**Staphylococcus aureus** enterotoxin type B (SEB) and alpha-toxin induced apoptosis in KB cell line

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**ARTICLE INFO**

**Keywords:** *Staphylococcus aureus*, enterotoxin type B, α-toxin, apoptosis, oral cancer, KB cell line, gene expression

**Original Article**

Introduction: According to global cancer statistics, oral cancer is the 11th most prevalent cancer worldwide. Despite the availability of numerous modern treatments for oral cancer, a complete reduction of mortality rates has not been achieved. Bacterial toxins have potential applications in inducing apoptosis or targeting tumor cells for destruction. The present study aimed to investigate the impact of *Staphylococcus aureus seb* and α-toxin genes on apoptotic-related gene mRNA expression, as well as apoptosis induction in KB cell lines, focusing on BAX, RB, BCL-2, and BAG-1 genes. Methods: The transfection of KB cells was performed using Lipofectamine 2000 to introduce pcDNA3.1 (+)-seb, pcDNA3.1 (+)-α-toxin, or an empty pcDNA3.1 (+) plasmid. The cells were cultured in DMEM supplemented with 10% FBS and 800 mg/L of G418 to select cells containing the plasmids. Subsequently, real-time RT-PCR was performed to measure the mRNA expression levels of BAX, RB, BCL-2, and BAG-1 genes. Cell apoptosis was assessed using Annexin V/PI staining and flow cytometry. Results: The seb and α-toxin significantly alter the expression of apoptotic-related genes in the KB cell line. In transfected KB cells, there was a significant increase in the mRNA expression of BAX and RB genes and a substantial decrease in the mRNA expression of BCL-2 and BAG-1 compared to the control group. Annexin test and flow cytometry analysis revealed that seb was more effective than α-toxin in inducing apoptosis. Conclusions: The seb and α-toxin genes of *S. aureus* exhibit an inhibitory effect on the growth, proliferation, and invasion of oral cancer cells by modulating gene expression in the apoptotic pathway. Hence, these toxins are promising for controlling and treating human oral cancer.

**INTRODUCTION**

Cancer represents a significant global health burden, with oral cancer constituting a prevalent malignancy that affects the oral cavity [1]. While oropharyngeal cancer incidence is higher in developed countries, developing nations are also experiencing an increase in the prevalence of the disease [2]. Oral cancer risk factors include tobacco and alcohol use, exposure to UV radiation, viral infections, poor oral hygiene, genetic predisposition, and male sex [3]. Radiation therapy is the most commonly used treatment, but there is an urgent need for new cancer medications [4].

Bacterial infections have been reported to possess anti-tumor properties and may serve as potential anticancer agents due to their immunogenicity, cytotoxicity, and survival ability in anoxic tumor environments [5]. Bacterial toxins have been used in cancer treatment for almost a century, and researchers have discovered that bacterial infection may inadvertently suppress certain cancers [6]. Toxins produced by bacteria effectively induce tumor death and promote cancer vaccination, as they can cause cell death and alter biological processes such as proliferation, differentiation, and apoptosis [7-12].

*S. aureus*, a highly virulent human pathogen, has been found to produce toxins with potential anticancer properties [13]. Toxins produced by *S. aureus* include pore-forming, exfoliative, and superantigen toxins, and most clinical *S. aureus* strains produce alpha-toxin. This protein monomer becomes lethal after oligomerizing into a heptameric form on the host cell's plasma membrane [14]. The alpha toxin has been shown to induce apoptosis in both endothelial cells and T-cells isolated from...
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Caucasian male. After

ts were subjected to
pecific primers as

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peripheral blood and in Jurkat T cells by releasing
cytokine C and increasing caspase activity [15].
Staphylococcal enterotoxins, known for causing food
poisoning and diarrhoea, have also been found to induce
apoptosis and potentially possess anticancer effects [16].

Oral squamous cell carcinoma (OSCC) is the most
prevalent malignant neoplasm of the oral cavity, and the
KB cell line was established from an epidermoid
carcinoma of the mouth in an adult Caucasian male [17].
This study examined the impact of S. aureus seb and
alpha-toxin genes on apoptosis-related genes, including
BAX, BCL-2, BAG-1, and RB, in the KB cell line.

MATERIAL AND METHODS

Preparation of recombinant vectors. The seb
(Accession: AM158256.1) and a-toxin genes (Accession:
MZ398129.1) from S. aureus were retrieved from GenBank
(http://www.ncbi.nlm.nih.gov/Genbank/), and
codon optimization was performed using Gene Runner
version 6.5.52 for expression of these genes in human
cells. Restriction sites for BamHI and EcoRI were inserted
at the N- and C-terminals of the genes, respectively.
GenRay Biotechnology (Dongjing Town Songjiang,
Shanghai, China) constructed two recombinant vectors,
pcDNA3.1(+)-seb and pcDNA3.1(+)-a-toxin. TOP10F
chemically competent cells were used to amplify
the recombinant plasmids and the empty pcDNA3.1(+) vector.
A commercial kit (YTA, Yekta Tajhiz Azma, Iran)
was used to extract the plasmids. Confirmation of the
recombinant plasmids was performed using PCR, double
enzymatic digestion, and sequencing.

Cell lines transfection. The KB cell line (CCL-17),
a type of human mouth epidermal carcinoma, was obtained
from the National Cell Bank of Pasteur Institute of Iran.
The KB cell line (CCL-17) was maintained in Dulbecco’s
modified Eagle’s medium (DMEM; Bio-IDEA-Iran)
supplemented with 10% heat-inactivated fetal bovine
serum (hi-FBS; Bio-IDEA-Iran) and 100 U/mL penicillin-
streptomycin (Bio-IDEA-Iran). The KB cells were
incubated in a 37 °C humidified atmosphere containing
5% CO2 using a CO2 incubator to maintain steady-state
conditions. Each well of a 6-well plate (SPL Life
Sciences; South Korea) was seeded with 300,000 cells.
The KB cells were transfected with Lipofectamine 2000
reagent (Invitrogen, USA) when they reached 85%
confluency.

The first and second wells were transfected with
recombinant plasmids, pcDNA3.1(+)-seb and
pcDNA3.1(+)-a-toxin, respectively. In addition, the third
well was lipofected with an empty pcDNA3.1(+) vector
(control plasmid), while the fourth was left untransfected.
Each transfected well was supplemented with 2 μg of
plasmid and 6 μL of Lipofectamine 2000 reagent per well
in serum-free DMEM medium containing 100 U/mL
penicillin and 100 μg/mL streptomycin (Sigma-Aldrich,
St. Louis, MO, USA). Four hours after transfection,
the medium was replaced with a fresh medium containing 800
mg/L G418 and 10% FBS, and the cells were selected for
stable cell line generation over three weeks.

RNA extraction and cDNA synthesis. Total RNA
was extracted from KB cells transfected with recombinant
or control plasmids using RNX-Plus reagent (SinaClon,
Iran) following the manufacturer’s instructions. The
concentration and quality of the extracted RNA were
assessed using a Nanodrop ND-1000 spectrophotometer
(Nanodrop Technologies, Wilmington, DE, USA) at
wavelengths of 260 nm and 280 nm. The Revert Aid First
Strand cDNA Synthesis Kit (Thermo Fisher Scientific,
USA) was used to synthesize cDNA from 1000 ng of
RNA per sample, following the manufacturer’s
instructions.

Detection of seb and a-toxin mRNAs by RT-PCR.
PCR reactions were carried out in a 20 μL volume
consisting of 1 μL cDNA, 2 mM MgCl2, 0.2 mM dNTPs,
0.5 units of Taq DNA polymerase (SinaClon), and 0.5 μM
of each forward and reverse primer (seb-F/R and a-toxin-
F/R primers, respectively, as listed in Table 1). The
reaction mixture was subjected to an initial denaturation
step at 95 °C for 3 min. PCR amplification was carried out
using an Eppendorf Mastercycler gradient (Eppendorf,
Germany) with the following cycling conditions: initial
denaturation at 94 °C for 1 min, annealing at the
temperatures specified in Table 1 for 1 min, extension at
72 °C for 1 min for 30 cycles, and a final extension step
at 72 °C for 5 min. The PCR products were subjected to
electrophoresis on a 1.5% agarose gel for analysis.

Real-time RT-PCR. Real-time PCR was performed
using a Rotor-Gene 6000 machine (Corbett Life Science,
Concorde, NSW, Australia) and SYBR® Premix Ex
TaqTM II kit (TaKaRa, Japan) with specific primers as
listed in Table 1. Amplification for each gene was
performed in duplicate for samples in a 20 μL reaction
volume. No template controls (NTCs) were also included
in each run. The amplification conditions for
quantification were as follows: initial denaturation at 95
°C for 4 min, followed by 40 cycles of denaturation at 95
°C for 15 s, annealing at the temperatures specified in
Table 1 for 20 s, and extension at 72 °C for 20 s. After
amplification, a melting curve analysis was performed to
confirm the specificity of the PCR product. GAPDH
was used as the internal reference gene for all experimental
quantification. Relative quantification analysis was
performed using the comparative Ct method (2-ΔΔCt) [24].
The qPCR products were analyzed by electrophoresis on
a 2% agarose gel to confirm the expected product sizes.

Flow cytometry. Apoptotic cell death induced by
recombinant vectors (pcDNA3.1(+)-seb and
pcDNA3.1(+)-a-toxin) and empty pcDNA3.1(+) was
quantified using the FITC Annexin V Apoptosis
Detection Kit I (556547, BD Biosciences Pharmingen,
USA) and flow cytometry. All experiments were
performed in duplicate. Cell lines (KB) were harvested
and washed twice with ice-cold phosphate-buffered saline
(PBS). The cells were then resuspended in 100 μL of 1X
binding buffer (provided in the kit) and stained with 5 μL of FITC-conjugated Annexin-V (10 mg/mL) and 10 μL of propidium iodide (50 mg/mL) in a flow cytometry microtube. After incubation at room temperature (25 °C) in the dark for 15 min, 400 μL of binding buffer was added to the cells, and the samples were analyzed using a flow cytometry instrument (FACSCalibur, Becton Dickinson).

Table 1. Primers used for real-time PCR amplification of genes in KB cells.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer's name</th>
<th>Sequence</th>
<th>Ta (°C)</th>
<th>Product length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>F</td>
<td>5'-GCCAAAAAGGCTACATCTCTTGCA-3'</td>
<td>65</td>
<td>183</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>5'-GTTGAGGATCTGGCTGGTGAGAG-3'</td>
<td>65</td>
<td>171</td>
</tr>
<tr>
<td>Seb</td>
<td>F</td>
<td>5'-AGGACACAAAGCTGGCAACATAC-3'</td>
<td>≤0.036</td>
<td>227</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>5'-TACGTGCTGTCGCTGGTGGAGAG-3'</td>
<td>65</td>
<td>227</td>
</tr>
<tr>
<td>α-toxin</td>
<td>F</td>
<td>5'-ACACCTGCTCGTCATTCACC-3'</td>
<td>67</td>
<td>183</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>5'-ATGGTGCTTGTTGCGAAAATCT-3'</td>
<td>65</td>
<td>227</td>
</tr>
<tr>
<td>BAG-1</td>
<td>F</td>
<td>5'-ACGACTCTTCATGTATCCCTCAG-3'</td>
<td>64</td>
<td>227</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>5'-TAGTTCACCTTCCTTCGTGGGACGT-3'</td>
<td>65</td>
<td>227</td>
</tr>
<tr>
<td>BCL-2</td>
<td>F</td>
<td>5'-GACGACTTCTCCCGCGCTAC-3'</td>
<td>65</td>
<td>245</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>5'-CGGTTAGTGATGTCATGATCCAC-3'</td>
<td>65</td>
<td>220</td>
</tr>
<tr>
<td>RB</td>
<td>F</td>
<td>5'-GCAAATTGGAAAGGACATGGACAG-3'</td>
<td>65</td>
<td>220</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>5'-TTGAGCACAATGGGAGGAGGAG-3'</td>
<td>65</td>
<td>234</td>
</tr>
<tr>
<td>BAX</td>
<td>F</td>
<td>5'-AGGCTTTTTCTCGAGGGAGCAG-3'</td>
<td>65</td>
<td>234</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>5'-GCCCTCAAGATGGAGGAGGAG-3'</td>
<td>65</td>
<td>234</td>
</tr>
</tbody>
</table>

Statistical analysis. The KB cell transfection was performed in duplicate using the corresponding plasmids. The real-time PCR, data, and flow cytometry assays were performed, and the results were presented as mean ± standard deviation (SD). The results were analyzed using one-way analysis, and the significance of the differences between sample means was determined. Statistical significance was defined as P ≤ 0.05. Statistically significant differences were not considered for duplicate samples.

RESULTS

Confirmation of recombinant plasmids. The pcDNA3.1(+)-seb and pcDNA3.1(+)-α-toxin recombinant plasmids were confirmed via PCR, followed by double-enzyme digestion, and sequencing. The seb and α-toxin genes were amplified using specific primers (Table 1), resulting in PCR products of 171-bp and 227-bp for seb and α-toxin, respectively (data not shown). Furthermore, the pcDNA3.1(+)-seb recombinant plasmid was confirmed via restriction enzyme analysis using BamHI and EcoRV, resulting in an 813 bp fragment for the full-length seb gene on agarose gel electrophoresis. In addition, digestion of pcDNA3.1(+)-α-toxin with BamHI/EcoRV and subsequent electrophoresis revealed a 963-bp fragment for α-toxin (Fig. 1A and B). Furthermore, DNA sequencing was performed to confirm recombinant plasmids (data not shown).

Eukaryotic expression of S. aureus toxin genes. KB cells were transfected with pcDNA3.1(+)-seb and pcDNA3.1(+)-α-toxin expression plasmids and were harvested three weeks after transfection. RT-PCR analysis revealed successful amplification of 171 bp and 227 bp fragments for seb and α-toxin genes, respectively, indicating successful transfection of the recombinant plasmids into KB cells (Fig. 1A).

α-toxin effects on gene expression in KB cells. Analysis of apoptosis-related gene expression in KB cells transfected with pcDNA3.1(+)-α-toxin demonstrated statistically significant changes in the expression levels of BAX, BCL-2, RB, and BAG-1 genes compared to control cells (P < 0.049; Fig. 3). Our findings indicate upregulation of the pro-apoptotic BAX and RB genes by approximately 2.5- and 7-fold, respectively, in α-toxin-treated KB cells compared to the control group (KB cells transfected with empty plasmid). Moreover, the expression levels of BCL-2 and BAG-1 genes showed statistically significant differences between the α-toxin-treated and control groups. The levels of BCL-2 and BAG-1 in treated KB cells decreased significantly compared to the control group (P < 0.033; Fig. 3).
Fig 1. Characterization of recombinant plasmids via restriction enzyme digestion. A) Agarose gel electrophoresis of BamHI/EcoRV-digested pcDNA3.1(+)-seb, with 1Kb DNA ladder as a size marker. Lane 1 corresponds to the digested pcDNA3.1(+)-seb plasmid. B) Agarose gel electrophoresis of BamHI/EcoRV-digested pcDNA3.1(+)-α-toxin, with 1Kb DNA ladder as a size marker. Lane 1 corresponds to the digested pcDNA3.1(+)-α-toxin plasmid.

Fig 2. Statistical analysis of mRNA expression levels of BAX, BCL-2, RB, and BAG-1 genes in KB cells treated with seb (treatment group) and control group (empty vector). All changes in gene expression were statistically significant.

**Induction of apoptotic death in KB cells by S. aureus toxins.** In the present study, Annexin V/PI staining and flow cytometry were utilized to analyze the proportion of cells undergoing early apoptosis, late apoptosis, and cell death. The apoptotic rates of KB cells transfected with pcDNA3.1(+)-seb were significantly higher than those of untreated control cells. The percentages of cells undergoing early and late apoptosis in seb-treated cells were 61.36% and 14.40%, respectively (Fig. 4A). Cell death, attributed to both apoptosis and necrosis, occurred in 76.00% of treated cells. The mortality rate in the control group was significantly lower (6.46%) compared to the treated KB cells (Fig. 4B). Additionally, as demonstrated in Fig. 4A, the percentage of viable cells in the seb-treated group decreased significantly (24.00%) (P<0.0065).

Moreover, following the transfection of KB cells with pcDNA3.1(+)-α-toxin, there was a significant increase in the cell death percentage of KB cells. The rate of viable cells in α-toxin-treated KB cells and control cells were 40.90% and 93.54%, respectively (Fig. 4B and 4C). Our findings show early and late apoptosis occurred in 58.54% of α-toxin treated cells. Thus, the apoptosis difference between treated and control cells was statistically significant (P<0.029).
Enterotoxin type B (SEB) and alpha-toxin

DISCUSSION

Cancer is a leading cause of death worldwide, affecting individuals across all age groups. Although current cancer therapies, including chemotherapy, radiation, and surgery, are widely available, they have not yet successfully eliminated cancer-related deaths [25]. Bacterial toxins have been studied to a limited extent for cancer therapy. Bacterial toxins can either destroy cells or, at low concentrations, alter cellular processes that regulate proliferation, differentiation, and apoptosis [26]. *S. aureus* bacteria produces approximately 20 types of staphylococcal enterotoxins, commonly called SEs [27]. In addition, *S. aureus* produces other types of enterotoxins, including SEK, SEL, SEM, SEN, SEO, and SEU, which are classified into antigenic groups known as staphylococcal enterotoxin type A to J (SEA to SEJ) [27].

Genes involved in cell proliferation and differentiation include *BAX*, *RB*, *BCL-2*, and *BAG-1*. The involvement of these genes in either activating or preventing apoptosis is highly probable [29-30].

Moreover, alpha-toxin is released by over 95% of clinically tested *S. aureus* strains. This water-soluble toxin can form beta-barrels in the body. The toxicity of the poison is achieved through oligomerization of the toxin on the host cell membrane, creating a toxic heptameric structure. This toxin affects various human cell types, including endothelial cells, epithelial cells, T cells, macrophages, and monocytes [28].

For this investigation, KB cells were transfected with either pcDNA3.1(+)toxin or pcDNA3.1(+)seb recombinant vectors, and the mRNA expression levels of *BAX*, *RB*, *BCL-2*, and *BAG-1* genes were assessed using...
real-time reverse transcription polymerase chain reaction (PCR) technique. Furthermore, Annexin V/PI labeling and flow cytometry were employed to determine cell viability. Our study confirms the practical expression of α-toxin or seb genes introduced into the pcDNA3.1(+) vector in transfected KB cells. Quantitative analysis revealed a significant increase of BAX and RB pro-apoptotic gene mRNAs in transfected KB cells than in control cells transfected with an empty pcDNA3.1(+) plasmid. KB cells transfected with pcDNA3.1(+)–sea or α-toxin recombinant vectors showed a statistically significant decrease in BCL-2 and BAG-1 expression compared to the control group.

Numerous studies have been conducted to investigate the impact of S. aureus on the expression of apoptotic genes. According to a study by Zhang et al. (2017), S. aureus toxins can impact the expression of genes such as BAX and BCL-2 [22]. This review suggests that overexpression of BCL-2 may mitigate the apoptotic effects of S. aureus-produced α-toxin. Our results were consistent with these findings, as we observed the downregulation of the BCL-2 gene and increased apoptosis in KB cell lines in response to α-toxin. Busbee et al. (215) conducted a study examining the effects of seb injection in rats [31]. Elevated levels of aspartate transaminase enzyme have been linked to heightened activity of inflammatory cytokines and the induction of apoptosis through the caspase-2-dependent apoptotic pathway. Research into the expression of genes involved in apoptosis indicated that seb could reduce BCL-2 expression in liver cells, leading to an increase in apoptosis. Our survey findings are consistent with prior research, as we observed the downregulation of BCL-2 in KB cell lines of oral cancer in response to seb. Sakagami reported that paclitaxel, an antimiotic chemotherapeutic drug, induced apoptosis in human oral cancer cells by downregulating BCL-2 expression and increasing BAX expression [32]. Our study suggests that seb and S. aureus-produced α-toxins may induce apoptosis in oral cancer KB cell lines similarly. Another study published in 2009 indicates that in malignancies where RB is not irreversibly inactivated, it may be possible to restore RB activity to halt further carcinogenesis [33]. Our investigation revealed that treatment with seb and S. aureus-produced α-toxin positively impacted the expression of the RB gene in treated cells. However, it is worth noting that the S. aureus-produced α-toxin appears more effective than the other two toxins, as it induces up-regulation of the RB gene. Subsequently, seb and S. aureus-produced α-toxin treatments increased apoptosis in the KB cell lines.

Shindoh et al. (2000) reported increased BAG-1 expression in tumor tissue compared to the adjacent normal epithelium in 60-80% of the samples in a review of oral squamous cell carcinomas [34]. Our findings not only corroborated the results of their study but also demonstrated that treatment with S. aureus-produced α-toxin and seb can reduce BAG-1 expression in oral cancer KB cell lines, resulting in increased apoptosis.

Furthermore, we conducted flow cytometry assays on cells exposed to seb or S. aureus-produced α-toxin. The percentage of total apoptosis was 75.76% and 58.54% in cells treated with seb and S. aureus-produced α-toxin, respectively. The viable cells were also 24% and 40.90% in the seb-treated and S. aureus-produced α-toxin-treated groups, respectively.

The flow cytometry experiment results indicate that the percentage of apoptosis induced by seb is higher than that induced by S. aureus-produced α-toxin. However, treatment with S. aureus-produced α-toxin resulted in a more significant increase in RB gene expression than treatment with seb. The up-regulation of BAX and down-regulation of BCL-2 and BAG-1 induced by both seb and S. aureus-produced α-toxin had similar effects.

Our findings demonstrate that seb and S. aureus-produced α-toxin up-regulate the RB and BAX genes and down-regulate BCL-2 and BAG-1 in oral cancer KB cell lines. Consequently, our data imply that these two S. aureus toxins may hold promise as in vivo inhibitors of cancer cell development and as a potential therapy for oral cancer. In conclusion, further investigation of additional oral cancer cell lines and animal tumor models is warranted based on our findings.

ACKNOWLEDGEMENT

This study was conducted as part of a M.Sc. thesis. We want to thank the Deputy of Research of the Shahrekord Branch, Islamic Azad University. Additionally, we would like to extend our appreciation to the staff of the Biotechnology Research Center at Shahrekord Branch, Islamic Azad University.

CONFLICT OF INTEREST

The authors declare no conflicts of interest associated with this manuscript.

REFERENCES

Enterotoxin type B (SEB) and alpha-toxin


