First report of a haemagglutinin-induced apoptotic pathway in breast cancer cells

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Synopsis

A dimeric 64 kDa HA (haemagglutinin) was isolated with a high yield from dried Phaseolus vulgaris cultivar ‘French bean number 35’ seeds. It inhibited the proliferation of hepatoma HepG2 cells and breast cancer MCF-7 cells with an IC50 of 100 and 2 μM respectively. After exposure of MCF-7 cells to the HA for 24 h, a number of changes were detected in the cells. Growth arrest in the G0/G1 and G2/M phases was observed. The number of cells undergoing early apoptosis and late apoptosis increased. Disruption of the mitochondrial transmembrane potential and disorganization of the inner mitochondrial membrane were induced. Western-blot analysis disclosed that the HA induced apoptosis through the death receptor-mediated pathway.

Key words: apoptosis, breast cancer cell, French bean, haemagglutinin, lectin, p53

INTRODUCTION

Lectins and HAs (haemagglutinins) are carbohydrate-binding proteins present in diverse organisms [1–3]. They display a number of biological activities such as antitumour [1], antifungal [4], antiviral [5] and antibacterial [6] activities. Lectins and HAs, which are usually abundant storage proteins in leguminous plants [7], act as defensive proteins against insects [8], animals [9] and phytopathogenic micro-organisms [7].

Lectins and HAs have been investigated for potential application as antiviral [10], and antitumour agents. Phytohaemagglutinin, pokeweed mitogen, soya-bean agglutinin and wheatgerm agglutinin restrict tumour growth and improve the life expectancy of the host [11]. Galectins play important roles in immune and inflammatory responses, tumour development and progression, neural degeneration, atherosclerosis, diabetes, and wound repair. They may be applied as therapeutic agents for inflammatory diseases, cancers etc. [12].

In order to increase its potential for clinical application, the detailed apoptotic mechanism should be studied. There are two main pathways of apoptosis: the death receptor-mediated pathway and mitochondrial pathway. Several plant lectins can induce tumour cell apoptosis [13–16]. However, there is a scarcity of reports pertaining to the pathway involved [17–21].

There are very few reports in the literature about the induction of apoptosis in MCF-7 cells by HAs. No information about the detailed apoptotic pathway involved is available. Thus the objective of the present study was to isolate an HA from Phaseolus vulgaris cultivar ‘French bean number 35’, and to investigate in detail the apoptotic pathway it induces in MCF-7 cells. The results revealed that the apoptosis involves a death receptor-mediated pathway. The investigation of the apoptotic pathway in MCF-7 cells indicates its potential for clinical application as an antitumour drug.

MATERIALS AND METHODS

Purification of HA

Dried seeds of P. vulgaris cv. ‘French bean number 35’ (100 g) from China were homogenized. After centrifugation, the supernatant was applied on a Blue-Gel column (20 mM Tris/HCl, pH 7.2). Adsorbed proteins were eluted with 0.5 M NaCl, dialysed, and then chromatographed on a DEAE-Sepharose column (20 mM Tris/HCl, pH 7.2). Adsorbed proteins were first eluted with 1 M NaCl. The adsorbed fraction was dialysed and freeze-dried before chromatography on a Superdex 75 HR10/30 column.
Assay for haemagglutinating activity

The haemagglutinating assay was conducted as described in [14].

Inhibition of HA-induced haemagglutination by carbohydrates

The haemagglutinating inhibition tests were performed by using D-mannose, D-glucosamine, D-sorbitol, D-galacturonic acid, D-xylose, D-fructose, D-galactose, D-fucose, D-raffinose, D-glucose, α-lactose, L-arabinose, mannitol sucrose and D-glucuronic acid [14]. Glycoproteins, including pregnant mare serum gonadotropin, human chorionic gonadotropin and ovalbumin, were also tested.

Assay of antiproliferative activity

The assay of the antiproliferative activity of the isolated HA was carried out by testing its inhibitory effect on the growth of human hepatoma HepG2 cells, human breast cancer MCF-7 cells and normal embryonic liver WRL68 cells. The cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum and 1% penicillin–streptomycin, in a humidified atmosphere of 5% CO₂ at 37°C. The cells (5 × 10⁵ cells/100 μl per well) were seeded on to a 96-well culture plate and serial dilutions of a solution of the HA or doxorubicin (as a positive control) in 100 μl of medium were added. A solution of RPMI 1640 medium was added as a negative control. After incubation for 48 h, the cells were harvested and stained with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide]. The attenuation of the samples at 595 nm was measured and was directly correlated to the level of its inhibitory activity. All the values reported are the means for triplicate experiments [22].

Annexin V and propidium iodide staining

Phosphatidyserine externalization (loss of membrane asymmetry) was examined by using the annexin V–FITC and propidium iodide staining method. Cells (5 × 10⁵) were plated on to a 6-well culture plate and incubated with various concentrations of the HA for 24 h. The cells were trypsinized before spinning down (2000 g, 4 min) and were washed with PBS and then centrifuged (2000 g, 4 min). Cells were resuspended in 250 μl of binding buffer (0.01 M Hepes, pH 7.4, containing 140 mM NaCl and 25 mM CaCl₂) and then stained with 250 μl of binding buffer containing 2.5 μl of annexin V solution (BD Pharmingen) and 0.5 μl of propidium iodide (6 mg/ml) (Sigma) in the dark at room temperature (25°C) for 20 min before analysis using a FACS Sort flow cytometer (Becton Dickinson, Cowley, U.K.). The signal was detected with a FL-1 (530 nm) channel and data analysis was conducted by using the program WinMDI (version 2.8; Joseph Trotter, La Jolla, CA, U.S.A.) [14].

Measurement of mitochondrial transmembrane potential by JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetracyethylrhodaminecarbocyanine iodide) staining

The fluorescent dye JC-1 was employed for the determination of mitochondrial transmembrane potential [23]. Briefly, cells (5 × 10⁵) were treated with various concentrations of the HA for 24 h before harvesting. They were then washed twice with PBS. The cell pellets were resuspended in 500 μl of plain RPMI 1640 medium containing 2.5 μg/ml JC-1 dye, and left in the dark for 15 min at 37°C. The mitochondrial depolarization patterns of the cells were observed by using a FACS Sort flow cytometer.

Cell cycle analysis

Cells (5 × 10⁵ per well) were seeded on to 6-well plates and incubated in the presence of various concentrations of the HA for 24 h. They were then harvested, washed with PBS and fixed in 70% (v/v) ice-cold ethanol at 4°C for 2 h. Afterwards, the cells were washed twice with PBS and resuspended in 1 ml of stain solution (20 μg/ml propidium iodide in PBS, containing 1% Triton X-100 and 10 μg/ml RNase A) at 37°C in the dark for 30 min. Fluorescence emitted from the propidium iodide–DNA complex was examined by flow cytometry [24].

DNA fragmentation detection

Cells collected after HA treatment for 24 h were resuspended in cell lysis buffer and incubated at 37°C for 2 h. Then, saturated NaCl solution was added. After vortex-mixing and centrifugation, DNA was precipitated with ice-cold 100% ethanol. After centrifugation, the pellet obtained was rinsed in 75% ethanol, air-dried, and dissolved in a buffer containing RNase A. Samples were then analysed by agarose-gel electrophoresis [25].

Subcellular localization of bimane-labelled (fluorescent) HA in MCF-7 cells

The freeze-dried HA was dissolved in 2 mM Mes buffer (pH 5.0) in the fluorescent dye bimane amine (final concentration: [150 mM ammonium bicarbonate, pH 7.2], which had been calibrated with molecular mass markers. The first fraction represented purified HA (64 kDa). The homogeneity was attested to be a single peak in every amino acid sequencing cycle using Edman degradation and by a single band in SDS/PAGE (32 kDa).
10 mM) (Invitrogen) and the linker EDC [N-ethyl-N’-(3-dimethylaminopropyl)carbodi-imide; final concentration 2 mM; Invitrogen]. The mixture was incubated at room temperature for 2 h with gentle stirring before centrifugation (10 min, 14,000 rev./min) to remove any precipitated protein. An Amicon filter [Millipore; MWCO (molecular-mass cut-off)=5000] was used to remove salts, unbound bimane amine and EDC from the labelled protein [26]. The Bradford protein assay was used to determine the protein concentration. The MCF-7 cells (60,000 in 2 ml of RPMI 1640 medium) were seeded in a cell-growth chamber and incubated with 15 μM of labelled HA for 24 h. MCF-7 cells without HA treatment acted as a negative control. Cells were washed with PBS twice before analysis with an SP5 confocal microscope (Leica Microsystems, Heidelberg, Germany) using λex = 388 nm, and blue fluorescence images was captured.

**Western-blot analysis of whole-cell lysate**

Cells (1×10⁷) were treated with HA for 24 h. The cells were disrupted in 200 μl of lysis buffer (4.9 mM MgCl₂, 100 mM NaVO₃, 10 % Triton X-100, 100 mM PMSF, 2.1 mg/ml aprotinin and 1 mg/ml leupeptin in PBS) on ice for 15 min. The lysates were boiled for 10 min and centrifuged (14,000 g, 10 min). The supernatants were saved. Protein concentration was quantified by using the Bradford assay. The supernatant (30 μg of protein) was mixed with the loading dye, subjected to SDS/15 % PAGE and the corresponding HRP (horseradish peroxidase)-conjugated secondary antibody (Santa Cruz Biotechnology) for 90 min at 4°C. Detection was performed using an ECL® kit (GE Healthcare) [27].

**Western-blot analysis of mitochondrial and cytosolic fractions**

HA-treated cells (1×10⁷) were harvested, washed by PBS, and resuspended in 50 μl of buffer A (250 mM sucrose containing 75 mM NaCl, 1 mM NaH₂PO₄, 8 mM Na₂HPO₄, 100 mM PMSF, 2.1 mg/ml aprotinin, 1 mg/ml leupeptin and 0.5 mg/ml digitonin in PBS). The cell suspensions were vortex-mixed and centrifuged (2000 g, 1 min). The supernatants obtained constituted the cytosolic fraction. The pellets were resuspended in 50 μl of buffer B (250 mM sucrose, 75 mM NaCl, 1 mM NaH₂PO₄, 8 mM Na₂HPO₄, 100 mM PMSF, 2.1 mg/ml aprotinin, 1 mg/ml leupeptin and 5 mg/ml digitonin in PBS) and centrifuged (2000 g, 1 min), and the supernatants were saved as the mitochondrial fraction [27]. Western-blot analysis was conducted using specific primary antibodies [mouse monoclonal anti-cytochrome c antibody; mouse monoclonal anti-human β-actin and anti-AIF (apoptosis-inducing factor) antibodies; Santa Cruz Biotechnology], as described above.

**RESULTS**

The French bean extract was resolved on Blue-Gel into fractions B1 and B2 (Figure 1A). Fraction B2 was separated on DEAE-Sepharose into fractions D1 and D2 (Figure 1B). Fraction D2 was subsequently separated on Superdex 75 HR10/30 into a major fraction S1 with haemagglutinating activity (Figure 1C). Fraction S1 represented purified HA as witnessed by a single 32 kDa band in SDS/PAGE (results not shown). There was no inhibition when the following sugars or glycoproteins were tested up to 100 mM: D-glucosamine, mannitol, D-xylene, sucrose, D-fucose, D-raffinose, α-lactose, D-fructose, L-arabinose, D-galacturonic acid, D-galactose, D-mannose, D-glucuronic acid, D-glucose, D-sorbitol, pregnant mare serum gonadotropin, human chorionic gonadotropin and ovalbumin.

The French bean HA reduced the viability of MCF-7 cells and HepG2 cells, with an IC₅₀ of 2.0±0.3 and 100±15 μM (mean ± S.D., n = 3) respectively. By comparison, the positive control doxorubicin exhibited an antiproliferative activity towards these tumour cells with an IC₅₀ of 5.0±2.1 and 10±3.3 μM (mean ± S.D., n = 3) respectively. French bean HA (up to 100 μM) had no antiproliferative effect on normal embryonic liver WRL68 cells (Figure 2).

For investigating the mode of cell death, cell cycle analysis was performed at different concentrations of the HA. When compared with the control, the distribution of cells in various phases of the cell cycle was affected by treatment with the HA for 24 h: in the G₀/G₁ phase and G₂/M phase arrests were discerned when the following sugars or glycoproteins were tested up to 45 μM HA. G₀/G₁ phase and G₂/M phase proportions were increased as the concentration of HA approached 45 μM HA. G₀/G₁ phase and G₂/M phase arrests were discerned in a dose-dependent manner (Figure 3).

To corroborate that cell death induced by the HA was associated with apoptosis, phosphatidylserine externalization onto the cell surface was investigated by using annexin V-FITC/propidium iodide staining. The results disclosed that the proportion of annexin V-stained cells representing both the early and late apoptotic cells increased as the concentration of HA applied was increased (Figure 4A). At low concentrations of HA (0.5, 1.5 and 5 μM), a substantial percentage of cells was already present in the early phase of apoptosis. Exposure to higher concentrations (15 and 45 μM) of the HA ensued in a shift of the cell population to late apoptotic/necrotic stage. The table in Figure 4(A) shows that the percentage of MCF-7 cells undergoing apoptosis increased as the HA concentration increased.

Early cellular apoptosis is escorted by disruption of mitochondrial membrane, resulting in a rapid dissipation of the electrochemical gradient [28]. The effect of the HA on mitochondrial transmembrane potential of MCF-7 cells was studied by using a mitochonerdron-specific dye, JC-1. Flow cytometric analysis
Figure 1  Purification of French bean HA
(A) Affinity chromatography of French bean seed extract on Blue-Gel column (5 cm × 13 cm). (B) Anion-exchange chromatography of fraction B2 on a DEAE-Sepharose column (5 cm × 15 cm). (C) Gel filtration of fraction D2 on a Superdex 75 HR10/30. S1 represents the purified French bean HA.

Figure 2  Effects of French bean HA on inhibition of proliferation of hepatoma HepG2 cells, breast cancer MCF-7 cells and normal embryonic liver WRL68 cells
Values are expressed as means ± S.D. (n = 3). The Figure shows the results of one of three experiments.

revealed that more cells became susceptible to mitochondrial membrane depolarization when the HA concentration was elevated (Figure 5). A remarkable attenuation (39.3 %) of mitochondrial transmembrane potential occurred in cells exposed to 45 μM HA.

The subcellular localization of bimane-labelled (fluorescent) HA was investigated. The labelled HA was not detected on the cell surface or inside the cells under an SP5 confocal microscope (results not shown). The results suggested that the HA was not taken up by the MCF-7 cells.

As the concentration of the HA increased, there was a dose-dependent increase in the expression of FAS, truncated Bid, p53, Bak, cytosolic cytochrome c, cytosolic AIF and truncated lamin A/C. In contrast, there was a dose-dependent reduction in the expression level of the anti-apoptotic factor, Bcl-2, and the pro-forms of upstream initiator caspases, pro-caspase 8 and pro-caspase 9. The expressions of Bax and total cytochrome c and total AIF remained unaltered (Figure 6).

The percentage of cells undergoing apoptosis/necrosis underwent a decline upon the addition of the caspase 8 inhibitor Z-IETD-FMK. The results are summarized in the table in Figure 4(B). After exposure to Z-IETD-FMK, an approx. 60 % decrease in the percentages of HA-treated MCF-7 cells undergoing apoptosis/necrosis was observed, indicating that Fas and caspase 8 are involved in the apoptotic pathway.

DNA fragmentation was not observed in HA-treated MCF-7 cells (results not shown).

The proposed pathway of the apoptosis induced by French bean HA is presented in Figure 7. Accompanying the dose-dependent increase in the expression of p53, morphological changes were observed (Figure 8).

DISCUSSION

French bean HA brings about G0/G1 phase and G2/M phase arrest in MCF-7 cells. It appears that apoptosis is induced by the HA
Apoptosis in MCF-7 cells by haemagglutinin

Figure 3 Cell cycle analysis of MCF-7 cells after HA treatment

Cells were treated with French bean HA (0, 3, 15 and 45 μM) for 24 h. Cells were harvested and stained with propidium iodide and subjected to flow cytometric analysis. The table summarizes the percentages of cells in each phase of the cell cycle after HA treatment. Data in the same vertical column but different rows, referring to the same phase of the cell cycle and different HA concentrations, that bear different superscripts are significantly different ($P < 0.05$) from one another when analysed by ANOVA followed by Duncan’s multiple range test. Values are means ± S.D. ($n = 3$). The Figure shows the results of one of three experiments.

<table>
<thead>
<tr>
<th>Hemagglutinin (μM)</th>
<th>G0/G1 (%)</th>
<th>S (%)</th>
<th>G2/M (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>42.5 ± 2.8 $^a$</td>
<td>42.7 ± 4.6 $^a$</td>
<td>14.8 ± 1.6 $^a$</td>
</tr>
<tr>
<td>3</td>
<td>43.6 ± 3.8 $^a$</td>
<td>37.5 ± 3.3 $^b$</td>
<td>18.9 ± 2.5 $^b$</td>
</tr>
<tr>
<td>15</td>
<td>52.5 ± 2.9 $^b$</td>
<td>21.9 ± 3.0 $^c$</td>
<td>25.6 ± 3.3 $^c$</td>
</tr>
<tr>
<td>45</td>
<td>55.5 ± 3.2 $^b$</td>
<td>14.5 ± 1.4 $^d$</td>
<td>30.1 ± 4.0 $^d$</td>
</tr>
</tbody>
</table>

due to the release of cytochrome $c$ from the mitochondria. It has been reported that cytochrome $c$ elicits G1 and G2/M cell cycle arrest and apoptosis in murine J774 cells [29]. In addition, from the results of the annexin V–FITC/propidium iodide staining experiment, the antiproliferative effect of the HA on MCF-7 cells is a consequence of apoptosis.

Consistent with the loss of mitochondrial transmembrane potential, an efflux of some mitochondrial inter-membrane space.
A

<table>
<thead>
<tr>
<th>Concentration of hemagglutinin (μM)</th>
<th>Normal cells</th>
<th>Cells undergoing early apoptosis</th>
<th>Cells undergoing late apoptosis/ necrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>74.7 %</td>
<td>4.1 %</td>
<td>20.9 %</td>
</tr>
<tr>
<td>0.5</td>
<td>66.8 %</td>
<td>11.1 %</td>
<td>22.1 %</td>
</tr>
<tr>
<td>1.5</td>
<td>38.0 %</td>
<td>34.9 %</td>
<td>27.1 %</td>
</tr>
<tr>
<td>5</td>
<td>25.4 %</td>
<td>43.1 %</td>
<td>31.4 %</td>
</tr>
<tr>
<td>15</td>
<td>8.4 %</td>
<td>50.4 %</td>
<td>41.1 %</td>
</tr>
<tr>
<td>45</td>
<td>10.8 %</td>
<td>44.2 %</td>
<td>45.0 %</td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>Concentration of hemagglutinin (μM)</th>
<th>% of apoptotic / necrotic cells in absence of caspase-8 inhibitor</th>
<th>% of apoptotic / necrotic cells in presence of caspase-8 inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>25.0</td>
<td>3.5</td>
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<td>5</td>
<td>74.5</td>
<td>11.6</td>
</tr>
<tr>
<td>45</td>
<td>89.2</td>
<td>11.5</td>
</tr>
</tbody>
</table>
Apoptosis in MCF-7 cells by haemagglutinin

Figure 5  Depolarization of mitochondrial membrane in HA-treated MCF-7 cells
Cells were incubated in the presence of different concentrations of French bean HA (0, 0.5, 1.5, 5, 15 and 45 μM) for 24 h. The cells collected were stained with JC-1 dye. Fluorescence emission was measured by a flow cytometer. Region R1 corresponds to the percentage of cells with depolarized mitochondrial membrane. The results shown here are from one of three experiments.

proteins, like cytochrome c and AIF, is also considered as a critical regulatory process in mitochondrion-dependent apoptosis [30].

The augmented expression level of Fas signifies that Fas ligands are the death receptor involved in the apoptotic pathway. There is no bimane-labelled (fluorescent) HA located intracellularly indicating that the HA did not enter into the MCF-7 cells. The association between the HA and Fas ligand is probably achieved by protein–protein interaction. Subsequently this causes increased expression of Fas [31], and activation of caspase 8. The caspase-8 inhibitor, Z-IETD-FMK, was used to deduce the importance of FAS and caspase-8 in the pathway. The sharp decrease in the percentage of Z-IETD-FMK/HA-treated MCF-7 cells undergoing apoptosis/necrosis shows that FAS and caspase-8 are involved in the apoptotic pathway.

The Western blots demonstrate that the protein level of truncated Bid, a pro-apoptotic protein, surges abruptly as the HA concentration is raised. This is attributed to the processing of Bid into a truncated form, tBid. Simultaneously, down-regulation of Bcl-2 (anti-apoptotic protein), together with an up-regulation of p53 and Bak (pro-apoptotic proteins), are also observed in this apoptotic incident. The expression of Bax (pro-apoptotic proteins) remains unaltered. These prompt cytochrome c and AIF release from mitochondria. Caspase 9 is then activated, causing the truncation of lamin A/C that targets cell shrinkage and membrane blebbing, ultimately disposing the cells to apoptosis. DNA damage causes the release of p53 from the nucleus, which further increases the total expression of p53.

p53 plays a key role in cellular proliferation, differentiation and apoptosis [32]. Morphological alterations have been reported due

Figure 4  Induction of apoptosis by French bean HA in MCF-7 cells
(A) Annexin V–FITC/propidium iodide staining of MCF-7 cells treated with different concentrations of French bean HA (0, 0.5, 1.5, 5, 15 and 45 μM) for 24 h. Cells collected were subjected to staining and then analysed with a flow cytometer. The table in (A) summarizes the percentage of cells at each stage of apoptosis. The results shown here are from one of three experiments. (B) Annexin V–FITC/propidium iodide staining of Z-IETD-FMK-treated MCF-7 cells after incubation in the presence of different concentrations of HA. The table in (B) summarizes the percentage of cells undergoing apoptosis/necrosis. The decrease in the percentage of apoptotic/necrotic cells after Z-IETD-FMK treatment and incubation with HA indicated that FAS and caspase 8 are involved in the apoptotic pathway. The results shown here are from one of three experiments.
Western-blot analysis was performed with the antibodies as described in the Materials and methods section. β-Actin was used as an internal control. The increased expression level of Fas indicated that Fas ligands are the death receptors which activate caspase 8. Truncation of Bid, together with the increase in Bak and p53 and down-regulation of Bcl-2, prompted the mitochondrial release of cytochrome c and AIF. Caspase 9 was then activated, causing the truncation of lamin A/C, which targeted cell shrinkage and membrane blebbing, ultimately causing the cells to undergo apoptosis. The Figures shown here are from one of three experiments.

Hepatocellular carcinoma is resistant to Fas-mediated apoptosis [34]. It can explain why the antiproliferative effect of the HA is much more potent towards MCF-7 cells than HepG2 cells. It further proves that apoptosis is caused by interaction of the HA with Fas ligands.

Apoptosis in tumour cells can be brought about by (i) DNA fragmentation, (ii) chromatin condensation and/or (iii) cell shrinkage/membrane blebbing. DNA fragmentation in MCF-7 cells is indiscernible, probably because there is no caspase 3, which targets DNA fragmentation. Chromatin condensation is also caspase 3 dependent. It is likely that cell shrinkage/membrane blebbing occurs in MCF-7 cells undergoing apoptosis.

There are two main pathways of apoptosis, i.e. the death receptor-mediated pathway and the mitochondrial pathway. Many reports illustrate that the mitochondrial pathway is the prevailing pathway of apoptosis in MCF-7 cells [35–40]. By comparison, there are much fewer publications revealing that the death receptor-mediated pathway is the major apoptotic pathway in MCF-7 cells [31,41]. Oestradiol activates the mitochondrial apoptotic pathway in oestrogen-independent MCF-7:5C cells. It does so by stimulating pro-apoptotic Bax, Bak, Bim (Bcl-2-interacting mediator of cell death), Noxa, PUMA (p53 up-regulated modulator of apoptosis) and p53 protein expression to overexpression of p53 [33]. Morphological changes, probably also due to overexpression of p53, are observed in the HA-treated MCF-7 cells.

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Apoptosis in MCF-7 cells by haemagglutinin

Figure 8  Expression of p53 and morphological changes in MCF-7 cells in response to French bean HA

Western-blot analysis was performed with mouse monoclonal anti-p53. β-Actin was used as an internal control. Increased level of p53 after treatment with 15 μM HA induced morphological changes in MCF-7 cells. The results shown here are from one of three experiments.

and eliciting the loss of mitochondrial transmembrane potential and cytochrome c release [42]. However, there is very little information about the proteins that lie in the apoptotic pathway in MCF-7 cells. Although both pathways are implicated in apoptosis, the death receptor cannot be identified in apoptosis induced by cycloheptapeptide [43].

Truncated lamin targets cell shrinkage and membrane blebbing in tumour cells. There are two reports in the literature that describe that lamin B is involved in the apoptotic pathway in MCF-7 cells: one on bile acid derivatives [44] and another on β-lapachone [45]. In the present study, it has been observed that lamin A/C cleavage is involved in the apoptotic pathway induced in MCF-7 cells by French bean HA.

There are very few studies about the pathway involved in lectin/HA-induced apoptosis cells death in the literature. Nevertheless, information on cell lines other than MCF-7 is available. Wheatgerm agglutinin brings about a loss of transmembrane potential, disruption of the inner mitochondrial membrane, liberation of cytochrome c and caspase 9 activation in the Jurkat cell line. However, the mitochondrial apoptotic events are independent of Bax, Bak and Bcl-2 [17]. Targeting of galectin-1 to glycolipids on Fas and subsequent activation of the apoptotic death-receptor pathway occurs in the T-cell lines Jurkat and MOLT-4 [18]. Viscum album coloratum (mistletoe) agglutinin-induced apoptotic COLO cell death is attributed to the activation of caspases 2, 3, 8 and 9 and suppression of receptor-interacting protein, NF-κB (nuclear factor κB), XIAP (X-linked inhibitor of apoptosis protein) and Akt (also known as protein kinase B), partly through the TNFR1 [TNF (tumour necrosis factor) receptor 1] signalling pathway. Both the death receptor-mediated pathway (activation of caspases 2, 3 and 8) and the mitochondrial pathway (activation of caspases 2 and 9) appear to be involved in the action of mistletoe lectin. TNFR1 induces both receptor-mediated and mitochondria-mediated apoptosis [19]. Polygonatum cyrtonema lectin induces an up-regulation of Bax protein, a down-regulation of Bcl-XL and Bcl-2 proteins, collapse of mitochondrial membrane potential, release of cytochrome c and activation of caspases 9 and 3. Thus the lectin triggers apoptosis and autophagy via a mitochondrially mediated ROS (reactive oxygen species)–p38–p53 pathway in A375 cells [13]. TNFR-mediated apoptosis is the predominant pathway induced by Pouteria torta lectin (pouterin) in HeLa cells, by inducing up-regulation of TNFR1 and TRAF2 (TNFR-associated factor), down-regulation of TRADD (TNF-associated death domain), p65 NF-κB subunit and IAP1 (inhibitor of apoptosis protein 1), and finally, nuclear fragmentation. Pouterin also induces an overexpression of p21, which is an indicator of cell cycle arrest [21].

The present paper is the first report to demonstrate HA-induced apoptosis in MCF-7 cells and the concerned apoptotic pathway. Our findings are in sharp contrast to the large number of observations indicating that the mitochondrial pathway is the predominant pathway of the apoptosis caused by non-proteinaceous molecules in MCF-7 cells.

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REFERENCES


14 Lam, S. K., Han, Q. F. and Ng, T.B. (2009) Isolation and characterization of a lectin with potentially exploitable activities from caper (Capparis spinosa) seeds. Biosci. Rep. 29, 299–299


