Antimicrobial peptide Brevinin-2R induces the secretion of a pro-inflammatory cytokine in HepG2 cells

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Abstract

Introduction: Antimicrobial peptides as the body's defense strategy play an important role in resistance against infection of microorganism. These peptides are able to modulate the immune and inflammatory processes through the production of defensive molecules. Therefore, the modulatory effects of Brevinin-2R, an antimicrobial peptide extracted from the skin of the frog (*Rana ridibunda*), was evaluated in this study.
Materials and methods: MTT assay was conducted for evaluating the cytotoxic effect of Brevinin-2R on human liver carcinoma cells (HepG2). Then, real-time PCR was used to assess the inflammatory properties of this peptide.
Results: It was appeared that the Brevinin-2R has a low toxicity against HepG2 cancer cells and can reduce cell growth to 24%. Results of molecular analysis showed that Brevinin-2R increases the expression of IL-1b and IL-6 genes in cancer cells in a dose dependent manner.
Conclusion: The results show that Brevinin-2R has a regulatory role in inflammation through targeting the genes involved in the process.

Keywords: Brevinin-2R, Inflammation, Gene expression

Introduction

Antimicrobial peptides are parts of the endogenous immune to resist infection of microorganism (1). Widely, these peptides as endogenous antibiotics are able to inhibit the growth of pathogens (bacteria, fungi, viruses) (2, 3). So far, the peptides are purified from some invertebrates (insects, ticks) (4) and vertebrates (amphibians) (5, 6). Peptides are amphipathic molecules with hydrophobic and hydrophilic parts, they are positively charged due to the presence of some amino acids including histidine, lysine and arginine in their structure (7). The difference in amino acid composition, size and conformational structures leads to the diversity of the peptides (2). These peptides are involved in immunity and inflammation and can modulate the process through the production of defensive molecules (4). Various investigations have showed that these peptides have the effect of an increase in chemokine and cytokine's production (8, 9). Angiogenesis and wound healing process inflammation is an immune process which is created as a result of the attack pathogens or tissue damage.

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Macrophages play an important role in immune response and the release of nitric oxide (NO), TNF-α, interleukin-6, and -1β as a macrophage-released inflammatory mediators leads to an increase in defense capacity (10). Brevinin-2R is non-hemolytic peptide and a member of the Brevinin-2 family with 25-amino acid (KLKNFAKGVAQSLLNKASCKLSGQC) that derived from secretions of frog skin (11). This peptide has shown antibacterial activity against gram positive and negative bacteria (12). Also, the peptide has shown cytotoxic effects on various cancer cell lines, including Jurkat, BJAB, HT29/219, SW742 cells and etc. (11).This study was conducted to evaluate the effects of the peptide on viability of HepG2 cells and also to evaluate its effect on the expression of genes involved in inflammation include IL-1b and IL-6 genes.

Materials and methods

Cell culture: HepG2 cancer cell line were purchased from NCBI (National Cell Bank of Iran) and cultured in DMEM medium supplemented with 10% Fetal Bovine Serum (FBS) (Gibco, USA), L-glutamine (Sigma, USA) and 1% antibiotic (penicillin/ streptomycin) and incubated at 37 °C in a 5% CO2.

Cell proliferation assay: The cytotoxic effect of Brevinin-2R on the HepG2 cells was evaluated using MTT assay. The HepG2 cells were seeded in 96-well plates at a concentration of 5 × 103 cells/well and incubated for 24 h. After completion of the incubation period the cells were exposed to various concentrations of peptide (10, 20, 40 µg/ml) for 24, 48 and 72 h. After treatment period, the treatments were removed and MTT was added to each well and incubated for 4 hours at 37 ° C in the dark. After incubation, the MTT was removed and replaced with 100 µl DMSO and finally, the optical absorbance was measured at 570 nm with ELISA plate reader. All experiments were done in triplicate.

Peptide Synthesis: The desired peptide (Brevinin-2R) was synthesized chemically using peptide synthesizer (Applied Biosystems Model 432A) and then purified using chromatography (C8 column) method. The column was developed at a flow speed of 2 ml/min by a linear gradient of 5–45 %acetonitrile for 40 min containing 0.1 % TFA. Finally, the purity of Brevinin-2R was determined using an analytical column (4.6 × 250 mm, manufactured by Macherey-Nagel GmbH & Co., Du¨ren, Germany) (13).

Real time PCR: Real time PCR was conducted in order to detection of changes in inflammatory genes expression. Total cellular RNAs of treated (different concentration of Brevinin-2R) and untreated cells were extracted using the High Pure Isolation kit (Roche, Germany) according to the manufacturer's guidelines. Then the RNA was used to determine the quantity and purity at wavelength 260 and 280 nm using Nano drop spectrometer (Thermo-scientific, Wilmington, USA). 2µ of extracted RNA was reverse transcribed to cDNA using thermo scientific kit according to manufactures protocol. Briefly, cDNA synthesis was done in the presence of Oligo (dT)18 Primer, 5X Reaction Buffer, RiboLock RNase Inhibitor, dNTP, RevertAid RT and water nuclease-free then transferred to the Biotech Thermal Cycler (London, England) incubated for 60 min at 42°C and 70°C for 5 min. Next, in order to do the real time PCR, 2 µL of cDNA products was added to SYBER green, the appropriate forward and reverse primers and distilled water. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as housekeeping gene. The sequences of used primers are showed in the table 1.

Statistical analysis

Data was assessed by SPSS software, One way ANOVA, LSD test with significance level of $P<0.05$. All results repeated three times and presented as mean ± SD.

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Table 1. The sequences of primers used in the study.

Gene	Forward 5' to 3'	Revers 5' to 3'
GAPDH	5'CAAGGTCATCCATGACAACTTTG3'	5'GTCCACCACCCTGTTGCTGTAG3'
IL-1b	5' GCTTATTACAGTGGCAATGA3'	5' GTGGTCGGAGATTCGTAG3'
IL-6	5' TTCGGTCCAGTTGCCTTCTC 3'	5'GAGGTGAGTGGCTGTCTGTG3'

Results

Effect of Brevinin-2R on cell proliferation of HepG2 cells: To assess the effect of Brevinin-2R on viability of HepG2 cells, treatment with different concentrations of peptide were performed. As shown in Figure 1, Brevinin-2R showed no significant toxic effects on cells and inhibited the cell growth about 5 to 25% at concentrations of 10 to 40µg/ml.

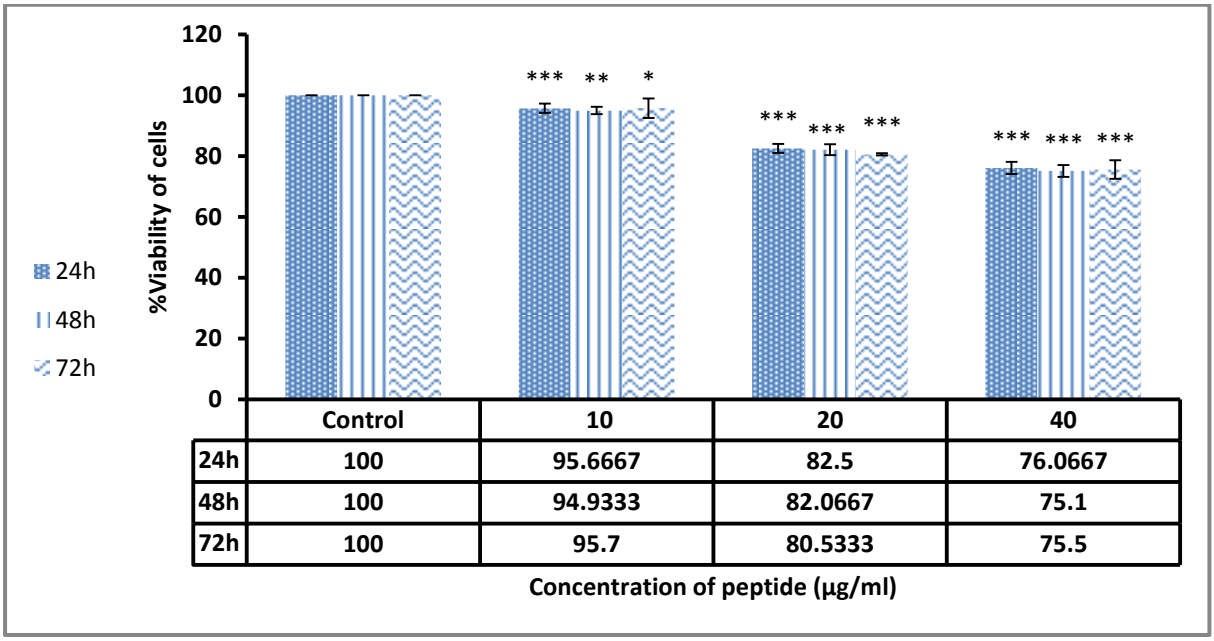
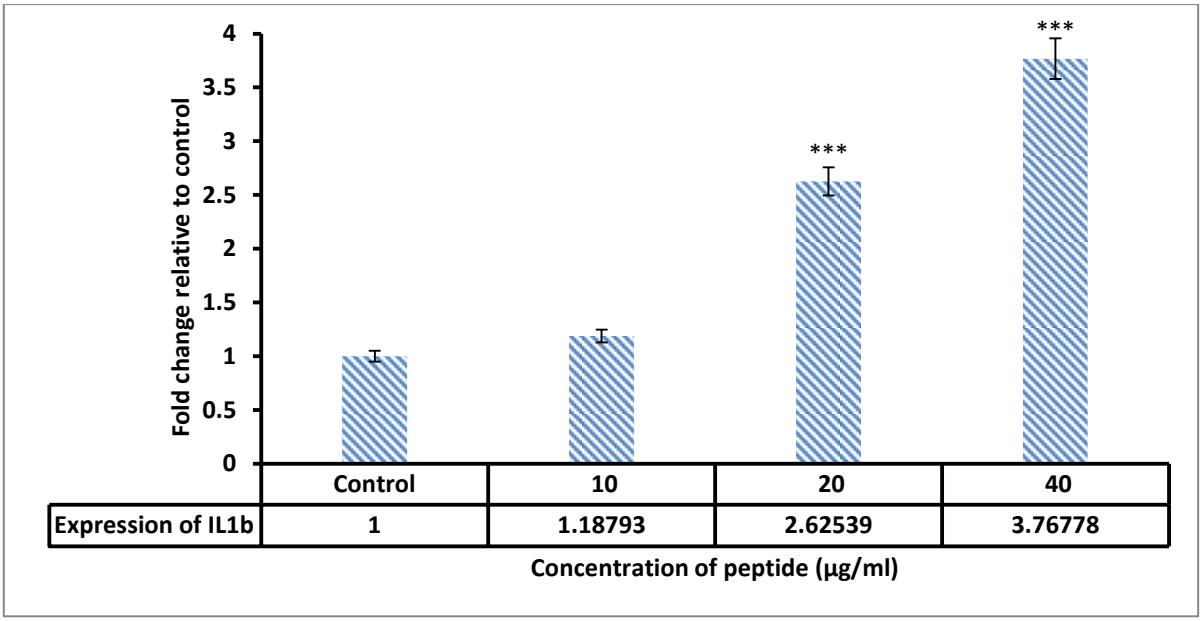


Figure 1. The effect of different concentrations Brevinin-2R on viability of HepG2 cells at 24, 48 and 72 h compared with control.

Regulatory effect of Brevinin-2R on expression levels inflammatory genes: Treatment of HepG2 cells with various concentration of B2R led to a significant increase in the expression of inflammatory genes in a dose dependent manner. As shown in Figure 2, the level of IL-1b gene expression is increased by increasing the concentration of peptide and there was observed a significant increase ($P< 0.05$) in gene expression at the concentrations of 20 and 40µg/ml. The effect of B2R on IL-6 expression in Hep2G cells is shown in Figure 2. As could be observed, with increasing the concentrations of peptide the expression level of IL-6 gene is also increased.

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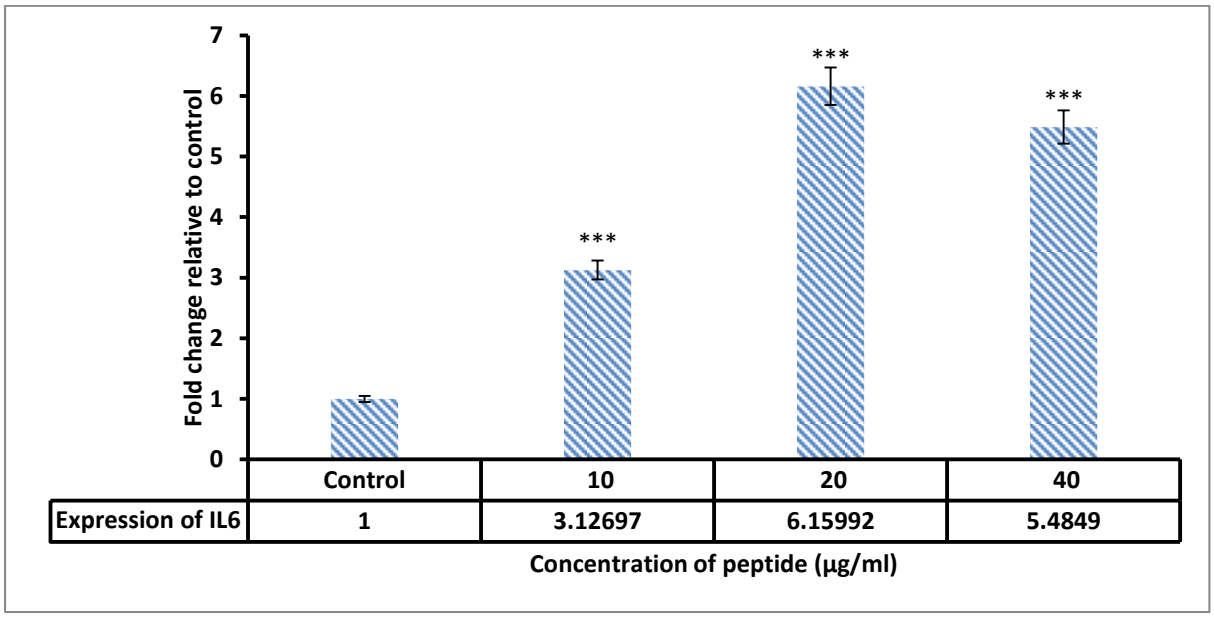


Figure 2. The effect of different concentrations of B2R on the expression level of IL-1b and IL-6 in HepG2 cells, 48 h after treatment. Exposure to the peptide led to increased expression of IL-1b and IL-6 genes.

Discussion

So far, many studies have been carried out on cytotoxic effect of peptides extracted from various sources on cancer cells. LfcinB is an antimicrobial peptide that exerts its cytotoxic effects against of cancer cells through interact with negatively charged membranes of target cells (14).

Gaegurin 6 (GGN6) is another type of peptide that has toxic effects against breast cancer lines. In a study that conducted by Kima et al in 2003 was shown that changes in amino acids, removing or adding certain areas in GGN6 can lead to change its anticancer effects (15). For example, the removal of the amino terminal region significantly reduced its anti-cancer activity while there was no particular change in the activity of the peptide by removing the carboxyl terminal region. In general, it is recommended that

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these peptides and derivatives can be considered as anti-cancer agents (15).

In another study in 2012 examined the cytotoxic effects of peptides extracted from venom on cancer (Hela) and normal cell (LK) lines. ICD-85 showed cytotoxic effects on tumor cells with IC50: 26.52 µg/ml so that by increasing peptide concentration, increased the percentage of inhibition of Hela cells. In this study, was compared the cytotoxic effect of ICD-85 on cancer with normal cells and the results showed that the minimum concentration of cytotoxic effect on normal cells (LK) 3,500 fold less compared to the same levels in cancer cells (Hela) (16).

In another study anti-cancer and cytotoxic effects of peptides Cecropin A and B were assessed on two cancer cell lines namely breast adenocarcinoma (MDA-MB-231) and human mesothelioma (M14K). The results showed that the toxicity of these peptides is dependent on the concentration and type of cancer cells and no association with the type of peptide. The M14K line cells had lower sensitivity to the action of Cecropins A and B than the MDA-MB-231 line cells (17).

The effects of peptides on the immune system and inflammation were investigated in various studies. Studies have shown that a wide range of anti-microbial peptides caused the release of pro-inflammatory cytokines and chemokines (18-3). In this study, we evaluated the effects of Brevinin-2R as an antimicrobial peptide on the activity of the two cytokine namely IL1b and IL6 in HepG2 cells. For this purpose, cells were treated with different concentrations of peptide (10, 20, and 40) then were analyzed using real time pcr. IL1b and IL6 showed an increase in expression 48 hours after treatment with B2R (Fig 2). Elssner and et al in 2004 investigated the effect of LL-37 on immune system and found that the peptide is able to increase the expression of IL-1b by monocytes (8) in another study that conducted by Yoshioka and et al in 2008 was shown that the expose of LAD2 cells with LL-37 peptide leads to the release of IL-1b from LAD2 cells (19).

rCRAMP is another form of the antibacterial peptide that exerts additive effects on the expression of IL-1b (as a pro-inflammatory cytokine) in glial cells (20).

Investigation the effect of B2R peptide on lung cancer cells (A549) showed that this peptide can lead to increased expression of IL-1b (as a cytokine) and IL8 (as a chemokine). The results showed that the expression of these genes in A549 cell line is dependent on the concentration and time (13). Niyonsaba and et al in 2010 investigated the effects of hBDs and LL-37 (as antimicrobial peptides) and showed that both peptides are able to induce the release of certain factors such as IL-2, IL-4, IL-6 (21).

The results of the present study is similar to the above studies, approved the regulatory effects of antimicrobial peptides on expression of pro- inflammatory factors.

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