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ORIGINAL ARTICLE

Apoptotic properties of polysaccharide isolated from fruiting bodies of medicinal mushroom *Fomes fomentarius* in human lung carcinoma cell line

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KEYWORDS

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Abstract Mushrooms are known to complement chemotherapy and radiation therapy by counteracting the side effects of cancer. Recently, there has been great interest in isolation of novel bioactive compounds from mushrooms due to their numerous health beneficial effects. Chemically water-extractable polysaccharide (MFKF-AP1 β), with a molecular weight of 12 kDa, was isolated from fruiting bodies of mushroom *Fomes fomentarius*. In this research, we investigated the anti-tumor effects of MFKF-AP1 β on human lung carcinoma A549 cells. Results showed that MFKF-AP1 β markedly inhibited A549 cell growth in a dose-dependent manner based on the amount of lactate dehydrogenase (LDH) released and morphological alterations. In addition, MFKF-AP1 β induced cellular apoptosis by causing single-stranded DNA breakage, as evidenced by apoptosis assay. Furthermore, MFKF-AP1 β (25–100 μ g/ml) significantly induced single-stranded DNA breakage in A549 cells, as shown by comet assay. Taken together, our results demonstrate that MFKF-AP1 β has strong anti-tumor effects mediated through induction of apoptosis. Therefore, MFKF-AP1 β could be useful in lung chemotherapy.

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1. Introduction

Lung cancer has become the most common cause of cancer-related death in both men and women, accounting for 28% of all cancer-related deaths in the U.S. (Cardenal et al., 1999). Although treatment of lung cancer has improved, mortality remains high in lung cancer patients. A growing body of research suggests that naturally occurring compounds can act as antioxidants as well as cancer preventative and therapeutic agents (Parkin et al., 1999; Rahman et al., 2005; Hashibe et al., 2007). Development of chemotherapeutic agents with maximal

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anti-tumor activity and minimal toxicity has become a favorable route for cancer management. In this context, mushrooms play a pivotal role as anti-tumor agents. Edible mushrooms have been used as health nutritional supplements for several centuries, and they complement chemotherapy and radiation therapy by countering the side effects of cancer (Mizuno et al., 1995).

The bioactive compounds in mushrooms responsible for their anti-tumor potential include polysaccharides, proteins, fats, ash, glycosides, alkaloids, volatile oils, tocopherols, phenolics, flavonoids, carotenoids, folates, ascorbic acid enzymes, and organic acids (Mizuno et al., 1992, 1995; Jong and Birmingham, 1993; Ferreira et al., 2009). Among these bioactive compounds, polysaccharides are the most well-known and most potent mushroom-derived substances with anti-tumor activity (Ferreira et al., 2010). In this study, we analyzed the anti-tumor activity of a polysaccharide (MFKF-AP1 β) isolated from fruiting bodies of the medicinal mushroom *Fomes fomentarius* in human lung cancer A549 cells.

F. fomentarius, a basidiomycete fungus, has been used as a traditional Chinese and Korean medicine for many centuries for the treatment of various diseases, including oral ulcers, gastroenteric disorders, hepatocirrhosis, inflammation, and various cancers. Recent studies have shown that *F. fomentarius* has antioxidant, anti-inflammatory, and anti-diabetic activities (Lee, 2005; Park et al., 2004). However, there are few reports on the apoptotic activity of *F. fomentarius* (Ito et al., 1976; Chen et al., 2008).

To discover novel agents from natural products that improve the therapeutic outcome of cancer, we tested the anti-tumor effects of a polysaccharide (MFKF-AP1 β) isolated from *F. fomentarius* on A549 lung cancer cells. Specifically, we tested the apoptotic activity of MFKF-AP1 β in lung carcinomas.

2. Materials and methods

2.1. Reagents

RPMI 1640, HEPES, staurosporine, Hoechst 33342, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide were purchased from Sigma–Aldrich, St. Louis, USA. Fetal bovine serum was supplied by Gibco, USA. All solvents were HPLC grade and supplied by J.T. Baker (Phillipsburg, NJ, USA).

2.2. Materials

Chemically water-extractable polysaccharide (MFKF-AP1 β) was isolated from hot water extracts of fruiting bodies of mushroom *F. fomentarius* by successive DEAE-Sepharose FF and concanavalin A-Sepharose 4B column chromatography. Molecular weight of MFKF-AP1 β was estimated to be about 12 kDa by high performance liquid chromatography (HPLC) (Park et al., 2013). Solutions of MFKF-AP1 β (25–100 μ g/ml) were prepared in a water/methanol mixture (1:1, v/v).

2.3. Cell culture

Lung cancer cells A549 (KCLB) were cultivated in RPMI 1640 medium supplemented with 25 mM HEPES buffer, 25 mM sodium bicarbonate, 300 mM L-glutamate, and 10%

heat-inactivated fetal bovine serum (Gibco, USA) in a CO₂ incubator (5% CO₂ in air) at 37 °C. For the experiment, cells were cultured in 12-well plates at 1×10^6 cells/well. Cells were treated with several concentrations of MFKF-AP1 β (25, 50, and 100 μ g/ml) for 24 h. As a positive control, 1 μ M staurosporine (Sigma, St. Louis, USA) was added for 4 h to induce apoptosis. Cells were then centrifuged (600g \times 3 min) and harvested.

2.4. MTT assay and cell morphology

Cell viability was measured by MTT bioassay. Briefly, A549 cells (1×10^6 cells/well) were seeded in each well of a 96-well plate for 24 h. After incubation with different concentrations of MFKF-AP1 β for 24 h, 10 μ l of MTT solution (5 mg/ml on PBS) dissolved in PBS was added and incubated for 4 h. After color development, 100 μ l of DMSO was applied. Absorbance was measured using an ELISA plate reader (Bio-Tek Instrument Co., WA, USA) at 540 nm. The viability% was measured using formula-

Sample absorbance/Control absorbance \times 100.

2.5. Lactate dehydrogenase (LDH) assay

A549 cells (1×10^6 cells/well) were seeded in each well of a 96-well plate for 24 h. After incubation with different concentrations of MFKF-AP1 β for 24 h, medium was collected and cleared by centrifugation. After supernatants were collected, cytotoxicity was quantified by measuring the amount of total LDH released by cells using an LDH assay kit (Sigma–Aldrich, St. Louis, USA) according to the manufacturer's protocol. Briefly, LDH, a glycolytic enzyme, is concerned with the reduction of pyruvic acid in the presence of dihydronicotinamide adenine dinucleotide (NADH). LDH assay reagent was added to supernatants and incubated for up to 30 min at room temperature in the dark, after which the reaction was stopped by adding 1 N HCl. The absorbance of samples was measured at 450 nm.

2.6. Assay for nuclear apoptosis (Hoechst staining)

To determine DNA chromatin morphological features, cells were treated with Hoechst 33342 stain according to Diaz-Ruiz et al., 2001. Briefly, cells were cultured and treated for 24 h with different concentrations of MFKF-AP1 β and staurosporine (1 μ M) for 4 h. After washing twice in PBS, cells were fixed with cold 4% formaldehyde. Cells were then washed with PBS again and incubated with Hoechst 33342 (1 μ g/ml) at 37 °C for 10 min. After washing with PBS, cells were analyzed under a fluorescence microscope (Nikon Eclipse TS100 Epi-fluorescence microscope, Japan).

2.7. Comet assay

To evaluate DNA damage, comet assay was performed according to Singh et al. (1988), with some modifications. A549 lung cancer cells treated with MFKF-AP1 β and staurosporine were harvested by washing twice with phosphate-buffered saline (PBS) and then suspended in 70 μ l of 1% (w/v) low melting point agarose in PBS, pH 7.4, at

37 °C. The cell solution was immediately pipetted onto frosted glass microscope slides pre-coated with a layer of 1% (w/v) normal melting point agarose, after which the third layer composed of 75 μ l of 0.5% (w/v) low melting point agarose similarly prepared in PBS was applied. The agarose was allowed to set for 10 min on ice. To remove cellular proteins, slides were immersed in lysis solution (2.5 M NaCl, 0.1 M Na₂EDTA, 10 mM Tris, pH 10.0, with NaOH, 10% v/v dimethyl sulfoxide, and 1% v/v Triton X-100) at 4 °C for 1 h. Slides were then placed in a electrophoresis tank containing electrophoresis buffer (0.3 M NaOH and 1 mM Na₂EDTA) for 30 min before electrophoresis at 40 V for 40 min. The slides were then washed with neutralizing solution (0.4 M Tris-HCl, pH 7.5) for 20 min before staining with 50 μ l of ethidium bromide (20 mg/ml), and analyzed visually with a Nikon EPI fluorescence microscope (Japan).

2.8. Measurement of single-stranded DNA

As DNA in apoptotic cells is sensitive to formamide, denatured DNA was detected by a monoclonal antibody against single-stranded DNA using an ApoStrand™ ELISA apoptosis detection kit (Enzo Life Sciences, Plymouth Meeting, PA, USA) according to the manufacturer's protocol. Briefly, 1.0×10^6 cells were cultured in a 96-well microplate and treated with different concentrations of MFKF-AP1 β . After 12 h, cells were fixed and dried for attachment to the plate surface. Cells were then treated with formamide and heated at 56 °C for 30 min, followed by incubation with an antibody mixture for 30 min after blocking non-specific binding sites. After washing, peroxidase substrate was added to each well, and the absorbance was measured at 405 nm using an ELISA reader (Bio-Tek Instrument Co., WA, USA).

2.9. Statistical analysis

Each experimental condition was analyzed at least in triplicate ($n = 3$). Data were expressed as the mean \pm standard deviation (S.D.). Statistical analyses were carried out using Student's *t*-test. Differences were considered statistically significant at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3. Results

3.1. Anti-proliferative effects of MFKF-AP1 β on A549 cells

We characterized the anti-proliferative effects of MFKF-AP1 β on A549 cells by measuring cell viability using MTT assay. Cultures of A549 cells were treated with MFKF-AP1 β at concentrations of 25, 50, and 100 μ g/ml for 24 h. As shown in Fig. 1, MFKF-AP1 β inhibited proliferation of A549 cells. Cell viability was lowest ($59.22 \pm 5.17\%$) after 24 h of MFKF-AP1 β treatment at a concentration of 100 μ g/ml, as compared to MFKF-AP1 β concentrations of 25 and 50 μ g/ml ($64.46 \pm 1.48\%$ and $65.73 \pm 1.24\%$, respectively).

3.2. Cytotoxic effects of MFKF-AP1 β

To better characterize the cytotoxicity of MFKF-AP1 β in human lung cancer cells (A549), we performed cellular lactate

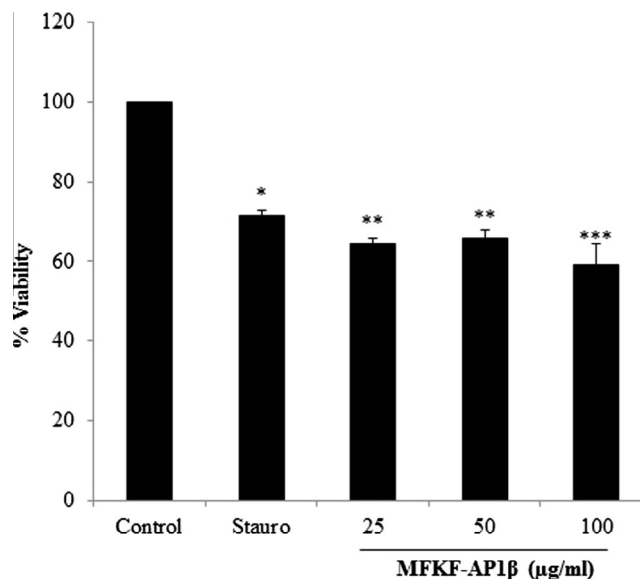


Figure 1 Effect of MFKF-AP1 β on viability of A549 lung cancer cells. The viability of cells pretreated with 1 μ M staurosporine (positive control) and MFKF-AP1 β at different concentrations (25, 50, and 100 μ g/ml) for 24 h was estimated using MTT assay after being cultured for 24 h. Data are represented as the mean \pm SD of three independent experiments. An asterisk indicates * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

dehydrogenase (LDH) release assay. In this experiment, intracellular LDH released into culture medium was an indicator of cytotoxicity. A549 cells treated with 25, 50, and 100 μ g/ml of MFKF-AP1 β for 24 h showed a dose-dependent increase in LDH activity, indicating cellular damage. Further, LDH release into culture medium was highest upon treatment with 100 μ g/ml of MFKF-AP1 β ($43.74 \pm 1.19\%$), which induced A549 cell death through cytotoxicity (Fig. 2). A significantly lower amount of LDH was released at concentrations of 25 and 50 μ g/ml ($39.91 \pm 1.19\%$ and $40.25 \pm 1.29\%$, respectively).

3.3. MFKF-AP1 β induces significant morphological alterations

The cytotoxic effects of MFKF-AP1 β were also reflected in the morphological appearance of A549 cells after 24 h (Fig. 3). Treatment with 1 μ M staurosporine and 100 μ g/ml of MFKF-AP1 β had a marked effect on cellular morphology, as cells became more round and refractile under a microscope. Cells treated with 100 μ g/ml of MFKF-AP1 β became apoptotic bodies and eventually detached from the surface, whereas untreated cells (control) remained well spread with a flattened morphology. Treatment with 50 μ g/ml of MFKF-AP1 β had a comparatively weaker effect, and 25 μ g/ml of MFKF-AP1 β had no discernible effect. These results suggest that MFKF-AP1 β -treated human lung cancer cells undergo cell death and exhibit morphological features suggestive of apoptosis.

3.4. MFKF-AP1 β potentiates cellular apoptosis

To determine whether or not the above results can be attributed to apoptosis of A549 cells, we performed chromatin condensation assay using Hoechst 33342 visualized by

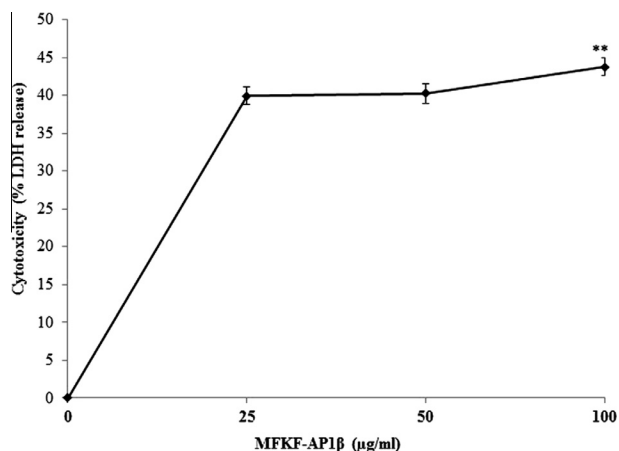


Figure 2 Lactate dehydrogenase (LDH) release assay. There was a significant increase in LDH release from lung cancer cells as measured by colorimetric assay. Pretreatment with 1 μ M staurosporine (positive control) and MFKF-API β at different concentrations (25, 50, and 100 μ g/ml) for 24 h significantly increased LDH release from A549 cells as a measure of cytotoxicity. Data are represented as the mean \pm SD of three independent experiments. An asterisk indicates ** $p < 0.01$.

fluorescence microscopy. Hoechst staining assay (Fig. 4) revealed a greater number of apoptotic cells showing characteristic morphologic alterations such as nucleic shrinkage and formation of pycnonuclei following treatment with 100 μ g/ml of MFKF-API β and staurosporine for 24 h compared to the control group.

3.5. MFKF-API β induces DNA fragmentation in A549 cells

Degradation of DNA into multiple internucleosomal fragments of 180–200 base pairs is a distinct biochemical hallmark

of apoptosis. To detect this, we performed single cell gel electrophoresis (comet assay), which is a sensitive technique that allows detection of DNA strand breaks. Cells treated with staurosporine and MFKF-API β showed comet formation in a dose-dependent manner, whereas untreated cells did not show any comet-like appearance (Fig. 5). To further determine whether or not DNA fragmentation is typical of apoptosis, we performed apoptosis detection assay using an ApoStrand™ ELISA apoptosis detection kit, which detects *in vitro* single-stranded DNA breaks induced by MFKF-API β treatment. The sensitivity of this assay reflects changes in chromatin structure associated with apoptosis, such as chromatin condensation and digestion of proteins stabilizing DNA. As shown in Fig. 6, treatment with MFKF-API β at concentrations of 25, 50, and 100 μ g/ml for 24 h significantly increased the apoptotic rate of A549 cells in a dose-dependent manner. Taken together, these results indicate that, apart from its cytotoxic effects, MFKF-API β can induce apoptosis in A549 cells.

4. Discussion

Cancer remains one of the major causes of death worldwide. The most effective treatment strategy for cancer is cytotoxic agent-based chemotherapy, which increases patient survival but also has side effects that severely limit its clinical effectiveness such as acquisition of drug resistance (Appel et al., 2007; Rutkoski and Raines, 2008; Lal et al., 2011). Therefore, novel therapeutic strategies involving natural compounds could reduce the cumulative effects of chemotherapy by enhancing anti-tumor activities. The present paper evaluated the effects of MFKF-API β , a dietary polysaccharide from medicinal mushroom *F. fomentarius*, on a human lung adenocarcinoma cell line (A549) in relation to cell growth inhibition and apoptosis induction. The anti-tumor activities of polysaccharides isolated from medicinal fungi are very attractive due to their low toxicity in normal cells and their apparent lack of side effects in clinical patients (Fukushima, 1989).

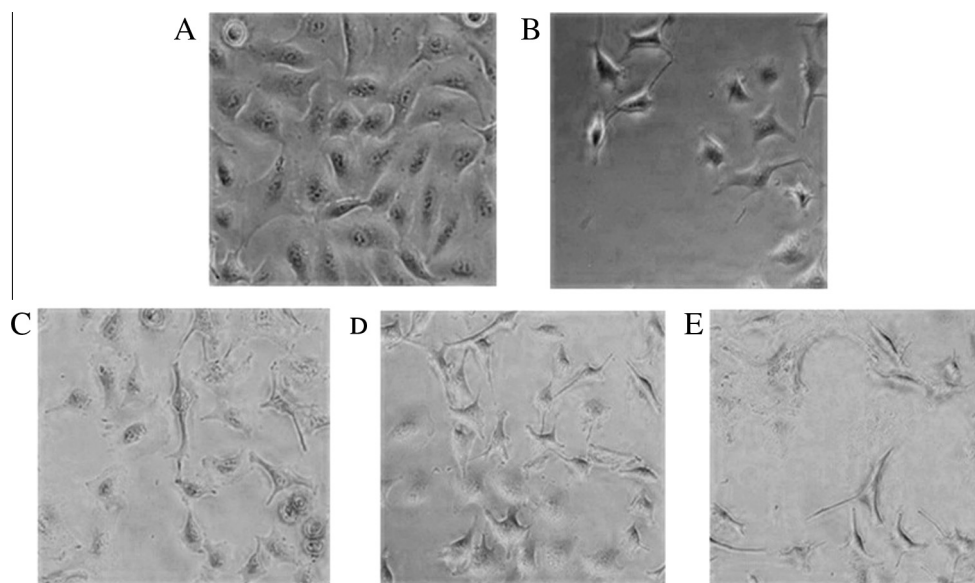


Figure 3 Morphological changes induced by (A) control, (B) 1 μ M staurosporine, (C) 25 μ g/ml of MFKF-API β , (D) 50 μ g/ml of MFKF-API β , and (E) 100 μ g/ml of MFKF-API β in A549 lung cancer cells after 24 h of treatment.

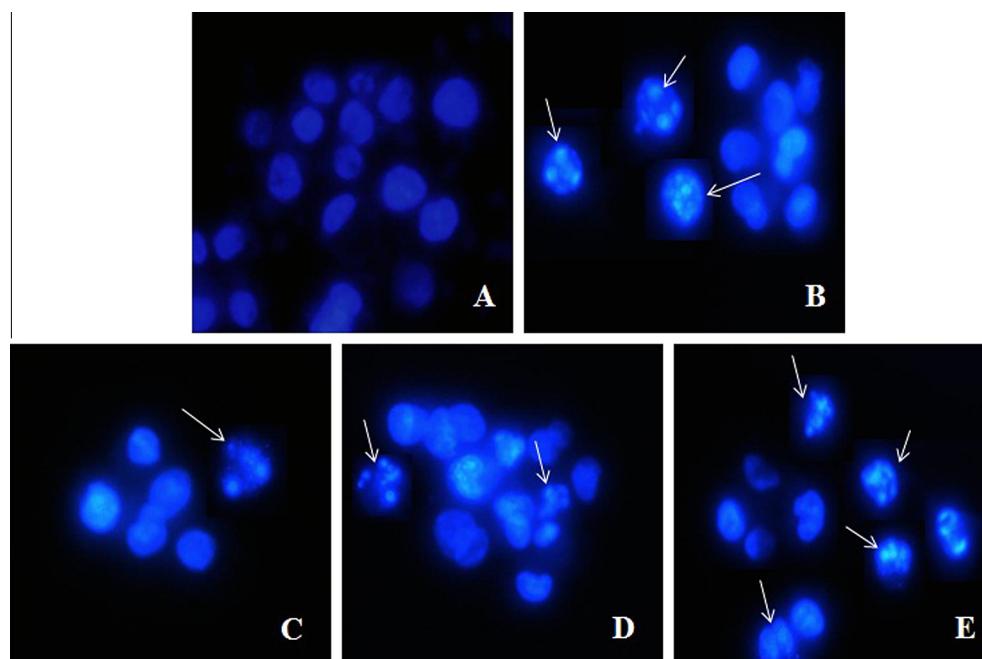


Figure 4 Representative nuclear stained images of A549 cells following treatment with different concentrations of MFKF-AP1 β (25, 50, and 100 $\mu\text{g/ml}$) and 1 μM staurosporine observed under a fluorescence microscope at a magnification of 40 \times . (A) Control; (B) treated with staurosporine; (C–E) MFKF-AP1 β 25, 50, and 100 $\mu\text{g/ml}$, respectively.

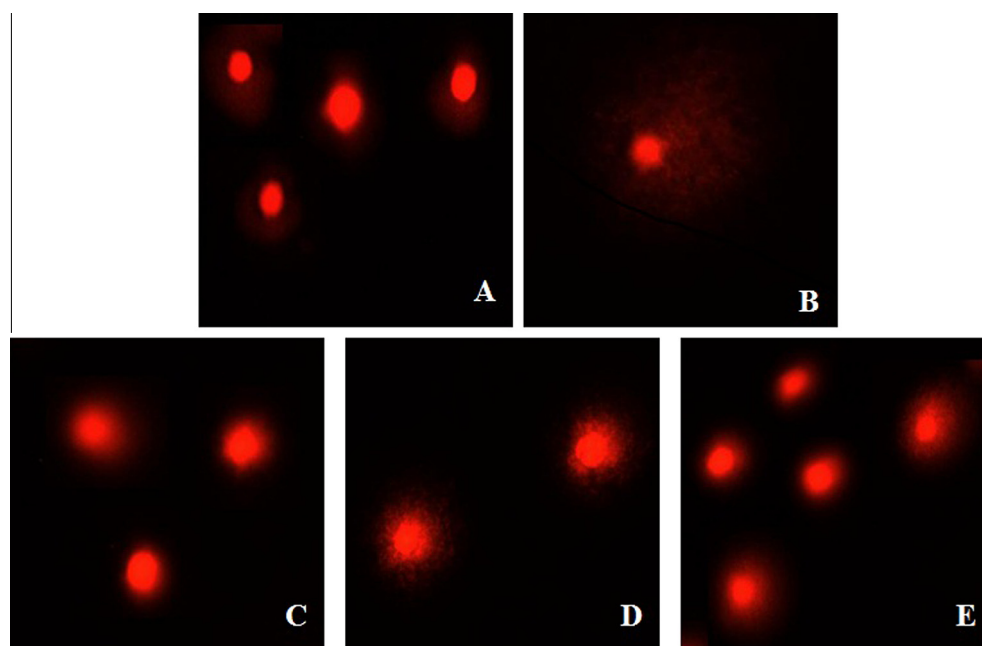


Figure 5 Representative Comet images of A549 cells following treatment with different concentrations of MFKF-AP1 β (25, 50, and 100 $\mu\text{g/ml}$) and staurosporine (1 μM). (A) Control; (B) treated with staurosporine; (C–E) MFKF-AP1 β (25, 50, and 100 $\mu\text{g/ml}$, respectively).

In this study, we first reported a cell growth inhibitory effect for MFKF-AP1 β in lung cancer cells. Exposure to MFKF-AP1 β for 24 h resulted in dose-dependent inhibition of A549 cell growth, as evidenced by cell morphological alterations (Fig. 3). Further, MFKF-AP1 β reduced cellular metabolic activity of A549 cells and potentiated cytotoxic activity via LDH release into the medium (Fig. 3). Up to this point,

the mode of cell death responsible for these results was unconfirmed. Autophagy, apoptosis, and necrosis are all different types of cell death, and apoptosis is the desired mode of cell death in chemotherapy (Amos et al., 1998). Recently, studies have extensively focused on the apoptotic potential of compounds as anti-tumor agents (Rosse et al., 1998). In the current study, we investigated whether or not the cytotoxic effects of

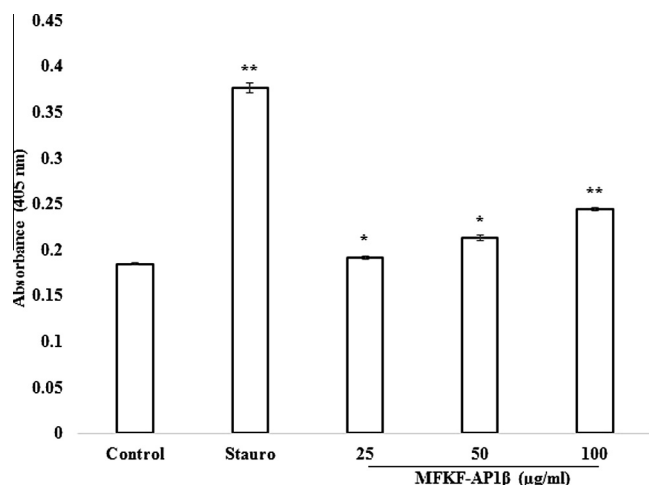


Figure 6 Induction of cell death by MFKF-API β . A549 cells were treated with 1 μ M staurosporine and MFKF-API β (25, 50, and 100 μ g/ml), after which amount of single-stranded DNA was detected by an ApoStrand™ ELISA apoptosis detection kit. An asterisk indicates * $p < 0.05$, ** $p < 0.01$.

MFKF-API β on A549 cells were mediated via apoptosis by examining cellular changes associated with apoptosis using comet assay, Hoechst staining, and apoptosis assay.

Induction of apoptosis is a common mechanism of anti-tumor drugs (Ahmad et al., 2010). Here, we detected apoptotic cells by comet assay with ethidium bromide staining as well as apoptosis assay, which detects formation of single-stranded DNA breaks (Figs. 5 and 6, respectively). MFKF-API β -treated cells exhibited a dose-dependent increase in nuclear chromatin staining, as visualized by Hoechst 33342 (Fig. 4), including morphological characteristics of apoptotic cells such as increased nuclear condensation, nuclear shrinkage, and pycnonuclei formation. In the current study, both Hoechst staining and comet assay confirmed that proportions of apoptotic cells dramatically increased in a dose-dependent manner upon MFKF-API β treatment compared with staurosporine or control cells (Figs. 4 and 5). Therefore, MFKF-API β can potentiate cytotoxicity by promoting apoptosis in cancer cells.

The ability of polysaccharides to induce apoptosis in cancer cells can differ greatly according to their chemical composition and configuration as well as their physical properties. Although it is difficult to correlate the structure and anti-tumor activity of complex polysaccharides, a relationship can be inferred. It has been suggested that structural features in the main chain of the glucan such as β -(1 \rightarrow 3) linkages as well as β -(1 \rightarrow 6) branch points are needed for anti-tumor activity (Miyazaki and Nishijima, 1981; Chihara, 1992). High molecular weight glucans appear to be more effective than low molecular weight species (Mizuno, 1996, 1999a,b). The polysaccharide used in the present study was a partially purified polysaccharide fraction with an average molecular weight of over 12 kDa. Its exact mechanism of action for activating apoptosis remains to be identified.

5. Conclusion

In conclusion, our studies prove that MFKF-API β induces cell death in A549 lung carcinoma cells in a dose-dependent

manner as well as apoptosis by elevation of LDH release, disruption of cellular morphology, and induction of nuclear damage. However, further investigation about the relationship between MFKF-API β -mediated cytotoxic and apoptotic pathways is needed.

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