. AL-Amri

[1]Department of Biology,

[2]Department of Biochemistry, Faculty of Science, King Abdul Aziz University, Jeddah, Saudi Arabia

Corresponding Author: Faten A. Khorshid, Tissue Culture Unit, King Fahd Medical Research Center, King Abdul Aziz University, P.O. Box 80216, Postal Code 21589, Jeddah, Saudi Arabia Tel: +9662 6401000/23490, KFMRC 25289, 25109 Fax: +9662 6952076

ABSTRACT

PM 701, a natural liquid product, has anticancer activity. The bio-active fraction G, which is coded PMF has recently been isolated from PM 701. PMF was proved to have cytotoxic properties against cancer cells. The target of this study was to investigate the apoptotic effect of PMF in the human cancer cell lines. PMF at concentration of 60 and 100 μg mL^{-1} were incubated with cells during 48 h culture. Metabolic activity were measured colorimetrically by MTT assay (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide). Apoptotic cell death was also determined by the TUNEL method (terminal deoxynucleotidyl transferase (TdT)-biotin nick end-labeling). The results showed that PMF induced apoptosis in association with increased number of TUNEL positive cells. MTT results showed that PMF decrease cell proliferation via inhibiting metabolic cell activities. We conclude that PMF has anti-cancer effects by increasing apoptosis and altering cellular metabolic activity.

Key words: Breast carcinoma, colorectal cancer cells, glioma cells, liver carcinoma, leukemia cells, lung cancer cells

INTRODUCTION

Apoptosis, programmed cell death, plays a critical role in the cyclic changes and maintenance of homeostasis in multicellular organisms (Schwartzman and Cidlowski, 1993). However, failure to undergo apoptosis is one of the mechanisms associated with oncogenesis and chemoresistance of transformed cells (Green and Reed, 1998). Apoptosis is characterized by DNA fragmentation and chromatin condensation and differs from necrosis, which is characterized by ruptured cell membranes and swollen nuclei (Nagata and Golstein, 1995; Steller, 1995). Impairment of apoptotic signaling enables tumor cells to avoid apoptotic cell death and grow into tumor masses that are resistant to apoptosis (Wyllie *et al*., 1999). Apoptosis is also an important phenomenon in chemotherapy-induced killing of tumor cells. Several recent reports have indicated that many anti-cancer drugs act through the induction of apoptosis to prevent tumor promotion and progression (Reed, 2002). In addition, many natural products used in cancer chemotherapy work through enhancing apoptotic pathways (Bhalla *et al*., 1993; Friesen *et al*., 1996).

In the present study, we investigated the Apoptotic effect of PMF when incubated with different cell lines of human cancer using TUNEL technique. MTT assay was performed to monitor of cancer cells metabolic activity.

MATERIALS AND METHODS

The project was conducted between 2007-2009 in Tissue culture unit King Fahd Medical Research Center in King Abdul Aziz University, Jeddah.

Materials: PMF (fraction of PM701) was prepared according to method reported by Khorshid (2009). Dulbecco's Modified Eagle Medium (DMEM) and RPMI media were purchased from MP Biomedicals Inc, USA. Fetal Calf Serum (FCS) was obtained from Gibco, Canada. *Situ* cell death detection kit, fluorescein and DNase I, grade I were purchased from Roche, Germany. Triton X-100 solution was obtained from Bio-Rad Richmond, USA. Paraformaldehyde powder 95-100% was purchased from Panreac PRS, Barcelona Spain. All other chemicals were purchased from Sigma, USA.

Cell lines: Six cancer cell lines were used in this study, namely human hepatocellular liver carcinoma (HePG2), human lung cancer (A549), glioma cells (U251), colorectal cancer cells (HCT116), mice leukemia cells (L1210) and breast carcinoma (MCF7).

The L1210 and A549 cell lines were obtained by cell strain from American Type Culture Collection (ATCC) (Rockville, Maryland). HepG2, U251, HCT116 and MCF7 cell lines were purchased from National Cancer Institute, Cairo University, Egypt.

Normal fibroblast cell line (HFS) was also used, which is obtained from human foreskin after circumcision operations (Surgical Clinic King Abdul Aziz, University Hospital, Jeddah, Saudi Arabia) and available in the cell bank of the TCU, KFMRC, King Abdulaziz (KAU), Jeddah.

Cell culture: The RPMI media was used for L1210 cell line. All other cell lines were grown in DMEM media supplemented with 10% FCS, penicillin (100 μ mL^{-1}) and streptomycin (100 μg mL^{-1}) in culture flasks at 37°C in 5% humidified CO_2 incubator. Cells were fed until confluence (Khorshid and Moshref, 2006).

Cell viability and growth assay: The 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay is used to measure the cytotoxic effects of many antitumor agents on cultured cells (Olivieri *et al*., 2000; Surazynski *et al*., 2001). MTT assay was performed here to evaluate the survival rate of cells treated with PMF. The MTT assay is a colorimetric assay based on the tetrazolium salt MTT that detects cell viability.

MTT was dissolved in Dulbecco's Phosphate Buffered Saline (PBS) at 5 mg mL^{-1} and filtered through a 0.22 μm filter to sterilize and remove the small amount of insoluble residue then stored at 2-8°C for frequent use.

Cells were seeded at a density of 6×10^3 per well in 100 μL culture media into a 96-well plate for 24 h in a humidified CO_2 incubator at 37°C. According to pilot studies, PMF was inoculated to the cells at concentration of 60-100 μg mL^{-1} for cancer and normal cell lines. Cells were then re-incubated for 48 h in a humidified CO_2 incubator at 37°C. After the time of incubation, 20 μL of stock MTT solution was added to all wells for the assay. After a further period of incubation (2 h), the media was aspirated from the wells as completely as possible without disturbing the formazan crystals. Then, 200 μL of isopropanol was added under dark to each well for dissolving the resulting precipitate. The concentration of the dye was then measured at 570 nm on plate reader (Microplate Reader Model 450; Bio-Rad). The optical density obtained was directly related to the viability of cells.

TUNEL assay for apoptosis: The assay was first described by Gavrieli *et al.* (1992). The cleavage of cellular DNA during apoptosis yields double stranded, low molecular weight DNA fragments and single strand breaks in high molecular weight DNA. The principle of this assay is to identify these breaks by labeling of DNA strand breaks at the free 3'-OH DNA by terminal deoxynucleotidyl transferase (TdT). This is called TdT-mediated dUTP nick end labeling (TUNEL) technique. The reaction mixture also contains fluorescein labels which are incorporated into nucleotide polymers and can be detected by fluorescence microscopy.

Cells were incubated at 37°C for 24 h. Fixation solution (pH 7.4) was prepared by dissolving 2 g of paraformaldehyde in PBS. Washing buffer was PBS (pH 7.4) and the permeabilisation solution contained 0.1% Triton X-100 in 0.1% sodium citrate. The TUNEL reaction mixture was prepared immediately before use and kept on ice until use. After cancerous cells were treated with different concentrations of 60-100 μg mL^{-1}, they fixed at 48 h with 200 μL paraformaldehyde solution for 30 min, rinsed with PBS and permeabilised with 200 μL of permealisation solution 10 min at 4°C. The wells were washed twice and the reaction mixture then added and incubated for 1 h at 37°C incubator in the dark. Each well was washed three times before analysis by fluorescence microscopy (BH2 U-PMTVC, Japan, connected with Nikon camera FX-35A HFXII, Japan). Apoptotic cells appear as fluorescent green in color.

Statistical analysis: The data were expressed as the mean (±SD) of the optical density obtained from three independent experiments (each experiment was performed in three replicate wells). Statistical analysis was performed with ANOVA using SPSS software (11.0). $p<0.05$ was considered significant.

RESULTS

Effect of PMF on cell viability of cancer cells: The apoptotic effect of PMF was tested using MTT test on six different cancer cells. In general, all treated cells showed reduction in cancer cells viability which differs according to cell type and concentration of the drug. To confirm that PMF has no effect on normal cells, HFS was tested with the PMF. The overall reduction in cell viability and the significant difference between treated and non treated cells are shown in Table 1.

This experiment indicates that there is significant reduction of proliferation of cancer cells which explained by the presence of apoptotic cells examined with MTT.

Effect of PMF on apoptosis induction of cancer cells: To determine the occurrence of apoptosis in six different cancer cells treated with the PMF, the cells were stained with TUNEL. After treatment with different concentrations of PMF (60-100 μg mL^{-1}) for 48 h, the numbers of

Table 1: Effect of PMF on cell survival of different cancer cells

Cell type	% of cell survival ±SD	% of cell death ±SD	p-value
HFS	105±20	None	0.43
HepG2	89±6	11±6	<0.0018
A549	87±4	13±4	<0.0027
U251	81±6	19±6	<0.001
HCT116	74±10	26±10	<0.0001
L1210	70±7	30±7	<0.0001
MCF7	69±19	31±19	<0.0001

Table 2: The percentage of TUNEL positive cancer cells treated with different concentrations of PMF

Concentration of PMF	Cell type	No. of TUNEL positive cells ±SD	p-value
100 μg mL^{-1}	HepG2	46.0±5.3	<.000
60 μg mL^{-1}	A549	51.6±10.4	<.000
100 μg mL^{-1}	U251	54.3±8.4	<.000
100 μg mL^{-1}	HCT116	66.3±5.5	<.000
60 μg mL^{-1}	L1210	76.3±3.2	<.000
100 μg mL^{-1}	MCF7	86.7±7.6	<.000

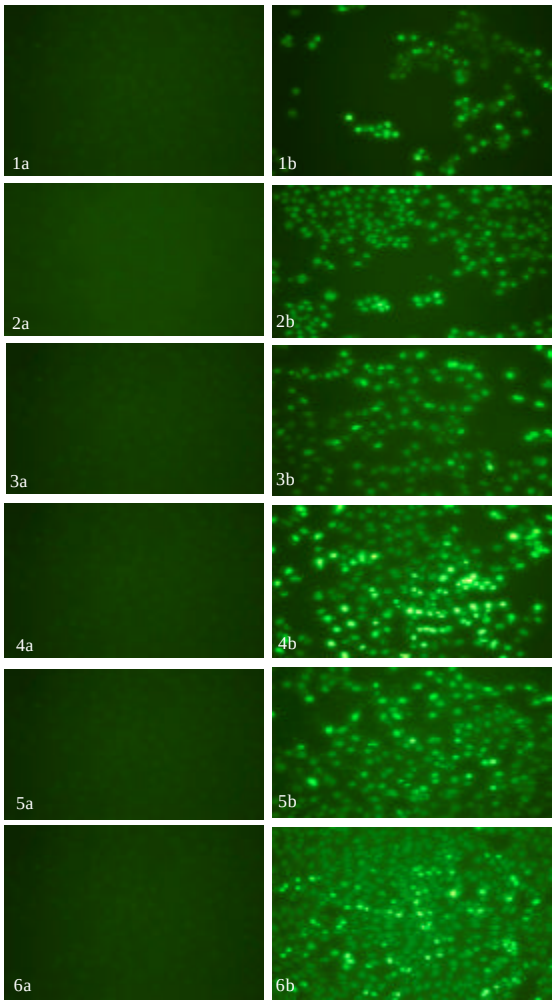

Fig. 1: Apoptosis in cancer cells was analyzed by visualizing TUNEL positive cells. The images illustrate untreated cancer cells (a) compared with treated cancer cells (b) note there are no TUNEL positive cells in untreated cancer cells (a), where apoptotic cells appear TUNEL positive in treated cultures with PMF (b): (1) is HepG2, (2) is A549, (3) is U251, (4) is HCT116, (5) is L1210 and (6) is MCF7

TUNEL positive cells in all treated cancer cell lines (Fig. 1) were significantly greater when compared with the control cancer cells. However, in MCF7 cells treated with 100 μg mL^{-1} of PMF, the number of TUNEL positive cells was greater than in the other cancer cell lines (Table 2).

DISCUSSION

In the previous studies, PM 701 was proved to have potential anticancer activity via selective programmed cell death in human lung cancer (A549) and leukemia cells (L1210) at tissue culture level, Conversely, it has no affect on normal cultured skin fibroblasts (Khorshid *et al.*, 2005; Khorshid and Moshref, 2006; Moshref *et al.*, 2006). This implies that PM701 may have a selectively killing effect on cancer cells with a reparative effect on normal dividing cells. It was effective in limiting of metastasic spreading effect of leukemia cells in animal models (Moshref *et al.*, 2006). Furthermore, PM 701 had considered safe as potentially anticancer agent with minimal or even negligible effects on vital organs such as liver and kidney (Khorshid, 2009).

The bio-guided extraction of PM 701 gives PMF fraction (Khorshid, 2009). Our previous investigations was undertaken to evaluate the cytotoxic activity of PMF against human cancer cells. PMF was able to inhibit significantly the proliferation of A549 cells without affect the normal HFS cells (Khorshid, 2009). PMF and its sub-fractions demonstrated a high cytotoxic activity against HepG2, HCT116 and U251 cancer cell lines and were not cytotoxic to human normal cells (Khorshid *et al.*, 2009).

In the present study, the effect of PMF on the cell survival and apoptosis of cancer cells were investigated using two different methods MTT to investigate the effect of PMF on the cell survival of cancer cells and TUNEL to study the number of apoptotic cells of treated cancer cells. The data demonstrated that PMF treatment was associated with a decreased cell survival and increased cell death by apoptosis. The cells were treated with different concentrations of PMF and it was observed that PMF had effect on both cell survival and apoptosis as demonstrated by the MTT and TUNEL assays. PMF was more effective on MCF7 cell growth and also cell death than other cell types.

CONCLUSIONS

In conclusion, it has still not been clearly demonstrated which mechanisms are responsible for apoptosis in PMF-treated tumor cell lines. Further *in vivo* studies are needed to establish the role of PMF as therapeutic agents for cancer.

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