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Antimicrobial and proinflammatory effects of two vipericidins

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ABSTRACT

Hospital infections allied to bacterial resistance to antibiotics have become a major worldwide problem. In this context, antimicrobial peptides (AMPs) are presented as an alternative in the control of these resistant organisms. Besides antimicrobial effects, these molecules play a crucial role in immunity by acting as immunomodulators. These peptides can activate inflammatory cells to produce pro- and anti-inflammatory mediators. In this study we will show the activity against multi-drug resistant bacteria (MDRB) of two cathelicidins from South American pit vipers *Bothrops atrox* and *Crotalus durissus terrificus,* named batroxicidin and crotalicidin. It was observed that both peptides showed activity against MDRB and presented no hemolytic or cytotoxic activity. In addition, the ability of peptides to modulate the production of cytokines TNF-α, IL-10 and IL-6 was analyzed using Raw 264.7 cells in the presence of IFN-γ stimuli, and multi-resistant *E. coli* and *K. pneumoniae* antigens. An up-expression or down-expression of TNF-α, as well as the IL-10 mediator, was observed. The cytokine IL-6, on the other hand, presented only a down-regulation for Raw 264.7 cell groups. In conclusion, the results demonstrate that both peptides presented a predominantly proinflammatory characteristic to the inflammatory mediators dosed. Overall, even presenting a proinflammatory characteristic, these peptides are still promising for future research and development of new potential antimicrobial molecules.

1. Introduction

Nosocomial infections have become one of the most common complications seen in hospitalized patients, increasing mortality and morbidity rates [1]. Concomitantly with nosocomial infections, the incidence of microorganisms resistant to conventional antibiotics has been shown to be an aggravating factor. In this context, where conventional antibiotics are becoming limited against resistant microorganisms, antimicrobial peptides (AMPs) represent an alternative for pathogen control. Antimicrobial peptides are short organic molecules (2–50 amino acid residues), which compose the innate immune system [2,3]. Moreover, AMPs may also have immunomodulatory activity, which is complex and includes anti-infective immune modulation, such as the induction of cytokines and chemokines [4].

New approaches to treating infection, like AMPs, can involve induction of the immunologic system, to act against pathogens. Innate immunity can be responsible for preventing the host from succumbing to infection, considering the frequent exposure to pathogens [5]. When the immune system is stimulated to induce the production of chemokines, a regulation in the production of proinflammatory cytokines for the promotion of wound healing is one way in which infections can be controlled [5]. Since such a process has no direct action on pathogens, the chances of developing resistance are considered low [5]. In this context, several AMPs or host defense peptides have demonstrated the ability to activate the host immune system.

Among the several AMP classes and host defense peptides, the cathelicidin-related antimicrobial peptides have been widely studied [6]. Cathelicidins from several animals, including pit vipers, present

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antimicrobial activity [7,8]. Recently, two new vipericidins named batroxicidin and crotalicidin [9], identified from South America pit vipers *Bothrops atrox* and *Crotalus durissus terrificus,* have been demonstrated to have pronounced antimicrobial activity against several bacteria and presented a low hemolysis rate, thus showing great biotechnological potential. Moreover, no incidences of immunomodulatory activity have been described for either of them. In addition to a description of activity against multi-drug resistant bacteria (MDRB), this work sheds some light on the immunomodulatory effects of two vipericidins, using heat killed antigens from MDRB in Raw 264.7 cell line.

2. Material and methods

2.1. Peptide purity determination

The peptides used in this study were purchased from *Aminotech* Ltda (São Paulo, Brazil) (> 95% of purity). The mass and sequence of the peptides were confirmed by *Matrix Assisted Laser Desorption Ionization – Time of Flight* (*MALDI-ToF*) on mass spectrometer Ultraflex MALDI-TOF III (Bruker Daltonics) [10]. The synthetic peptide concentrations were determined using measurements of UV-spectroscopy at 205, 215 and 225 wavelength [11].

2.2. In vitro antimicrobial assays

Primarily, the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) for the American Type Culture Collection (ATCC) bacteria*, K. pneumoniae 13883, P. aeruginosa 27853, E. coli 25922 and S. aureus 25923*, besides the clinically isolated MDRBs, *K. pneumoniae 1445333, K. pneumoniae 1410503, E. coli 2101123* and *E. coli 1812446,* were established. All strains were grown in Mueller-Hinton (*Himedia*) broth overnight at 37 °C. MIC measurements were performed based on National Committee for Clinical Laboratory Standards (NCCLS) [12].

2.3. Cell culture and experimental groups

In order to analyze the vipericidins' immunomodulatory effects, Raw 264.7 cell lineage was obtained from the Rio de Janeiro Cell Bank (BCRJ code 0212) [13]. Cell cultures were incubated (5×10^5 per well) in 96-well culture plates, in supplemented minimum essential medium alpha medium (α-MEM) in a 5% [14] CO_2 incubator at 37 °C and 95% humidity. During cell experiments, cultures were submitted to different stimuli, such as the peptides batroxicidin and crotalicidin (16 $\mu g.mL^{-1}$), heat killed (HK) antigens from *K. pneumoniae 1410503* and *E. coli 1812446*, with and without IFN-γ.

2.4. Preparation and testing of heat killed microbial antigens (HK)

Experimental groups determined for the cytotoxicity and immunomodulation experiments involved the use of stimuli with heat killed antigens (HK) specific to the previously used microorganisms. The antigens were prepared from the same microorganisms used in the antimicrobial assays: *K. pneumoniae* 1410503 and *E. coli* 1812446, since these showed a greater susceptibility to the AMP tested. The preparation of these microorganisms involved the collection of freshly picked colonies that were re-suspended in PBS buffer (separately) and quantified by O.D. with further counting in a Neubauer chamber (density 1×10^5 $UFC.mL^{-1}$) for the bacteria. Subsequently, they were heated at 120 °C for 50 min and stored at −20 °C [118, 119]. The death of these microorganisms was confirmed from inoculation of an aliquot of each sample in liquid medium and incubation for more than 24 h. Antigens were tested prior to the cytotoxicity and immunomodulation assays. This test defined the concentration of antigen sufficient to stimulate the response of pre-osteoclast Raw 264.7 cells by analyzing nitrite production in the supernatant culture without promoting cell death. Thus, the concentration for cellular challenge of 1×10^6 $UFC.mL^{-1}$ was defined.

2.5. Cell viability

In order to assess cell viability after 24 h, the colorimetric MTT assay (Sigma-Aldrich, USA) was used. The MTT is a colorimetric method based on the ability of live cells to reduce the yellow salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide) to formazan [16]. After an incubation period, 155 µl of the supernatant was removed and 10 µl of MTT ($mg.mL^{-1}$) per well was added to the plates. The plates were incubated for 3–4 h in 5% CO_2 at 37 °C and 95% humidity. Further, 60 µl of dimethylsuldoxide (DMSO; Sigma-Aldrich, USA) per well was added in order to block the reaction, followed by 595 nm absorbance reading (Bio-Tek Powerwave HT, USA). Cell viability was expressed in absorbance, compared to Raw 264.7 cells, represented by 100% live Raw cells [15].

2.6. Cytokine and nitric oxide production

Thus, nitrite production was evaluated in cell culture supernatant by Griess reaction, as described previously, with some adjustments [16]. The dosage of the inflammatory mediators in the Raw 264.7 cells, using only the IFN-γ and antigen stimuli (*E. coli* 1812446 and *K. pneumoniae* 1410503), was performed previously, aiming to observe the regular production of these mediators by the cells without the presence of the peptides under study. Cytokines produced from culture supernatants were assessed after 6 h for MCP-1, 24 h for IL-6, TNF-α, GM-CSF and 72 h for IL-10 [17–19], by the enzyme-linked immunoabsorbent assay (ELISA) method, using the respective murine ELISA development kit (Peprotech, USA). Cytokine levels were expressed as picograms per milliliter ($pg.mL^{-1}$).

2.7. Statistical analysis

Experimental groups were analyzed in biological and technical replicates in 96-well culture plates, and the data were presented as mean and error deviation (mean ± SEM). Before the analysis, the normality of the data was assessed by Kolmogorov-Smirnov test. An analysis of variance (one way ANOVA) was used for comparisons between the studied conditions. Additionally, a statistical *post hoc* Bonferroni test was applied. Statistical tests were performed in GraphPad Prism 5.0 software (Instat California, USA), considering $p < 0.05$.

3. Results

3.1. Antimicrobial activity

MIC and MBC for the vipericidins were shown to be promising against ATCC bacteria, and most importantly against the MDRB, *K. pneumoniae* 1445333*, K. pneumoniae* 1410503*, E. coli* 2101123 and *E. coli* 1812446. As demonstrated in Table 1, the peptide crotalicidin is more active against ATCC bacteria *K. pneumoniae* 13883 (4 $\mu g.mL^{-1}$) and *E. coli* 25922 (2 $\mu g.mL^{-1}$) than batroxicidin (8 $\mu g.mL^{-1}$ for both bacteria), but less active against *P. aeruginosa* 27853 (16 and 8 $\mu g.mL^{-1}$). On the other hand, the peptide batroxicidin was shown to be as active as crotalicidin against both clinical isolated *K. pneumoniae* and *E. coli* 2101123, presenting a MIC of 16 $\mu g.mL^{-1}$ and 8 $\mu g.mL^{-1}$ respectively. For the clinical isolate *E. coli* 1812446, batroxicidin was demonstrated to be more active than crotalicidin (4 and 16 $\mu g.mL^{-1}$).

3.2. Cell viability and inflammatory mediators

The cell viability evaluation performed after 24 h of exposure to crotalicidin and batroxicidin demonstrated no cytotoxicity for any of