In Vitro Study of the Toxicity Effects of Bacillus anthracis Protective Antigen

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Abstract

Anthrax, a common disease of human and cattle, is caused by Bacillus anthracis infection. Protective antigen (PA) from Bacillus anthracis is a potent immunogen, which has been of interest in the development of new candidate vaccines against the disease. In this study, the toxicity effects of this antigen on prokaryotic (Escherichia coli and Staphylococcus aureus) and eukaryotic (MCF-7) cells were investigated. Antibacterial effects of the recombinant PA were analyzed using MTT and MIC (Minimum Inhibitory Concentration) assays. Cytotoxicity effect of the recombinant protein (in concentrations ranging from 0.5 to 2 μg/ml) on MCF-7 cell line was analyzed using MTT, Neutral red uptake, and comet assays. MCF-7 cells' oxidative stress following the treatment with PA (0.5-2 μg/ml) was analyzed by NO assay, reduced glutathione assay (GSH), and catalase activity assay. MTT and MIC assays showed that PA has a low inhibitory effect on Escherichia coli and no inhibitory effect on Staphylococcus aureus. Cell cytotoxicity assays indicated that the antigen significantly inhibits the growth of MCF-7 cells. Comet assay also showed that the antigen induces apoptosis in MCF-7 cells. According to nitrite oxide, reduced glutathione, and catalase activity assays, PA has not a significant effect on MCF-7 cells in comparison to the control (P<0.001). Protective antigen has no significant inhibitory effect on the growth of bacterial cells. However, it significantly inhibits the growth of the breast cancer cells (P<0.001). The effect of PA on breast cancer cells is pharmacologically important so that the antigen can be considered as a candidate anticancer molecule.

Keywords: Bacillus anthracis, Protective Antigen, Antibacterial, Anticancer, Cytotoxicity

Introduction

Anthrax is a common disease of humans and chattels which is caused by Bacillus anthracis infection. Humans are infected through the direct contact to sick chattels or their products such as kips, hairs, or wool, so, people working with chattels, such as veterinarians, ranchers, microbiologists, tillers, pastors, and labors working in the slaughterhouses should be immunized against the disease. Okinaka and Brey in different studies demonstrated pXO1 (toxins coding gene) and pXO2 (capsule coding gene) plasmids are needed for Bacillus anthracis pathogenicity [1, 2]. To Dixon and Dai, have shown three proteins (Edema Factor (EF), Protective Antigen (PA), and Lethal Factor (LF)) are encoded by pXO1 or PBA1 plasmids [3, 4]. Protective antigen, which is coded by Pag gene, is a long and flat protein with no cysteine residues. This protein is responsible for the transfer of LF and EF proteins into the cytosol [5]. In 2001, demonstrated that the immunogenicity of a portion of PA following administration with aluminum hydroxide adjuvant to A/J mice was comparable to the immunogenicity level of the whole recombinant PA [6]. Another study by Yogendra et al., in 2001 showed that mice immunized with a mutant strain of Bacillus anthracis lacked the domain 4 of PA, has lower immunity level, so, PA is immunodominant antigen and can be used for the recombinant vaccine design [7]. To demonstrate toxicity of antigen in prokaryotic and eukaryotic cells and investigating the safety of the antigen as a candidate vaccine, in the present study the toxicity effects of the recombinant PA on two prokaryotic cells (Escherichia coli, Staphylococcus aureus) and one eukaryotic cell line (MCF-7) were analyzed.

Materials and Methods

PA protein and cells
Recombinant PA was obtained from Imam Hossain University, investigated by Mehr-Azin et al., 2011 [8]. Escherichia coli (ATCC 25922) as Gram-negative bacteria and Staphylococcus aureus (ATCC 25923) as Gram-positive bacteria were purchased from Persian Type Culture Collection (PTCC) and MCF-7 cell line was purchased from Iranian Biological Resource Center.

Antigen preparation
Phosphate buffer in which the antigen was dissolved passed through a 0.2 micron filter before doing the toxicity tests. To remove possible contaminants in PBS (pH 7.4) before doing the tests, Antibiotic-Antimycotic 1% (Invitrogen, USA) was added to the buffer and stored one night at 4°C. In this study, protein assay was performed using the Bradford method.

Determination of the antibacterial effects of PA using MTT assay
MTT assay is colorimetric method based on the reduction and breakdown of tetrazolium yellow crystals by dehydro-
genases in the cytoplasm and make them insoluble purple crystals. DMSO was used to solve the crystals [9]. Accordingly, bacterial cells were cultured in Mueller-Hinton broth (Sigma, USA) for 5 hours and when the concentration reached to the concentration equivalent to 0.5 McFarland (the absorbance at 600 nm was 0.08-0.1), 5μL of the bacterial culture was added to each well of a 96-well plate [10]. Recombinant PA (0.28, 0.56, 1.12, 2.25, and 4.5 μg/ml) was added to wells and the final volume for each well was set to 100 μl by addition of appropriate volume of Mueller-Hinton broth. Tetracycline antibiotic (50 μg/ml) was used as positive control and bacterial suspension was used as negative control, also Mueller-Hinton broth was used as the blank. All samples were incubated at 37°C for 23 hours. Then, 5 μl MTT (5 mg/ml) (Sigma, USA) was added to each well and incubated at 37°C for an hour and then 100 μl DMSO was added and incubated for additional 2 hours. The absorbance was measured at 595 nm and the assay was repeated three times for each concentration. Bacterial cells viability was calculated using below equation [10]:

Viability = (OD sample-OD blank)/(OD negative control-OD blank) ×100  

(1)

**Determination of the antibacterial effects of PA using MIC assay**

According to Lu et al., (2002) MIC assay protocol was similar to MTT assay [11], but after incubation of bacteria with different concentrations of PA at 37°C for 24 hours, the absorbance was measured at 600 nm using a plate reader (Biotek, USA). The assay was repeated three times for each concentration and inhibitory effect of PA on bacterial cells was calculated using equation 2 [12]:

MIC = (1- (OD sample-OD blank)/(OD negative control (without antibiotic)-OD blank)) × 100  

(2)

**Assessment of the cytotoxicity effects of PA using MTT assay**

DMEM-F12 media (Gibco, USA) containing 10% fetal bovine serum (FBS) (Gibco, USA) was used for MCF-7 cells culture. Cells were cultured in 25T flasks and incubated at 37°C and 5% CO2. The media was changed every 2-3 days and hemocytometer was used for counting the cell growth.

For cytotoxicity effects of PA, MTT assay was done using the standard protocol [13]. Briefly, 3×10⁴ cells were seeded in 96-well plate containing 100 μl media (without serum) and incubated overnight in 5% CO2 and 80% humidity at 37°C. Then, the media was discarded and fresh media containing 0.5, 1, and 2 μg/ml of recombinant PA were added and the plate was incubated for 24 hours at 37°C. 5 μl MTT dye (5 mg/ml) was added and incubated for 1 hour in dark condition at 37°C. Then, 100 μl DMSO was added to every well and incubated 2 hours in the same condition. Media was used as the blank and untreated cells were used as the negative control. The absorbance was measured at 570 nm.

The assay was repeated three times for every concentration. Bacterial cells viability was calculated using equation 3 [14]:

Viability = (OD sample-OD blank)/(OD negative control-OD blank) × 100  

(3)

**Assessment of the cytotoxicity effects of PA using neutral red uptake assay**

Neutral red uptake assay protocol was similar to MTT assay [13, 15], but after incubation of cells with recombinant PA for 23 hours, 1 μl neutral red dye (5 μg/ml) was added to every well and incubated at 37°C. After the formation of red crystals, the supernatant was discarded and the pellet washed by PBS two times. About 100 μl fixation buffer (formaldehyde 37%, CaCl2 10%, water) was added to every well and incubated for 1 minute and then 100 μl solubilizing buffer (acetic acid 5%) was added and incubated for 20 min in dark condition in a shaker incubator and finally, the absorbance was read at 570 nm. The mortality rate was measured as equation 4:

Mortality = (1- (OD sample-OD blank)/(OD control-OD blank)) × 100  

(4)

**Assessment of apoptosis effect of the recombinant PA using alkaline comet assay**

Alkaline comet assay, also called single-cell gel electrophoresis assay, is a good method for DNA fragmentation analysis in cells. The assay was performed according to Sajad et al., [16]. Briefly, 12×10⁴ cells were seeded in a 24-well plate containing 300 μl media (without serum) and incubated overnight in 5% CO2 and 80% humidity at 37°C. Then, the media was discarded and 300 μl of fresh media containing 0.5, 1, and 2 μg/ml of recombinant PA were added and incubated for 24 hours at 37°C. Cells then trypsinized and harvested in fresh 1.5 ml tubes and centrifuged at 1500 rpm for 5 min at 4°C and washed with PBS (pH 7.4) two times. 200 μl PBS was added to tubes and cells were separated by a needle. Slides were covered by normal melting agarose (1%) and incubated for 10 min at 4°C. Cell suspensions were mixed with low melting agarose 1% (1:2 ratios) and were added to the slides. To form one cell layer, a glass lamel was placed on every slide. In order to lyse the cells and distract the nuclei, all slides were incubated for 16-18 hours in fresh and cold lysis buffer (NaCl 2.5 M, EDTA 100 mM, Tris 10 mM, NaOH 0.2 M, Triton X-100 %1, and pH 10) at 4°C. Then, slides were washed two times with electrophoresis buffer for 20 min and incubated in fresh electrophoresis buffer for 40 min at 4°C. The electrophoresis was done in 25 V and 300 mA for 45 min at 4°C. For neutralization, slides were incubated for 10 min in neutralizing buffer (Tris 0.04M pH7.5). Then slides were incubated in 100 μl ethidium bromide (20 μg/ml) for 10 min at room temperature. Slides were washed two times (10 min each) with water and analyzed by fluorescent microscope. For each sample at least 100 pictures were captured and pictures were analyzed by one way ANOVA test.
Reactive nitrogen species assay
To perform NO assay 2×10⁴ cells were seeded in 96-well plate containing 100 μl media (without serum) and incubated overnight in 5% CO₂, 80% humidity, and 37°C. Then, old media was discarded and fresh media containing 0.5, 1, and 2 μg/ml of recombinant PA were added and incubated for 24 hours at 37°C. Then, media was transferred to a flat 96-well plate and the absorbance was measured at 570 nm using a plate reader. The results were converted into activity using the equation 6.

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\text{Catalase activity} = \frac{(\text{OD} \times 1.998)}{(\text{mg protein of sample})}
\]  

Statistical analysis
Results were reported as Mean ± SD and data were analyzed using GraphPad InStat software (version 3, 2003). Treated cells and controls were analyzed using One-way ANOVA and tukey tests. *P<0.05 was considered as significant. Microsoft Excel software (2013 version) was used for plotting the curves.

Results
Antibacterial effects of recombinant PA using MTT assay
Antibacterial effects of different concentrations of PA (0.28, 0.56, 1.12, 2.25, and 4.5 μg/ml) on E. coli and S. aureus were analyzed in vitro. Different concentrations of PA had significant inhibitory effects on E. coli cells’ viability in comparison to the control. However, the viability rate between different concentrations was not significant (Fig. 1).

![MTT assay results](image)

**Figure 1.** Viability of E. coli and S. aureus after treatment with different concentrations of the recombinant PA using MTT assay. Results were compared with the negative control. (*) p<0.05

Determination of the antibacterial effects of PA using MIC assay
MIC assay was used to analyze the antibacterial effects of recombinant PA in vitro. 0.28, 0.56, 1, 12, 2.25, and 4.5 μg/ml of recombinant PA had 12.3, 34.3, 36.1, 37, and 34% inhibitory effects on E. coli growth, respectively. It indicates that PA significantly inhibits the growth of E. coli cells. For S. aureus, 0.28, 0.56, 1.12, 2.25 and 4.5 μg/ml of recombinant PA had 5.8, 6.6, 9.16, 8.16, and 0
percent inhibitory effect, respectively. Which indicates that PA did not significantly inhibit the growth of *S. aureus* as compared with the control (Fig. 2).

Figure 2. Determination of the inhibitory effect of recombinant PA on *E. coli* and *S. aureus* using MIC assay. Results were compared with the negative control. (*p<0.05) (**)p<0.01), (***)p<0.001)

**Cytotoxicity effects of the recombinant PA on MCF-7 cells using MTT assay**

In this study, the cytotoxicity effects of different concentrations of recombinant PA (0.5, 1, and 2 μg/ml) on MCF-7 cell line were analyzed. The viability of MCF-7 cells was 62.3, 33.3, and 20.5 percent in the presence of 0.5, 1, and 2 μg/ml of recombinant PA, respectively. The results indicates that the recombinant PA significantly inhibits the growth of MCF-7 cells (Fig. 3).

Figure 3. The inhibitory effects of different concentrations of the recombinant PA on MCF-7 cells using MTT assay. Results were compared with the negative control. (***)p<0.001)

**Cytotoxicity effects of the recombinant PA antigen on MCF-7 cells using neutral red uptake assay**

Growth inhibition of MCF-7 cells following addition of 0.5, 1, and 2 μg/ml of recombinant PA to the media was obtained and the results showed that the recombinant PA protein significantly inhibited the growth of MCF-7 cells, as compared with control (Fig. 4).

Figure 4. The inhibitory effects of different concentrations of recombinant PA on MCF-7 cells using neutral red uptake assay. Results are compared with the negative control. (***)p<0.001)

**Apoptosis effects of the recombinant PA using alkaline comet assay**

Alkaline comet assay was used for analyzing apoptosis induction by recombinant PA. So the results demonstrate that PA significantly induced the apoptosis of MCF-7 cells as compared with the control (Figure 5 and 6).

Figure 5. Analysis of Apoptosis effect of the recombinant PA on MCF-7 cells using alkaline comet assay. Results are compared with the negative control. (*p<0.05), (**)p<0.01), (***)p<0.001)

**Reactive nitrogen species assay**

The amount of nitrite oxide released from MCF-7 cells treated with different concentrations of the recombinant PA, 0.5, 1, and 2 μg/ml, was 39.12, 38.57, and 36.17
μM/ml, respectively. In comparison to the control, the recombinant PA did not significantly induced the release of NO from MCF-7 cells (Fig. 7).

![Nitrite release](image1.png)

**Figure 7.** Nitrite oxide release from MCF-7 cells following recombinant PA treatment using reactive nitrogen species assay. Results are compared with the control.

**Assessment of Reduced glutathione (GSH)**

GSH content of MCF-7 cells after the treatment with 0.5, 1, and 2 μg/ml of recombinant PA were 10.45, 9.98, and 9.98 μg, respectively. The released GSH from treated cells was not significant as compared with the control (10.85 μg GSH) (Fig. 8).

![Estimation of GSH](image2.png)

**Figure 8.** GSH release from MCF-7 cells using reduced glutathione (GSH) assay. Results are compared with the control.

**Catalase enzyme activity assay**

Catalase enzyme activity following treatment of cells with 0.5, 1, and 2 μg/ml of recombinant PA was assayed. The enzyme activity in MCF-7 cells was decreased after 24 hours treatment, in a dose dependent manner. Catalase activity was 839.265, 830.14, and 784.53 μM of hydrogen peroxide consumed/min/mg protein for 0.5, 1, and 2 μg/ml of recombinant PA antigen and not significant as compared with the control (860 μM of hydrogen peroxide consumed/min/mg protein) (Fig. 9).

![Estimation of catalase](image3.png)

**Figure 9.** Catalase activity following recombinant PA treatment. Results are compared with the control (ns: not significant).

**Discussion**

According to our results, PA has significant inhibitory effects on MCF-7 cell line. This antigen also has partial inhibitory effects on Escherichia coli and no inhibitory effect on Staphylococcus aureus. In the present study, the toxicity effects of PA were analyzed on *E. coli*, *S. aureus*, and MCF-7 cells. Antibacterial effects of the protein were analyzed for the first time using MTT reduction and MIC assays. Results showed that PA has a partial inhibitory effect on *E. coli* and no inhibitory effect on *S. aureus*, while it was cytotoxic on MCF-7 cells. Results of MTT and neutral red uptake assays demonstrated that different concentrations of recombinant PA (0.5, 1, and 2 μg/ml) significantly inhibited the growth of the breast cancer (MCF-7) cells. Also, the results of alkaline comet assay showed that the protein significantly induced the apoptosis in MCF-7 cells. The comparison of MTT, neutral red uptake, and comet assay results indicated that PA induces apoptosis and necrosis in these cells. The results of NO, GSH, and catalase assays showed that PA could not change the oxidation and reduction potential of MCF-7 cells, so nitrite oxide has no role in the induction of cell apoptosis.

The results of this study were compatible with a study by Ching-Gong et al., (1996), which showed that Bacillus anthracis LF toxin can induce apoptosis and necrosis in macrophages in a dose dependent manner [17]. A study by Liu et al., (2000) indicated that mutated protective antigen (PA) in which the furin protease cleavage site was replaced by specific sequences, which are selectively cleaved by MMPs, can induce toxicity in MMP-overexpressing cells [18]. A study by Park et al., (2002) showed that *B. anthracis* lethal factor (LF) selectively induces apoptosis in activated macrophages, which is in agreement with our results [19]. Rogers et al., represent that PA can inhibits angiogenesis and tumor growth [20]. Liu et al., (2017) demonstrated that PA can cause a pore in the cancer cells’ membrane and LF can enter the cytosol and induces the apoptosis, so the use of PA and LF proteins simultaneously have potent anti-tumor activity [21].
Also, in another study Liu et al., showed that recombinant and engineered tumor-targeted anthrax lethal toxin proteins strongly suppress the growth of solid tumors in mice. In this regard, native toxin receptors tumor endothelium marker-8 (TEM8) and capillary morphogenesis protein-2 (CMG2) act as the receptors for the PA domain of these toxins. [22] Compatible with our results, Khandia et al., (2017) demonstrated that recombinant lethal toxin of Bacillus anthracis had anti-proliferative activity on primary mammmary ductal carcinoma cells, so this toxin has therapeutic potential [23].

Conclusion
The results of this study demonstrated that the Bacillus anthracis’ protective antigen has a significant cytotoxic effect on cancer cells, so it can be considered as a candidate antitumor drug. However, more investigations are required. On the other hand, since this protein is considered as a candidate vaccine against B. anthracis, the safety of the protein should be noticed. Indeed, no significant growth inhibition was observed against bacterial samples so it seems the protein could not be important in developing of antibacterial drugs.

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References


