APOPTOTIC PATHWAYS IN HUMAN BREAST CANCER CELL MODELS (MCF-7 AND MDA-MB-231) INDUCED BY RICE BRAN DERIVED PENTAPEPTIDE

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ABSTRACT

Bioactive peptides derived from food sources with anti-proliferative properties against cancer have drawn more attention in recent years. A pentapeptide derived from rice bran has shown anti-proliferative property on human breast cancer cells. The objective of this study was to investigate the apoptotic pathways induced by the pentapeptide in breast cancer cell models (MCF-7 and MDA-MB-231). The growth inhibition activity of pentapeptide was evaluated by phenazine methosulfate-3-(4,5-dimethyl thiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTS) assay in a dose-dependent manner. The levels of molecular targets (TNF-α, Bax, Bcl-2, Fas, and erbB-2) were evaluated by ELISA kits. Pentapeptide showed growth inhibition activity of 80-85% on MCF-7 and MDA-MB-231 at a dosage of 1000 µg/mL with a significantly high reduction (80%) of MCF-7 cells even at a lower dosage – 400 µg/mL. Significant (p < 0.05) decreases in the levels of Bcl-2 and erbB-2 and increases in the levels of TNF-α and Bax were detected after pentapeptide treatment over an incubation period of 72 to 96 hrs. The results suggest that the rice bran pentapeptide inhibits human breast cancer cell proliferation by introducing apoptosis through Caspase-dependent pathways.

Keywords: Pentapeptide, breast cancer, caspase, biomarker, pathway

1. INTRODUCTION

Breast cancer is one of the leading causes of cancer related deaths and illnesses in the United States. Chemo, radiation, and immune therapies rely heavily on apoptosis to kill breast cancer cells. However, cells can subsequently survive and gain resistance to these treatments leading to a more aggressive cell variant with an inclination to metastasize despite initially responding to the therapy [1]. Recent studies on preparation of small molecular therapeutics’ (like peptides) and mechanism of action in treating, preventing, and management of various diseases have drawn the attention of science community. Among those are food derived peptides with potential antitumor activity where several of these biomolecules motivated clinical trials. In comparison with other chemotherapies, bioactive peptides possess the advantages of high affinity, strong target cells specificity, low toxicity, and high modification feasibility [2]. Apoptosis plays a critical role in the development of a variety of human diseases including cancer, immune system diseases, and neuro-degenerative disorders. Apoptosis is a multi-step, multi-path cell-death program that is inherent in every cell of the body. In the process of tumorigenesis, the ratio of proliferated cell and apoptotic cells is altered and uncontrolled followed by the invasion of tumor cells and metastatic potential [3].

Caspase, a group of cysteine-dependent aspartate-directed proteases, plays an important role in controlling cell apoptosis. The caspase cascade is the primary feature of cell apoptosis which may be activated by two pathways: the intrinsic and the extrinsic pathway [4]. Thus, the observation of the caspase cascade is considered the most important
benchmark for the characterization of the potential efficiency of certain cancer treatment [5]. The Bcl-2 family proteins are considered vital control point of mitochondrial apoptosis in cancer cells. The pro-apoptotic proteins (Bax, Bak, Bad, and Bid, etc.) enlarge the existing transition pore permeability which leads to the release of cytochrome C [6]. While the anti-apoptotic proteins (Bcl-2, Bcl-XL, Mcl-1, and Bfl-1/A1, etc.) located on the outer mitochondrial membrane and protect the cells by forming heterodimers with pro-apoptotic proteins [7]. The interactions between Bcl-2 family proteins affect cellular sensitivity to apoptosis and the ratio of pro-apoptotic proteins and anti-apoptotic proteins determines the fate of cells. TNF-α is a naturally occurring cytokine secreted by cells of the immune system and other systems [8]. Although TNF-α is cytotoxic to some tumor cells, it is rarely cytotoxic to normal cells. This unique property has led to numerous studies of TNF as a chemotherapeutic agent and apoptosis-inducing agent [9]. TNF can activate both apoptotic pathways and survival pathways inside the cell. Fas is a member of the TNF and nerve growth factor (NGF) receptor [10]. It is located on the cell surface and is activated by its ligand (Fas L) binding. Fas then forms the death-inducing signaling complex (DISC), which finally leads to the activation of effector caspases resulting in apoptosis in cells [11, 12]. In recent years, there is a growing interest in studying chemotherapies by targeting specific death receptors such as Fas, to induce apoptosis in human cancer cells.

As important biological indicator of cancer status and progression for the physiological state of the cell at a specific time, the above biomarkers are considered powerful tools for monitoring the course of cancer and gauging the efficacy of a novel therapeutic agent. Thus, the objective of this study was to determine the mechanistic pathways of pentapeptide-induced apoptosis in human breast cancer cell models. This study provided insight on the molecular mechanism of action of the pentapeptide against breast cancer cell lines and some preliminary results on its therapeutic/drug-like property.

2. MATERIALS AND METHODS

2.1. Materials

Pentapeptide (amino acids sequence: EQRPR) was prepared in the lab or received from Biomatik LLC. (Wilmington, Delaware, USA). Human breast cancer cell lines (MCF-7 and MDA-MB-231), Eagle's Minimum Essential Medium, and Leibovitz's L-15 Medium were purchased from ATCC (Manassas, VA, USA). Fetal bovine serum (FBS), insulin, and trypsin-EDTA solution were purchased from Sigma (MO, USA). The phenazine methosulfate 3-(4, 5-dimethyl thiazole-2-yl)-2, 5-diphenyl tetrazolium bromide (MTS) one solution kit and Caspase-Glo®8, 9 kits were obtained from Promega Corporation (Madison, WI, USA). The human ELISA kits for determining levels of Bcl-2 and ErbB-2 were supplied by Abcam Plc. (Cambridge, MA, USA). The TNF-α, Bax, and Fas human ELISA kits were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA), Enzo Life Science Inc. (Farmingdale, NY, USA), and RayBiotech, Inc. (Norcross, GA, USA), respectively.

2.2. Procedures

2.2.1. Cell lines and cell culture

The human breast cancer cell lines (MCF-7 and MDA-MB-231) were cultured following the recommend protocols from ATCC (Manassas, VA, USA). The MCF-7 was cultured in Eagle's Minimum Essential Medium with 0.01mg/mL bovine insulin and 10% FBS. Cells were grown at 37°C in a humidified 5% CO₂ incubator. The MDA-MB-231 was cultured in Leibovitz's L-15 Medium with 10% FBS and incubated at 37°C without CO₂.

2.2.2. Cell viability assay (MTS)

The cytotoxic effects of pentapeptide on the breast cancer cell lines (MCF-7 and MDA-MB-231) were evaluated by the MTS assay [13]. Cells were trypsinized from a 75 cm² flask and the cell numbers were determined using a hemocytometer. The cells were plated on a 96-well plate at a density of 5 x 10⁴ cells per well. After 24 h incubation for cells to attach, the spent media was removed. The pentapeptide at various concentration (50, 100, 200, 400, 500, and 1000 µg/mL), medium alone (negative control), and genistein at 400 µg/mL (positive control) were added to respective well with the final volume of 200 µL and incubated for 96 h (37°C, 5% CO₂). The MTS one solution reagent (40 µL/well) was added and incubated for 1 h. The absorbances of samples were measured at 490 nm using a microplate reader after adding 10% SDS for reaction termination. The cytotoxic effect of the pentapeptide
was expressed as a relative percentage of inhibition calculated as follows: Relative inhibition (%) = (A\text{490}_{Experiment} - A\text{490}_{Control}) \times 100

2.2.3. Assay for caspase-8 and -9 activities

The activities of caspase-8 and caspase-9 were determined by caspase-Glo® assay kits following the company protocol (Promega Corp., USA). The caspase-8, -9 kits reagents were prepared following the instruction and equilibrated to ambient temperature before use. The cells (MCF-7 and MDA-MB-231) were cultured and transferred into the white-walled 96-well plate with a density of 5 x 10^3 per well and allowed to attach for 24 hours. Then, the cells were treated with pentapeptide (1000 µg/mL) for 72 and 96 hours. The cells treated with media alone and genistein (400 µg/mL) in media were referred as negative and positive control. One hundred µl of Caspase-Glo® reagent was added into each well and gently mixed contents using a plate shaker at 300 rpm for 30 seconds, followed by 2 hours incubation at room temperature. The luminescence of each sample was measured using a plate-reading luminometer. The levels of activated caspase-8 and caspase-9 in cells were expressed as relative luminescence intensity.

2.2.4. Determination of TNF-α levels

The levels of TNF-α in pentapeptide-treated breast cancer cell lines (MCF-7 and MDA-MB-231) were determined using human TNF-α ELISA kit following the company protocol (Thermo Fisher Scientific Inc., MA, USA). Cells were cultured and treated with pentapeptide (1000 µg/mL) for 72 and 96 hrs. The culture supernatants were collected and transferred into a TNF-α antibody coated microplate and incubated for 1 hr at room temperature. The unbound materials were removed by washing buffer. Then, the biotinylated antibody reagent was added to each well and incubated for 30 min. The plate was washed using washing buffer three times and the streptavidin-HRP reagent was added and incubated for 30 min. The substrate reagent was added to each well to develop color which the absorbances can be measured at 450 nm using a microplate reader. The concentration of TNF-α in each sample was determined by standard curves plotted using standard TNF-α proteins.

2.2.5. Determination of Bcl-2 family proteins (Bax and Bcl-2) levels

Human ELISA kits were used to determine the levels of Bax and Bcl-2 in breast cancer cells according to the company protocols with similar principle. Cells (MCF-7 and MDA-MB-231) were cultured and incubated in the presence of pentapeptide (1000 µg/mL) for 72 and 96 hr. The cell lysate for each sample was prepared using lysis buffer following by centrifugation and added to a microplate with anti-human Bax or Bcl-2 coating antibodies and incubated for 2 hr. The biotin-conjugated anti-human Bax or Bcl-2 antibodies were added after a washing step. Then, the streptavidin-HRP is added and binds to the biotin conjugated anti-human Bax/Bcl-2 antibodies. The unbound streptavidin-HRP is removed after a wash step following by the incubation and the substrate solution reactive with HRP was added to each well. A colored product was formed in proportion to the amount of human Bax or Bcl-2 in the samples. The standard curves were prepared to determine the human Bax or Bcl-2 levels in each sample.

2.2.6. Determination of Fas and ErbB-2 levels

The levels of Fas and ErbB-2 in pentapeptide-treated MCF-7 and MDA-MB-231 cells were determined using respective human ELISA kits following the company protocols. Briefly, cells were cultured and treated with pentapeptide (1000 µg/mL) for 72 and 96 hr. The cell lysates were prepared using lysis buffer and placed in 96-well plates that coated with monoclonal detective antibodies and incubated for 2 hr. The unbound materials were removed by washing buffer. Then, the horseradish peroxidase conjugated streptavidin was added to bind to the antibodies in respective wells and incubated for 1 hr. The substrate reagent was added to each well and the absorbances were measured at 450 nm using a microplate reader. The concentrations of Fas and ErbB-2 were determined by standard curves plotted using standard proteins.

2.3. Statistical Analysis

All the treatments in assays were conducted in triplicates and the values were reported as means ± standard deviation. The cytotoxic effects of pentapeptide and levels of various molecular targets in human breast cancer cell lines were analyzed by analysis of variance (ANOVA) using the Statistical Analysis System (SAS 9.2 2000, SAS Institute Inc., Cary, N.C., U.S.A.). The Fisher’s protected least
significant difference (LSD) test was conducted to separate the means at $p \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1. Pentapeptide Inhibits the Growth of Human Breast Cancer Cell Lines

Attached human breast cancer cells (MCF-7 and MDA-MB-231) were treated with pentapeptide at various concentrations (50, 100, 200, 400, 500, and 1000 μg/mL). The anti-proliferative activities of pentapeptide at various concentrations have shown a dose-dependent pattern in both cell lines. The maximum growth inhibitions were achieved after treatment with pentapeptide (1000 μg/mL) on MCF-7 (90.9%) and MDA-MB-231 (87.0%) significantly differed from the untreated controls after the same incubation time (96 hrs). This concentration was used in all experiments that determined the levels of molecular targets (TNF-α, Bax, Bcl-2, Fas, and ErbB-2). The anti-proliferative activities of pentapeptide on both breast cancer cell lines were increased with the increasing levels of pentapeptide. There were no significant differences ($p > 0.05$) in growth inhibitions of pentapeptide at levels of 1000 μg/mL and 500 μg/mL on MCF-7 cells. These results suggested that the growth inhibition activity of pentapeptide saturated at a concentration of 500 μg/mL on MCF-7 cell line. The pentapeptide had shown relatively higher anti-proliferative activities on MCF-7 cells at each concentration than on MDA-MB-231 cells (Figure 1).

![Figure 1: The Growth Inhibition Effects of Pentapeptide at Various Concentrations (50, 100, 200, 400, 500, 1000 μg/mL) on MCF-7 and MDA-MB-231 Cells after 96 hours Incubation. The cell lines cultured with media alone are negative controls. Values are means ± standard deviation of three determinations. Values with the same letter are not significant different (P>0.05). The standard deviations are shown by the error bars.](image)

3.2. Pentapeptide Induces Apoptosis in Human Breast Cancer Cells (MCF-7 and MDA-MB-231) Through the Caspases-Dependent Pathway

To investigate the activations of caspase-induced apoptosis in pentapeptide treated human breast cancer cells, the relative intensities of caspase-8 and caspase-9 levels in MCF-7 and MDA-MB-231 cells were evaluated after treatment with 1000 μg/mL of pentapeptide for 72 and 96 hrs and the results are shown in Figures 2 and 3. As the positive control, the genistein (400 μg/mL) treated MCF-7 and MDA-MB-231 cells were also determined for comparison. Significant increases ($p < 0.05$) in levels of activated caspase-8 and caspase-9 were detected at 72 and 96 hrs after the pentapeptide treatment compared to the controls (MCF-7/MDA-MB-231 cultured with media). Pentapeptide induced significant ($p < 0.05$) increases in relative caspase-8 intensity in MCF-7 (from 5.2 to 7.7 folds) and MDA-MB-231 (from 3.8 to 6.9 folds) compared to the untreated cells (Figure 2). The caspase-9 was also significantly ($p < 0.05$) activated after the pentapeptide treatment in MCF-7 (from 2.2 to 2.7 folds) and MDA-MB-231 (from 2.0 to 2.2 folds) cells.

![Figure 2: The Relative Intensity of Activated Caspase-8 in Pentapeptide (1000 μg/mL) Treated MCF-7 and MDA-MB-231 Cells after Incubation for 72 and 96 hours. The cells cultured with media alone and genistein (400 μg/mL) are negative and positive controls, respectively. Values are means ± standard deviation of three determinations. Values with the same letter are not significant different (P>0.05). The standard deviations are shown by the error bars.](image)

Caspases are a group of cysteine proteases divided into two sub-categories including effectors such as
caspase-3 and -7 and initiators such as caspase-8 and -9 [14]. Caspase-dependent and caspase-independent pathways are two major cell apoptosis mechanisms. In the caspase-dependent pathway, the activations of effector caspase-3 and/or -7 are essential in both extrinsic and intrinsic pathways [15]. In the current study, the activated caspase-8 and -9 in cells reacted with aminoluciferin labeled substrates and the relative concentrations were determined by measuring the luminescence intensities. Pentapeptide treatment induced activation of caspase-8 and -9 in human breast cancer cell lines (MCF-7 and MDA-MB-231). Caspase-8 is activated through extrinsic pathway by interactions between ligands and death receptors. Caspase-9 is involved in intrinsic pathway and activated due to the mitochondrial cytochrome c leakage [16]. The results showed that caspase-8 and -9 activation induced apoptosis in MCF-7 and MDA-MB-231 cells by the pentapeptide (Figures 2 and 3). These results suggest that pentapeptide triggers apoptosis via a combination of intrinsic and extrinsic pathways.

### 3.3. The Mitochondrial-dependent Pathway Is Activated by Changing the Bax/Bcl-2 Ratio Involving the Activation of Caspase-9

To determine whether the mitochondrial apoptotic pathway is involved in pentapeptide induced apoptosis in MCF-7 and MDA-MB-231 cells, the expression of anti-apoptotic protein (Bcl-2) and pro-apoptotic protein (Bax) were investigated for indicated times (72 and 96 hrs) after pentapeptide (1000 µg/mL) treatment. Significant ($p < 0.05$) increases in Bax expression and decreases in Bcl-2 levels were observed in pentapeptide treated MCF-7 and MDA-MB-231 cells after 72 and 96 hr compared to the untreated cells (Figures 4 and 5). The expressions of activated Bax increased significantly ($p < 0.05$) in MCF-7 and MDA-MB-231 cells with 2.6 to 5.5 folds and 11.5 to 22.5 folds, respectively. Furthermore, more decreases in Bcl-2 level in MDA-MB-231 (from 6.5 to 16.8 ng/mL) than MCF-7 (from 11.0 to 12.1 ng/mL) were observed along with prolonged incubation time from 72 to 96 hrs.
The mitochondrial-mediated apoptotic pathway is activated through the disruption of mitochondrial membrane potential leading to the change of permeability by opening of transition pores and release of cytochrome C [17, 18]. Then, caspase-9 activates effector caspase-3, -7, and -6, which results in the activation of subsequent apoptosis [19]. The Bcl-2 family proteins, including pro-apoptotic proteins and anti-apoptotic proteins, play a curial role in determining the ultimate fate of cells and the ratio of Bax to Bcl-2 is also a critical element in apoptosis regulation. The pro-apoptotic protein (such as Bax, BAD, Bak, and Bok) inserts into the mitochondria membrane and release the cytochrome c via forming a large channel, while the anti-apoptotic proteins including Bcl-2, Bcl-xL, and Bcl-w could prevent this process [20]. Previous studies have demonstrated the importance of Bax/Bcl-2 ratio in regulating the mitochondria-mediated apoptotic pathway in human breast cancer models [17, 21 - 24]. Our data showed that pentapeptide treatment increased the expression of Bax while down-regulated the Bcl-2 levels, which happened along with the increased levels of caspase-9 and apoptosis in MCF-7 and MDA-MB-231 cells. Therefore, these results indicated that the possible mechanism of pentapeptide-induced apoptosis involved the mitochondrial membrane potential change via controlling the Bax/Bcl-2 ratio.

3.4. Pentapeptide Induced Apoptosis Is Promoted Via A TNF-α and Fas-Dependent Pathway and the Activation of Caspase-8

To assess whether death receptor-mediated pathway is involved in pentapeptide-induced apoptosis, the TNF-α and Fas protein levels were determined in MCF-7 and MDA-MB-231 cells after exposing to pentapeptide. TNF-α levels increased significantly (p < 0.05) after pentapeptide treatment for 72 and 96 hrs compared to negative controls (cell lines cultured only with fresh media) (Figure 6). Even though MCF-7 has shown significant (p < 0.05) lower TNF-α concentrations than in MDA-MB-231 cells, there were more folds of TNF-α activated in MCF-7 (3 folds) than in MDA-MB-231 cells (1.3 to 1.5 folds) after incubation in the presence of pentapeptide (1000 µg/mL) for 72 and 96 hrs. As positive control, genistein resulted in significant (p < 0.05) increases in Fas levels in MCF-7 and MDA-MB-231 cells, significant (p < 0.05). Fas increases were observed in pentapeptide treated cells in a time-dependent manner (Figure 7).

TNF and Fas are considered as members of the death domain receptor family, they activate caspases through their death receptor-activator complexes [25]. Studies have demonstrated that MCF-7 cells is sensitive to TNF-induced apoptosis and TNF-α is an effective inducer of apoptosis in MCF-7 cells [26 - 30]. The TNF-α and Fas-induced apoptotic pathway activated via the interaction of TNF-receptor (TNFR)-associated death domain (DD) and the TRADD, which leads to the recruitment of Fas-associated death domain (FADD) [31]. In turn, the FADD binds pro-caspase-8 and the activated caspase-8 triggers a protease cascade leading to apoptosis in cells [32]. Studies have shown some anticancer agents such as ellipticine, caffeic acid phenethyl ester, doxorubicin, and camptothecin initiate the apoptosis via increasing Fas expression and recruiting death domain receptors such as FADD [33 - 36]. In this study, pentapeptide treatment induced increases in levels of TNF-α, Fas, and Caspase-8 in MCF-7 and MDA-MB-231 cells; these data suggested that the pentapeptide stimulated apoptosis by triggering death receptor signaling involving the activation of TNF-α and Fas followed by the caspase cascade by caspase-8.
Figure 7: The Levels of Fas in Pentapeptide (1000 µg/mL) Treated MCF-7 and MDA-MB-231 Cells after Incubation for 72 and 96 hours. The cells cultured with media alone and genistein (400 µg/mL) are negative and positive controls, respectively. Values are means ± standard deviation of three determinations. Values with the same letter are not significant different (P>0.05). The standard deviations are shown by the error bars.

3.5. Pentapeptide Down-regulate the ErbB-2 Expression in Human Breast Cancer Cell Models

To study the possible regulation of ErbB-2 in pentapeptide induced apoptosis in human breast cancer cell lines, the ErbB-2 levels in MCF-7 and MDA-MB-231 were determined using ELISA assay. Pentapeptide led to significant (p < 0.05) decreases in levels of ErbB-2 in both cell lines. Besides, more down-regulating effects on ErbB-2 levels in MCF-7 cells (from 1.4 to 2.0 folds) than MDA-MB-231 (from 1.1 to 1.6 folds) were observed after pentapeptide treatment (Figure 8).

ErbB-2 is a member of receptor tyrosine kinases, its overexpression results in the resistant to apoptosis which may leads to the failure of chemotherapy of ErbB-positive cancer cells [37]. The high expression of ErbB2 promotes the cell growth by activation of Akt (a serine/threonine kinase), which inactivates pro-apoptotic proteins such as Bax, Bad, and caspase-9 through phosphorylation [38]. Previous studies suggested that down-regulation of ErbB-2 could efficiently suppress the breast tumor cell growth [39 - 42]. The results of MCF-7 and MDA-MB-231 cells treated with pentapeptide suggested that pentapeptide may enhance the apoptotic signal by suppressing the expression of ErbB-2. However, further research in molecular levels is required to explain the role of ErbB-2 in pentapeptide-induced apoptosis.

4. CONCLUSION

The pentapeptide with a sequencing of EQRPR has shown promising anti-proliferative activities against MCF-7 and MDA-MB-231 human breast cancer cell lines and there are at least two apoptotic pathways involved in pentapeptide-induced apoptosis. On one hand, the apoptosis is activated after the increased Bax/Bcl-2 ratio which leads to the release of cytochrome c in mitochondria-mediated pathway. On another hand, pentapeptide may induce apoptosis via death receptor-mediated apoptotic pathway by stimulating the expression of TNF-α, followed by forming the death-inducing signaling complex (DISC) through Fas expression, which results in the activation of caspase-8. Besides, the pentapeptide may amplify apoptotic signals by down-regulating the expression of ErbB-2. The findings in this study indicate potential therapeutic value of pentapeptide and further research in animal tumor models is necessary to confirm its anti-cancer activity in vivo.

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