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Gomesin acts in the immune system and promotes myeloid differentiation and monocyte/macrophage activation in mouse

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ABSTRACT

Due to the cytotoxic effect of antimicrobial peptides (AMP) against several microorganism and tumor cells has been proposed their association with the immune system. However, just a few reports have shown this relationship. In this study, mice were treated with gomesin, a β-hairpin AMP that exhibit high cytotoxicity against bacterial and tumor cells. Different effects in the immune system were observed, such as, decrease of $CD3^+$ in T lymphocytes (Control: 17.7 ± 1.4%; Gomesin: 7.67 ± 1.2%) and in hematopoietic progenitors and increase of hematopoietic stem cell (Control: 0.046 ± 0.004%; Gomesin: 0.067 ± 0.003%), $B220^+$ B lymphocytes (Control: 38.63 ± 1.5%; Gomesin: 47.83 ± 0.48%), and Mac-1^+F4/80^+ macrophages (Control: 11.76 ± 3.4%; Gomesin: 27.13 ± 4.0%). Additionally, macrophage increase was accompanied by an increase of macrophage phagocytosis (Control 20.85 ± 1.53; Gomesin 31.32 ± 1 Geometric mean), interleukin 6 (Control: 47.24 ± 1.9 ng/mL; Gomesin: 138.68 ± 33.68 ng/mL) and monocyte chemoattractant protein-1 (Control: 0.872 ± 0.093 ng/mL; Gomesin: 1.83 ± 0.067 ng/mL). Thus, this report showed immunomodulatory activity of gomesin in the immune system of mice.

1. Introduction

Antimicrobial peptides (AMPs) are present in nearly all organisms displaying diverse tridimensional structures. Due to the presence of AMPs in the most of species and its ability to kill several microorganism, AMPs have been associated to the immune system. AMPs with immunomodulatory activity are called of host defense peptides, these peptides are typically expressed in macrophages, neutrophils, mast cells, stromal bone marrow and mucosal epithelial cells [1–5]. The host defense peptides from cathelicidins and defensins families are the best characterized [3,5,6].

Among the known AMPs most of them are cationic, amphipathic and with molecular weights of less than 10 kDa [7]. Their mode of action has not been completely elucidated, however, several models of interaction with bacterial and eukaryotic plasma membrane have been proposed [8–10]. Despite the classical models of interaction of AMPs with bacterial membranes, such as barrel-stave model, toroidal pore model and carpet model [11], more complex actions seems to occurs in mammals cells. For instance, internalization into the cells seems to be particularly important in their cytotoxic effect [12–14]. Several reports have shown the internalization of AMPs and intracellular targets such organelles and DNA, but is unknown in detail how AMPs can affect internal cellular targets [11,13]. Cytotoxic effects in mammals cells occurs by several mechanism such as membrane permeabilization [12], caspase-dependent apoptosis [15], or autophagy [16]. When associated with the immune system AMPs have showed chemotactic activity against macrophages, neutrophils [17], dendritic cells [18], and degranulation of mast [19].

Among AMPs, we point out gomesin (Gm) that is a well-studied β-hairpin AMP containing 18 amino acid residues, including 4 cysteines engaged in two internal disulfide bridges, Cys^{2-15} and Cys^{6-11}. It contains two posttranslational modifications, a pyroglutamic acid (Z) as the N-terminus and an amidated arginine residue as the C-terminus. The entire sequence is ZCRRLCYKQRCVTYCRGR-NH_2. Gm was isolated from the hemocytes of the Brazilian spider *Acanthoscurria gomesiana*. Gm exhibits high cytotoxicity against bacteria, fungi, yeast, and parasites [20–24]. It is commonly

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assumed that the main antimicrobial mechanism of Gm, and other AMPs involves membrane permeabilization and destabilization. The main activity described of Gm in mammals cells is its cytotoxic activity against B16 melanoma tumor cells [25], neuroblastoma SH-SY5Y, pheochromocytoma PC12 cells [26], leukemic lineages [8], and cell hamster Chinese cells [13]. Intracellular mechanism triggered by Gm to induce cell death was related to endoplasmic reticulum disturbance, cytosolic Ca^{2+} increase, followed by an accumulation of Ca^{2+} in organelles, which induces loss of mitochondria potential leading to collapse of mitochondria, which culminates with the disruption of cellular membrane [13,26]. The structural characteristics of Gm are associated to cytotoxic activity and has been related with its disulfide bridges that are responsible to its β-hairpin conformation, degradation resistance, and interaction with cell membrane [27–29].

In this paper we decide to expand our acknowledge about the Gm molecule and evaluate its effect in the immune system in a murine model.

2. Experimental procedures

2.1. Peptide synthesis

Gm was synthesized manually by the solid-phase method on a 4-methylbenzhydrylamine-resin (MBHAR) (0.8 mM/g) using the *t*-Boc strategy [27]. Full deprotection and cleavage of the peptide from the resin were carried out using anhydrous hydrogen fluoride (HF) treatment with anisole and dimethyl sulfide (DMS) as scavengers at 0 °C for 1.5 h. Formation of disulfide bridges was achieved immediately after the HF cleavage and extraction of the crude peptide. The resulting peptide solution was kept at pH 6.8–7.0 and 5 °C for 72 h. Cyclization reactions were monitored by reverse-phase liquid chromatography coupled to an electrospray ionization mass spectrometer (LC/ESI–MS). Lyophilized crude peptides were purified by preparative RP-HPLC on a Vydac C_{18} column (25 × 250 mm, 300 Å pore size, and 15 μm particle size) in two steps. The first was performed by using triethylammonium phosphate (TEAP) pH 2.25 as solvent A and 60% acetonitrile (ACN) in A as solvent B. The second step was carried out using 0.1% trifluoroacetic acid (TFA) H_2O as solvent A and 60% ACN in A as solvent B. Pure peptides were characterized by amino acid analysis and by LC/ESI–MS.

2.2. Animals

In this study, 3-month-old male C57BL/6 mice were used. Animals were supplied by INFAR/UNIFESP Animal Facility. All experiments were approved by the Animal Care Ethics Committee of Universidade Federal de São Paulo (0225/10). Mice were euthanized by cerebral concussion and the bone marrow was extracted from femoral cavity.

2.3. In vivo assays

Experimental animals were daily intraperitoneally administered 100 μL of sterile saline, pH 7.4 of Gm at 1 or 3 mg/kg body weight for 3 days; the control animals were simultaneously administered equal sterile saline volumes.

2.4. Bone marrow immunophenotyping

Hematopoietic populations were identified by flow cytometry as described previously [30,31]. HSC cells were identified using a lineage marker cocktail (Lin: PE-conjugated B220, CD3, Ter-119, CD11b and Gr-1). Additionally, several antibodies (FLK-2-PE, IL-7R-PE, CD90-FITC, Sca-1-Cy7/PE and c-Kit-APC) were used to recognize a subset rich in HSCs: $Lin^-FLK\text{-}2^-Sca\text{-}1^+c\text{-}Kit^+Thy1.1^+$. Moreover, common myeloid progenitors (CMP): $Lin^-IL\text{-}7R^-c\text{-}Kit^+Sca\text{-}1^-CD34^+Fc\gamma R^{low}$); granulocyte/monocyte progenitor (GMP): $Lin^-IL\text{-}7R^-c\text{-}Kit^+Sca\text{-}1^-CD34^+Fc\gamma R^{high}$; megakaryocyte–erythroid progenitor (MEP): $Lin^-IL\text{-}7R^-c\text{-}Kit^+Sca\text{-}1^-CD34^-Fc\gamma R^{low}$ populations were identified using IL-7R-PE, Sca-1-PE, c-Kit-Cy7/PE, CD34-FITC and FCγR-APC antibodies. Mature populations were identified as myeloid cells: $Mac\text{-}1^+Gr\text{-}1^+F4/80^-$; monocyte/macrophage: $Mac\text{-}1^+F4/80^+Gr\text{-}1^-$; T cell: $CD3^+B220^-Gr\text{-}1^-$; B cells: $B220^+CD3^-Gr\text{-}1^-$. All antibodies were purchased from Becton Dickinson (Franklin Lakes, NJ, USA). We captured 300.000,00 events to evaluate hematopoietic progenitor's populations and 30.000,00 to evaluate mature populations. Flow cytometry was performed on a BD Accuri C6 cytometer (Becton Dickinson, USA). For data analyzes FlowJo v10 software (Tree StarInc., Ashand, OR) was used.

2.5. Colony forming unity (CFU) assay

CFU assays were performed by plating 2×10^4 murine bone marrow cells in methylcellulose-based medium with recombinant cytokines and EPO (Methocult M3434; StemCell Technologies, USA) in 35-mm diameter dishes. The cells were cultured in a fully humidified air incubator under 5% CO_2 at 37 °C for 7 or 14 days for murine and human cells, respectively. At the end of the incubation period, colonies of more than 50 cells were counted using a dark field microscope [30,31].

2.6. Macrophage phagocytosis assay

The phagocytic capacity of J774 macrophages was determined using heat-killed Escherichia coli labeled with the pH-dependent dye pHRODO (Life Technologies, Carlsbad, CA). After stimulation with Gm, about 2×10^8 labeled *E. coli* were added to 2×10^5 J774 cell line cultures and analyzed using flow cytometry (excitation: 488 nm; emission: 585/26 nm for pHRODO Red BioParticles conjugates), according to manufacturer's instructions. Flow cytometry was performed on a BD Accuri C6 cytometer (Becton Dickinson, USA). For data analyzes FlowJo v10 software (Tree StarInc., Ashand, OR) was used.

2.7. Quantification of Cytokines

The cytokines present in mouse plasma were measured using BD™ Cytometric Bead Array (CBA) Mouse Inflammation Kit, accordingly with manufacturer instructions. Briefly, blood was extracted by cardiac puncture, and the plasma obtained by centrifugation. Next, the anti-cytokines Beads were added to 300 μL of plasma, washed with PBS and labeled. A standard curve was obtained using cytokines standards. Flow cytometry was performed on an Accuri C6 cytometer (Becton Dickinson, USA). For data analyzes FlowJo v10 software (Tree StarInc., Ashand, OR) was used. Were quantified: interleukin 6 (IL-6), interleukin 10, monocyte chemoattractant protein 1 (MCP-1), interferon γ, Tumor necrosis factor, and interleukin 12.

2.8. Statistical analysis

All data represent at least three independent experiments and were expressed as mean ± standard error of the mean (SEM). Statistical analyzes were performed using Student's *t*-test for comparison between two groups, and analysis of variance (ANOVA) and Dunnett's post hoc test for multiple comparisons among groups. A probability value of $P < 0.05$ was considered significant.