INTRODUCTION

Damage to DNA and oxidative stress play important roles in various illnesses and pathological conditions, including carcinogenesis and aging, in humans. Cancer is the second leading cause of mortality worldwide, and scientific society and commercial sectors show very strong interests in discovering new anticancer agents from natural sources (Fouche et al., 2008). In recent years, some scientific evidences indicated that certain bioactive proteins and peptides could have several beneficial effects on human health (Möller et al., 2008).

Egg is an important source for proteins, lipids, vitamins, and minerals, and is an excellent source for biologically active substances (Mine, 2007). Phosvitin is a phosphoglycoprotein present in egg yolk and represents about 7% of yolk proteins (Abe et al., 1982; Itoh et al., 1983). The molecular weight of phosvitin ranges from 35 to 40 kDa and is composed of 217 amino acid residues. More than 50% of the amino acid residues in phosvitin are serine, and 90% of these are phosphorylated (Mecham and Olcott, 1949; Byrne et al., 1984; Clark, 1985). This specific structure makes phosvitin a very strong metal (iron, calcium, magnesium, and so on) chelator. Ko et al. (2011) demonstrated that most iron ions in egg yolk are bound to phosvitin, but the major functions of phosvitin in egg are not fully understood.

ABSTRACT Egg yolk phosvitin is one of the most phosphorylated proteins in nature, and thus has a strong metal-binding ability. The objective of this study was to evaluate the cytotoxic and antigenotoxic activities of phosvitin in vitro. Using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay, the cytotoxicity of phosvitin was evaluated in human cancer cell lines of various tissue origins, including the cervix (HeLa), breast (MCF-7), stomach (AGS), lung (A549 and SK-MES-1), liver (HepG2), and larynx (Hep-2). The growth of all cancer cell lines was inhibited in a dose-dependent manner by phosvitin. Among the cancer cell lines tested, MCF-7 and SK-MES-1 were the least sensitive and HeLa, AGS, and HepG2 were the most sensitive to phosvitin. The 50% inhibition of cell viability values of phosvitin were 5.38, 11.57, 4.78, 6.98, 11.82, 3.93, and 9.97 mg/mL for HeLa, MCF-7, AGS, A549, SK-MES-1, HepG2, and Hep-2, respectively. The protective effects of phosvitin against DNA damage in human leukocytes indicated that phosvitin showed protective effects against the oxidative stress-induced DNA damages in human leukocytes. These results suggested that phosvitin has a high potential to be used as an anticancer agent for humans.

Key words: phosvitin, cytotoxic activity, antigenotoxic activity, cell growth, DNA damage

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tyrosinase and melanin biosynthesis activities (Jung et al., 2012). However, there has been little study on the anticancer activity of phosvitin from egg yolk so far.

The objective of this study was to investigate the antigenotoxic activity of phosvitin against normal human peripheral blood mononuclear cells and cytotoxic activity of phosvitin against human cancer cell lines.

MATERIALS AND METHODS

Materials

Phosvitin was prepared from chicken egg yolk according to the method of Ko et al. (2011). RPMI 1640 medium, Dulbecco’s modified Eagle’s medium (DMEM) medium, minimum essential medium (MEM), fetal bovine serum (FBS), horse serum, and penicillin-streptomycin were purchased from Gibco-Life Sciences (Carlsbad, CA). Hydrogen peroxide, sodium chloride, sodium hydroxide, potassium chloride, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), and potassium phosphate were purchased from Sigma Aldrich Co. (St. Louis, MO). All other organic solvents and chemicals used were of analytical grades.

Cell Lines and Culture Conditions

Human normal and cancer cell lines were purchased from the Korean Cell Line Bank (Seoul National University, Seoul, Korea). Human cervix adenocarcinoma (HeLa) and human liver hepatoblastoma (HepG2) cells were grown in MEM containing 10% heat-inactivated FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL). Human breast adenocarcinoma (MCF-7), human stomach adenocarcinoma (AGS), and human lung adenocarcinoma (A549) cell lines were maintained in RPMI1640 medium containing 10% heat-inactivated FBS, penicillin, and streptomycin. Human lung carcinoma (SK-MES-1) and human larynx carcinoma (Hep-2) cells were grown in DMEM supplemented with 10% heat-inactivated FBS, penicillin, and streptomycin. A normal human lung fibroblastic cell line (MRC-5) was maintained in MEM supplemented with 10% heat-inactivated FBS, penicillin, and streptomycin. All cell lines were cultured at 37°C in a humidified incubator containing 5% CO₂. For cytotoxicity testing, cells were seeded in new dishes and grown to 80% confluency before treatment.

In Vitro Cytotoxicity Test (MTT Assay)

The cytotoxic effect of phosvitin was tested in vitro using the MTT assay as described by Moon et al. (2013). To determine the cytotoxic effects of phosvitin on normal and cancer cell lines, MRC-5, HeLa, MCF-7, AGS, A549, SK-MES-1, HepG2, and Hep-2 cells were treated with 2.5 to 40 mg/mL of phosvitin for 48 h. Cells (3 × 10³/well) were plated at 100 μL of medium/well in 96-well plates. After incubating overnight, phosvitin was added to each cell suspension and incubated at 37°C for 44 h. At the end of the incubation, MTT solution was added, and the plate was incubated for an additional 4 h. The supernatant was removed from each of the cell suspension, and 100 μL of dimethylsulfoxide was added to dissolve the colored formazan crystals produced from the reaction of cell formazan with MTT. The optical density of each solution was measured using a microplate reader at 540 nm (EL311, Bio-Tek Instruments Inc., Seoul, Korea). The experiments were performed in triplicates, and the assays were repeated 3 times (n = 3). The concentration required for a 50% inhibition of cell viability (IC₅₀) was determined graphically (Mosmann, 1983; Carmichael et al., 1987; Park et al., 1999).

DNA Damage Determination by Alkaline Comet Assay

Blood samples were collected in heparinized vials from a peripheral vein of 3 healthy human donors who had no history of smoking/drinking or chronic medications. Leukocytes were isolated as a fraction of mononuclear cells (containing lymphocytes and monocytes) by density gradient centrifugation with Histopaque-1077 at 250 × g for 5 min at 4°C. The leukocytes isolated were washed with 1 mL of PBS and used for the comet assay (Singh et al., 1988; Kim et al., 2012). Leukocytes were incubated with 50, 250, and 500 μg/mL of phosvitin, which were dissolved in distilled water for 30 min at 37°C in a dark incubator. For oxidative stimulus, they were then resuspended in a PBS containing 200 μM H₂O₂ for 5 min on ice. After treatment, the leukocyte samples were centrifuged at 250 × g for 5 min at 4°C and washed with PBS. The sample without oxidative stimulus (H₂O₂) was used as a negative control. The leukocytes were then mixed with 75 μL of 0.7% low-melting agarose gel, and added to a slide precoated with 1% agarose gel. The slide was then immersed in a lysis solution containing 2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% sodium laurylsarcosine, 1% Triton X-100, and 10% DMSO, and incubated for 1 h at 4°C. For the electrophoresis of DNA, the slide was placed in an electrophoresis tank containing a solution prepared with 300 mM NaOH and 10 mM Na₂EDTA (pH 13.0) and run for 20 min at 4°C at an electric current of 25 V/300 ± 3 mA. At the end of electrophoresis, the slide was washed 3 times with a neutralizing buffer (0.4 M Tris, pH 7.5) and then treated with ethanol for 5 min before staining with ethidium bromide solution (20 μg/mL). Measurements were made by image analysis (Komet version 5.0, Kinetic Imaging, Liverpool, UK) and fluorescence microscope (Leica DMLB, Bensheim, Germany), determining the percentage of fluorescence in the tail (tail intensity; 50 cells from each of 2 replicate slides).
**Statistical Analysis**

All cytotoxicity of phosvitin results were presented as mean ± SE, and statistical analysis was performed using the SPSS (Chicago, IL) package for Windows (version 18.0). The IC\(_{50}\) value was calculated by Soft Max Pro version 6.3. The mean values were compared using the one-way ANOVA (ANOVA) followed by Duncan’s multiple range tests (\(P < 0.05\)).

**RESULTS AND DISCUSSION**

**In Vitro Cytotoxicity of Phosvitin**

Different cells had a different sensitivity to the growth inhibition effect of phosvitin (Figure 1). The result, expressed as IC\(_{50}\), means the effective concentration of phosvitin required for 50% cytotoxic activity under the experimental conditions (Table 1). In the present study, the cytotoxic effect of phosvitin on normal cells (MRC-5) was significantly different from that on human cancer cells. At all concentrations of phosvitin, there was no more than 20% of cytotoxic activity against MRC-5 cells (data not shown).

Phosvitin showed a strong cytotoxic effect and the effect increased as the level of phosvitin increased. Phosvitin at 40 mg/mL displayed 94.63% cytotoxicity to HeLa cells (Figure 1A) and the IC\(_{50}\) value of phosvitin to HeLa cells was 5.38 mg/mL (Table 1). Phosvitin showed weak cytotoxic activity to MCF-7 cells at a low concentration (10 mg/mL), but it suppressed the growth of MCF-7 cells in a dose-dependent manner (Figure 1B). Phosvitin inhibited the growth of AGS cells by 40.84, 55.30, 62.03, 88.23, and 93.67% at concentrations of 2.5, 5, 10, 20, and 40 mg/mL, respectively. The IC\(_{50}\) value of phosvitin to AGS cells was 4.78 mg/mL (Figure 1C) and that of A549 cells was 6.98 mg/mL (Figure 1D). The SK-MES-1 cells were the least sensitive to phosvitin among the cancer cell lines tested. The IC\(_{50}\) value of phosvitin to SK-MES-1 cells was 11.825 mg/mL (Table 1), but increasing the concentration of phosvitin from 2.5 to 5 mg/mL resulted in no growth inhibition, as assessed by the MTT assay (Figure 1E). Phosvitin showed an IC\(_{50}\) value of 3.93 mg/mL to HepG2 cells, and >70% cytotoxicity was observed at the 10 mg/mL level. The effect of phosvitin to HepG2 cells was more pronounced than other cancer cells tested (\(P < 0.05\)). The IC\(_{50}\) value of phosvitin to Hep-2 cells was 9.97 mg/mL (Figure 1G), which is more resistant to phosvitin than HeLa, AGS, A549, and HepG2 cells.

Jung et al. (2012) demonstrated that cell viability decreased significantly in melanoma cells exposed to 1 mg/mL of phosvitin. However, no significant decrease in cell viability at the 5 to 500 μg/mL level was observed. Among the egg white proteins, ovotransferrin displayed relatively high cytotoxicity at 40 mg/mL. At lower concentrations (≤10 mg/mL), however, the cytotoxic effects of ovotransferrin were not significant in all cancer cell lines tested (Moon et al., 2012). Moon et al. (2012) also demonstrated that ovotransferrin hydrolysates have higher cytotoxic activity than native ovotransferrin. Compared with ovotransferrin, phosvitin showed cytotoxicity at much lower concentrations (e.g., IC\(_{50}\) value of 3.93 mg/mL to HepG2 cells). Oguro et al. (2001) demonstrated that glycopeptides of ovomucin had antitumor effect and inhibited tumor growth in a double-grafted tumor system in mice (Watanabe et al., 1998).

**Inhibitory Effects on H\(_2\)O\(_2\)-Induced DNA Damage**

The comet assay, also known as the single cell gel electrophoresis assay, has been used as one of the standard methods for assessing direct and indirect damages to DNA. Reactive oxygen species such as hydrogen peroxide, superoxide anion, singlet oxygen, and hydroxyl radical are well-known stress agents for DNA damages (Stohs and Bagchi, 1995). When oxidative stress continuously occurs, it causes DNA damage in cells and raises the risk of diseases such as aging or cancer (Ames et al., 1993).

The genotoxic effects of H\(_2\)O\(_2\) and the protective ability of phosvitin were assessed in normal human leukocytes using the comet assay (Figure 2). The protective effect of phosvitin against DNA damages increased in a dose-dependent manner over the range of 50 to 500 μg/mL. When phosvitin was added at the concentrations of 50 to 500 μg/mL, the tail portion (%) of DNA (damaged portion) was 23.62 to 15.48%, whereas that of the positive control was 35.04%, which was a significant reduction from the positive control. At a higher level of phosvitin (500 μg/mL), the damaged DNA was reduced by 55.8%. The concentrations of phosvitin that produced 50% reduction in DNA damage was 424.59 μg/mL.

Lee et al. (2002) reported that phosvitin inhibited lipid oxidation in phosphatidylcholine liposomes, muscle homogenates, and ground pork under the pH, temperature, and NaCl conditions expected in processed muscle foods. Ishikawa et al. (2004) demonstrated that the ability of phosvitin and its hydrolysates in inhibiting the iron-catalyzed hydroxyl radical formation. Ishikawa et al. (2005) reported that egg yolk phosvitin showed protective effects on DNA against UV-light-induced lipid peroxidation in the presence of ionic iron because phosvitin accelerated Fe(II)-dependent autoxidation by decreasing the availability of Fe(II) to participate in -OH-generating Fenton reactions. The ability of egg yolk phosvitin to chelate metal ions may be another important reason for its inhibitory activity on UV-light-induced DNA damage in mouse skin homogenates. Phosvitin is also reported to control tyrosinase activity and melanogenic enzyme expression (tyrosinase TRP-1, and TRP-2) by inhibiting MITF in B16F10 melanoma cells (Jung et al., 2012). The results...
Figure 1. The cytotoxic effects of phosvitin on various human cancer cell lines by 3-[4,5-dimethythiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. (A) Human cervix adenocarcinoma (HeLa) cell, (B) human breast adenocarcinoma (MCF-7) cell, (C) human stomach adenocarcinoma (AGS) cell, (D) human lung adenocarcinoma (A549) cell, (E) human lung carcinoma (SK-MES-1) cell, (F) human liver hepatoblastoma (HepG2) cell, and (G) human larynx carcinoma (Hep2) cell. Values are means with SE. Values not sharing the same letter (a–e) are significantly different ($P < 0.05$; n = 3). Cytotoxic effect (%) = 1 − (absorbance in the samples/absorbance in the control) × 100. Conc. = concentration.

Table 1. The concentrations inhibiting 50% of the cell growth (IC50) value of phosvitin on various human cancer cell lines

<table>
<thead>
<tr>
<th>Item</th>
<th>HeLa</th>
<th>MCF-7</th>
<th>AGS</th>
<th>A549</th>
<th>SK-MES-1</th>
<th>HepG2</th>
<th>Hep-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosvitin</td>
<td>5.38 ± 2.25</td>
<td>11.57 ± 1.19</td>
<td>4.78 ± 1.71</td>
<td>6.98 ± 1.82</td>
<td>11.82 ± 1.21</td>
<td>3.93 ± 0.96</td>
<td>9.97 ± 0.18</td>
</tr>
</tbody>
</table>

1Values are means with SE (n = 3).
2IC50 value means the effective concentration of phosvitin required for 50% cytotoxic activity under the experimental condition. HeLa, human cervix adenocarcinoma; MCF-7, human breast adenocarcinoma; AGS, human stomach adenocarcinoma; A549, human lung adenocarcinoma; SK-MES-1, human lung carcinoma; HepG2, human liver hepatoblastoma; Hep-2, human larynx carcinoma.
of this study indicated that phosvitin has antigenotoxicity and cytotoxic activity against several human cancer cell lines and has some potential to be used as an anticancer agent for humans.

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