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VGB3 Induces Apoptosis by Inhibiting Phosphorylation of NF-κB p65 at Serine 536 in the Human Umbilical Vein Endothelial Cells

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ABSTRACT

Background and objectives: Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) inhibition results in an increase in apoptosis. It has been demonstrated that NF-κB subunit p65 phosphorylation at the IκB kinase phosphorylation site serine 536 (Ser^{536}) is essential for the NF-κB nuclear translocation and activation. Therefore, NF-κB can be downregulated by suppressing its phosphorylation. The vascular endothelial growth factor receptor-2 (VEGFR-2) suppression could result in apoptosis induction. Therefore, targeting these pathways via VEGFR-2 inhibitors might have therapeutic potential for cancer treatment. It has been indicated that an antagonist peptide of VEGF, referred to as VGB3, could neutralize and recognize VEGFR2 in the tumoral and endothelial cells. This study aimed to induce apoptosis in human umbilical vein endothelial cells (HUVEC) cells through the inhibition of these signaling pathways.

Methods: Effects of different concentrations of VGB3 (1-200 ng/ml) were evaluated on the viability of HUVEC cells using MTT assay. In addition, downstream signaling pathways in HUVE cells were evaluated through quantitative assessment of protein expression via western blotting.

Results: The results demonstrated that VGB3 treatment inhibited the growth of HUVEC cells. Moreover, Bcl-2 was decreased in the cells treated with the VGB3 compared to the control. Furthermore, VGB3 significantly enhanced the cleaved-caspase7 levels, which is an indicator of apoptosis progression. Altogether, VGB3 enhanced apoptosis in HUVEC cells.

Conclusion: Our results indicate that the peptide might be a potential candidate for antitumor therapy via inhibiting the NF-κB pathway.

Keywords: Apoptosis, NF-kappa B, VEGFR-2.

INTRODUCTION

Several constitutively activated pathways have crucial roles in the growth and survival of cancer cells (1). These include PI3-kinase/AKT and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathways (2-4). Nowadays, NF-κB is broadly acknowledged as a vital positive regulator of tumor cell survival and proliferation through its capability to transcriptionally activate a large number of anti-apoptotic and pro-survival genes such as *Bcl-2*, *XIAP*, *Bcl-Xl*, *survivin*, *IkB-a*, *cIAP-2*, and *cIAP1* (5,6).

The IκB phosphorylation mediated by IκB kinase (IKK) was recognized as the main mechanism for regulation of the NF-κB nuclear translocation. It has been demonstrated that NF-κB subunit p65 phosphorylation at the IKK phosphorylation site serine 536 (Ser^{536}) is essential for the nuclear translocation and activation of NF-κB (7,8). Furthermore, it has been indicated that phosphorylation of Ser^{536} denotes a non-canonical pathway through which NF-κB could translocate to the nucleus independently of IκBα degradation (9–11). Consequently, cell-permeable peptides that suppress the phosphorylation of Ser^{536} also inhibit the NF-κB nuclear translocation in vitro (12) and in vivo (13).

Vascular endothelial growth factors (VEGFs) play important roles in regulating angiogenesis. Its receptor, vascular endothelial growth factor receptor-2 (VEGFR-2) displays a significant tyrosine kinase activity against pro-angiogenic signals and also has a role in regulating endothelial cell migration, proliferation, secretion, vascular permeability, and other endothelial tasks (14,15). Several studies have demonstrated that VEGFR-2 and VEGF are overexpressed in breast cancer, and could predict disparaging patient survival rates (16–18). It has been indicated that VEGFR-2 antibody could inhibit the VEGF activity by suppressing the signaling pathways of VEGFR-2, such as the downstream NF-κB pathway, demonstrating the possible application of anti-VEGFR-2 antibody in treating cancers (19). Hence, different therapeutic methods using anti-VEGF/VEGFR2 could be applied to enhance apoptosis and suppress the proliferation of cancerous cells (20). As a result, by inhibiting VEGFR-2, we can suppress the NF-κB pathway and induce apoptosis.

The most advanced VEGFR-2 inhibitors are Sunitinib, which is approved for treating advanced renal cell carcinoma (21) and gastrointestinal stromal tumors (22) and Sorafenib has been approved for patients with unresectable hepatocellular carcinoma (23) and metastatic renal cell carcinoma (24). Nonetheless, the clinical application of these drugs is still narrowed via various factors including side effects, acquired drug resistance, and toxicity (25,26). Peptides have developed as a novel generation of remedial treatments since they possess the benefits of proteins including high potency and specificity with those of small molecules, like bioavailability and stability. In previous studies, we described a peptide variant that binds to both VEGFR-2 and VEGFR-1, thereby suppressing VEGF-driven tube formation, migration, and proliferation of endothelial cells, leading to inhibition of metastasis and tumor growth in mice bearing 4T1 mammary carcinoma tumors (27). In earlier works, we reported a peptide variant, referred to as VGB3 that binds to both VEGFR-1 and VEGFR-2, which suppresses VEGF-driven migration, proliferation, and tube formation of HUVEC cells, and metastasis and tumor growth in murine 4T1 mammary carcinoma tumor model (27,28). The purpose of this study was to target human umbilical vein endothelial cells (HUVEC) cells via VGB3 and to indicate that apoptosis can be induced through inhibition of the NF-κB pathway.

MATERIALS AND METHODS

The 14-mer peptide with the sequence of 2HN-KAWAECRPPDEGLC-COOH (referred to as VGB3) was synthesized and purified by high-performance liquid chromatography to a purity of 90%, and later analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF), and confirmed by electrospray ionization mass spectrometry (ESI-MS) analysis (Shine Gene Biotechnologies, Inc., Shanghai, China). The HUVEC cell line was purchased from the National Cell Bank of Iran (NCBI) (Tehran, Iran). The cells were cultured in RPMI 1640 (Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (Gibco, USA) and containing 100 µg/ml streptomycin and 100 U/ml penicillin. The cells were retained at 37 °C in a 5% CO_2 humidified incubator.

The effect of VGB3 treatment in the presence

and absence of VEGF on HUVEC cells was assessed using MTT assay (M2128-1G; Sigma-Aldrich, St. Louis, USA). A total of 10,000 cells per well were seeded in a 96-well plate at 37 °C for 48 hours. Then, different dilutions of VGB3 in VEGF-positive and VEGF-negative cultures were used to treat the cells for 48 hours. Then, 20 µl of MTT solution (5 mg/ml) was added to each well. The plate was incubated at 37 °C in the dark for 2 hours. Subsequently, for the dissolution of formazan crystals, 100 µl of dimethyl sulfoxide was added to each well, and the plate was shaken for 15 minutes. All treatments were carried out in triplicate. The absorbance at 570 nm was read using a Microplate Reader (BioTek, Winooski, USA), and the results were presented as a percentage of the viability of control cells. The half-maximal inhibitory concentration (IC50) values at various time intervals in the presence and absence of VEGF were obtained using GraphPad Prism v8 software to be used as a treatment for the breast cancer cell lines for further experiments.
In order to carry out immunoblotting, 20 µg/ml of cells were lysed using 1 ml of radioimmunoprecipitation assay buffer containing a 1% protease inhibitor cocktail.
The lysed cells were centrifuged at 12,000 × g at 4°C for 20 minutes, and the supernatant was gathered in a clean 1.5 ml tube. The concentration of proteins was measured using a Bradford assay kit (Bio-Rad, Hercules, USA) based on the manufacturer's instructions. An equal amount of protein (~100 µg) was subjected to sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 12% acrylamide gel. The separated protein samples were transferred onto a polyvinylidene difluoride membrane (Roche, UK). The membrane was blocked with 3% (w/v) bovine serum albumin in phosphate-buffered saline with Tween 20 at room temperature for 1 hour. Consequently, the membranes were incubated overnight at 4 °C with primary antibodies (Santa Cruz Biotechnology, U.S.A) against Bcl-2 (sc-492), caspase7 (#9492), NF-κB (sc-8008), and p-NF-κB (sc-136548). Next, the membrane was treated with suitable horseradish peroxidase-conjugated secondary antibody (sc-516102; sc-2357) for 1 hour at room temperature. Protein bands were visualized using the increased chemiluminescence-plus kit (GE Healthcare, Chicago, USA).
The bands were normalized to β-actin, and the ImageJ v.1.52 software was used for determining the density of the bands. The blotting test was done in triplicate.
Statistical analyses were performed using GraphPad Prism 8 Scientific software (GraphPad Software, Inc., La Jolla, CA). The mean ± standard deviation (SD) was used to express the results. For multiple comparisons, a one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test was conducted. A *p*-value of less than 0.05 was regarded as statistically significant.

RESULTS

The cytotoxic effects of different doses of VGB3 (1-200 ng/ml) in the presence and absence of VEGF on HUVEC cells were investigated after 48 hours of incubation. As shown in figure 1, VGB3 treatment exhibited time- and dose-dependent cytotoxic activity toward HUVEC cells compared to the untreated control cells.

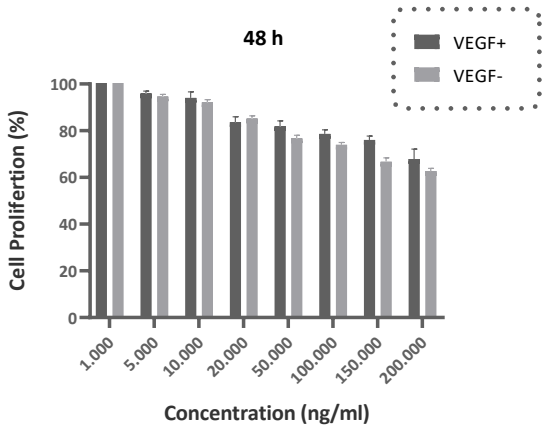

Figure 1- Diagram of HUVEC cells survival rate (percentage) after 48 hours of incubation with different concentrations of VGB3 in the presence and absence of VEGF. The significance level was set at <0.0001. Vertical lines indicate standard deviation.

The IC50 value of VGB3 after 48 hours was 22 ng/ml. Overall, the results indicated that treatment of HUVECs by VGB3 reduced their growth rate or proliferation. According to these results, the optimal concentration of VGB3 was considered to be 22 ng/ml and was used in subsequent tests.

To investigate whether NF-κB transcriptional activity decreases in response to VGB3 and whether the inhibition of this pathway results in apoptosis, the immunoblotting assay was performed on HUVEC cells (Figure 2a, b). After 24 hours of incubation with the indicated concentration of VGB3 (22 ng/ml), the cells were lysed, and aliquots were assessed for *in vitro* Bcl-2, pro-caspase7, cleaved-caspase7, p-NF-κB, and NF-κB activity. The western blotting data demonstrated that treating the HUVEC cells with VGB3 at the concentration of 22 ng/ml significantly increased the levels of p-NF-κB (p<0.0001). Moreover, the results indicated that VGB3 significantly enhanced the cleaved-caspase7 levels (p<0.0001), which is an indicator of apoptosis progression. Also, it has been observed that the levels of Bcl-2 which is an anti-apoptotic agent have been reduced after treatment with the peptide (p<0.0001).

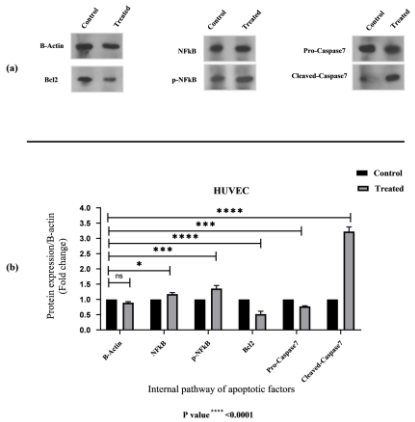

Figure 2- Western blot analysis of Bcl-2, pro-caspase7, cleaved-caspase7, NF-κB, and p-NF-κB protein expression after treatment with 22 ng/ml of VGB3 for 24 hours. (a) Western blotting was performed for Bcl2, pro-caspase7, cleaved-caspase7, NF-κB, and p-NF-κB, with β-actin as a loading control. (b) Ratios of Bcl-2, pro-caspase7, cleaved-caspase7, NF-κB, and p-NF-κB to β-actin. Values display mean ± SD of at least 3 independent experiments. (*p<0.05 vs. untreated control).

DISCUSSION

Based on the results obtained from previous studies, the VEGF antagonist peptide, VGB3, recognizes both VEGFR-2 and VEGFR-1. It has been shown that VGB3 treatment of 4T1 and HUVEC cell lines leads to down-regulation of PI3-kinase/AKT and NF-κB pathway (13). The general impact of AKT activation is cell survival. It has been formerly indicated that PI3-kinase/AKT pathway is also connected to other survival pathways, including the NF-κB pathway (29,30). It has been demonstrated that NF-κB subunit p65 phosphorylation at the Ser^{536} is required for the NF-κB nuclear translocation and activation (17). Accordingly, inhibiting NF-κB p65 phosphorylation can lead to the induction of apoptosis. Therefore, to investigate whether suppression of VEGFR-2 results in apoptosis, we evaluated the suppressing effect of VGB3 on the NF-κB pathway. For this purpose, first, HUVEC cells were treated with VGB3 for 48 hours. It was observed that the viability of HUVEC cells reduced after the treatment with different concentrations of VGB3 in a dose-dependent manner. Subsequently, the cells were treated with the peptide and the extracted proteins were western blotted with antibodies against Bcl-2, pro-caspase7, cleaved-caspase7, NF-κB, and p-NF-κB. The VGB3 treatment of HUVEC cells inactivated the NF-κB pathway. Similarly, the peptide considerably enhanced the levels of cleaved-caspase7 in the cells compared with the control. Caspase-7 is an effector caspase with important roles in mediating cell death signaling (31). These data confirm the blockade of VEGFR2-mediated signaling pathways.

We also established that in HUVEC cells, the expression levels of Bcl-2 have been significantly decreased. It has been indicated that inhibition of the NF-κB pathway via NF-κB p65 phosphorylation at serine 564 leads to the downregulation of Bcl-2 (32). Since Bcl-2 is a n anti-apoptosis agent, the reduced

expression of Bcl-2 in the peptide-treated cells could be attributed to the suppressive effect of the peptide on the NF-κB pathway. These results indicate that peptide-induced apoptosis in HUVEC cells occurs via an NF-κB-dependent pathway. Overall, the peptide exhibited VEGF antagonistic possessions, thereby blocking the signaling pathways induced by VEGF.

CONCLUSION

Our findings contribute to our understanding of the relationship between NF-κB and apoptosis. Phosphorylation of NF-κB p65 at serine 564 is crucial for the NF-κB activation. Therefore, targeting the NF-κB pathway at either the protein expression level or its transcription regulation capability can provide essential therapeutic answers to a difficult problem. Taking together, our results suggest that the peptide binds to VEGFR-2 cells and reduces the activity of VEGFR-2 tyrosine kinase. Suppression of kinase activity decreases the phosphorylation of the VEGFR-2 downstream target NF-κB, which in turn reduces the levels of Bcl-2 expression and ultimately apoptosis. This suggests that VGB3 is a unique peptide capable of exerting considerable apoptosis-inducing effects.

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Ethics approvals and consent to participate

Not applicable.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this article.

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