International Immunopharmacology 38 (2016) 204–213

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Human β-defensin-3 alleviates the progression of atherosclerosis accelerated by *Porphyromonas gingivalis* lipopolysaccharide

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ARTICLE INFO

Article history:
Received 19 December 2015
Received in revised form 1 June 2016
Accepted 2 June 2016
Available online xxxx

Keywords:
Human β-defensin-3
Atherosclerosis
Porphyromonas gingivalis
Lipopolysaccharide
Periodontitis

ABSTRACT

Background and aim: Porphyromonas gingivalis (*P. gingivalis*) lipopolysaccharide (LPS) is reported to be associated with the progression of atherosclerosis (AS). In this study, we explored the potential of human β-defensin-3 (hBD3), an antimicrobial peptide with immunomodulatory properties, to alleviate AS progression accelerated by *P. gingivalis* LPS and the mechanism underlying this effect.

Materials and methods: Apolipoprotein E-deficient mice were injected intraperitoneally with hBD3, *P. gingivalis* LPS, or hBD3 + *P. gingivalis* LPS. The aorta was assessed immunohistologically and mRNA levels of inflammatory cytokines were determined by quantitative PCR. Macrophages and vascular endothelial cells were stimulated *in vitro* to investigate the hBD3 target cells. Inflammatory cytokines in serum and cell culture supernatants were detected using cytometric bead arrays. Signaling pathways were investigated by Western blotting.

Results: In *P. gingivalis* LPS-treated mice, hBD3 significantly reduced serum IL-6 and TNF-α levels and aortic expression of ICAM-1, IL-6, and MCP-1 (mRNA and protein). The area and severity of atherosclerotic lesions were also diminished, with less advanced plaque formation, more continuous and distinct elastic lamina, and more normal smooth muscle cells arranged along the tunica media layer. *In vitro*, hBD3 decreased TNF-α, IL-1β, IL-6 secretion and downregulated TNF-α, IL-1β, IL-6, IL-8, VCAM-1, and IL-10 mRNA levels in macrophages. hBD3 did not influence TNF-α, IL-6, and IL-8 levels in HUVECs culture supernatants. Furthermore, hBD3 suppressed *P. gingivalis* LPS-induced activation of the NF-κB, p38 and JNK pathways.

Conclusion: hBD3 alleviates AS progression accelerated by *P. gingivalis* LPS in apolipoprotein E-deficient mice by downregulating the cytokine expression in macrophages *via* the MAPK and NF-κB signaling pathways.

1. Introduction

Porphyromonas gingivalis (*P. gingivalis*) is a major pathogen involved in the pathogenesis of periodontitis, which is a chronic inflammatory disease with a high incidence. *P. gingivalis* lipopolysaccharide (LPS) in periodontal lesion may invade into bloodstream and impact the systemic conditions negatively. Efforts have been made to understand the association between periodontal disease and systemic condition including atherosclerosis (AS), which remains a hot topic [1–5]. Evidence has been provided that elevated serum level of *P. gingivalis* LPS is associated with the progression of AS [6,7]. Long-term exposure to *P. gingivalis* LPS promotes the formation of AS plaque in Apolipoprotein E-deficient (ApoE −/−) mice [8]. *P. gingivalis* LPS augments the production of pro-inflammatory cytokines and facilitates monocyte adhesion to vascular endothelial cells, which are the initial events of AS [9–11]. In addition, many studies demonstrate that *P. gingivalis* LPS promotes macrophages transformation to foam cells. The expression of pro-inflammatory cytokines, growth factors, and adhesion molecules in macrophages is upregulated by *P. gingivalis* LPS [12–16]. Collectively, these findings indicate that *P. gingivalis* LPS from periodontal lesions may contribute to the development of AS.

Essentially, AS is a chronic inflammatory disease of the arterial wall with inflammation implicated in every stage of its progression [17,18]. Currently, treatment of AS depends predominantly on lipid-lowering drugs. Despite various therapies targeting serum lipoprotein levels, AS remains to be the most significant cause of death in the industrialized world [19]. As the fundamental role of inflammation in AS has been characterized, more and more attention has been paid to anti-inflammation therapy [20–23]. Agents targeting chronic systemic inflammatory conditions have shown promise in the treatment of AS both experimentally and clinically [24].

Human β-defensins (hBDs) are small, cationic host defense peptides with immunomodulatory properties and chemoattractant activity for human monocytes, lymphocytes and DCs, suggesting that they contribute to the link between innate and adaptive immunity [25,26]. Human

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http://dx.doi.org/10.1016/j.intimp.2016.06.003
1567-5769/

β-defensin-3 (hBD3) exerts diverse innate immune activities and is considered to be the most promising of its class in preventing inflammatory disease [27,28]. hBD3 is widely expressed in many tissues including oral and vascular epithelium, where it participates in inflammation and the initiation of innate immune responses [29–32]. Many studies have shown that hBD3 suppresses the inflammatory effect caused by *Escherichia coli* lipopolysaccharide (LPS) *in vitro* and *in vivo* [33–35]. In addition, hBD3 exerts anti-inflammatory effect against *Enterococcus faecalis* to induce TNF-α, IL-8, and ICAM-1 expression in human monocytic cell line, THP-1 [36].

Based on the anti-inflammatory effect and antimicrobial property of hBD3, we hypothesized that hBD3 may play a role in the development of AS. However, very limited evidence regarding the effect of hBD3 on AS is currently available.

In this study, we demonstrated that *P. gingivalis* LPS accelerates the progression of AS in ApoE −/− mice. And hBD3 alleviates these unfavorable effects by downregulating the expression of pro-inflammatory cytokine expression. Furthermore, target cells and related signaling pathways were investigated *in vitro* to elucidate the underlying mechanism of the anti-inflammatory effects of hBD3. Our results demonstrated that hBD3 downregulates inflammatory cytokine expression in macrophages rather than vascular endothelial cells. Both the mitogen-activated protein kinase (MAPK) and NF-κB signaling pathways participate in the anti-inflammatory effects of hBD3. Based on these observations, we speculated that hBD3 alleviates the progression of AS accelerated by *P. gingivalis* LPS from periodontal lesions.

2. Materials and methods

2.1. Reagents and instruments used

Ultrapure *P. gingivalis* LPS was purchased from Invivogen (Carlsbad, CA, USA). Recombinant human BD-3 was purchased from PEPROTECH (Rocky Hill, NJ, USA). Phosphate-buffered saline solution was purchased from Hyclone (Logan, UT, USA). BD Cytometric Bead Array Human Inflammatory Cytokines Kits and the Mouse Enhanced Sensitivity Flex Set of TNF and IL-6 were purchased from BD Biosciences. Anti-CD68 antibody and anti-MCP-1 antibody were from Wuhan Boster Biological Technology (Wuhan, China). Anti-ICAM-1 antibody, anti-IL-6 antibody, and anti-VCAM-1 antibody were from ProteinTech Group (Chicago, IL, USA). The following antibodies were purchased from Cell signaling (Cell Signaling Technology, MA, USA): anti-p44/42 MAPK (Erk1/2) antibody (catalog number 4695), anti-phosphorylated p44/42 MAPK (Erk1/2) antibody (catalog number 4370), anti-p38 MAPK antibody (catalog number 8690), anti-phosphorylated p38 MAPK antibody (catalog number 4511), anti-SAPK/JNK antibody (catalog number 9252), anti-phosphorylated JNK antibody (catalog number 4668), anti-phosphorylated IkappaB-alpha antibody (catalog number 2859), anti-phosphorylated NF-κB p65 antibody (catalog number 3033), and anti-NF-κB p65 antibody (catalog number 8242). The anti-GAPDH antibody was obtained from Bioworld (Nanjing, China).

2.2. ApoE −/− mice

Nine-week-old male ApoE −/− mice on a C57BL/6 background were purchased from the Department of Laboratory Animal Science, Beijing University (Beijing, China). The mice were maintained in a specific pathogen-free facility on a 12 h light: 12 h dark cycle at 25 °C. Throughout the treatment period, body weight and food intake were monitored at weekly intervals. All surgery on animals was performed in accordance with the Animal Ethics Committee of Nanjing University (China).

Firstly, we explored the role of hBD3 in the acute inflammatory response caused by *P. gingivalis* LPS. Twenty-four randomly selected mice were divided into four groups (n = 6 per group) and injected intraperitoneally with sterile phosphate-buffered saline, hBD3 (0.5 mg/kg, approximately 10 μg/mouse), *P. gingivalis* LPS (5 mg/kg, approximately 100 μg/mouse) or hBD3 + *P. gingivalis* LPS. Mice were euthanized 2 h later.

Based on the observation of the anti-inflammatory effect of hBD3, we conducted a long term experiment to investigate the role of hBD3 in AS. Forty randomly selected mice were divided into four groups and injected intraperitoneally with sterile phosphate-buffered saline, hBD3 solution (0.1 mg/kg, approximately 2 μg/mouse), *P. gingivalis* LPS (0.5 mg/kg, approximately 10 μg/mouse), or hBD3 + *P. gingivalis* LPS. Treatments were administered three times per week. Mice were fed a high-fat diet containing 10% lard, 4% milk powder, 2% cholesterol and 0.5% sodium cholate. Eight weeks later, the mice were euthanized and atherosclerotic plaques were observed.

2.3. Tissue harvesting and preparation

Blood was collected immediately after the mice were anesthetized by overdose of isoflurane and euthanized. Then they were dissected and perfused with 10 mL PBS *via* the left ventricle, followed by 10 mL of 4% phosphate-buffered paraformaldehyde phosphate. The aortic trees (from the aortic valve to the iliac bifurcation) were carefully dissected. Five of them in each group were preserved in 4% buffered paraformaldehyde phosphate and processed for *en face* morphometric analysis [37,38]. The other five were homogenized using a TissueLyser (Shanghai JingXin) at 65 Hz for 90 s in TRIzol (Sigma, MO, USA) and stored at −80 °C until further processing for quantitative PCR. The proximal aortas together with hearts of all mice were fixed in 4% phosphate-buffered paraformaldehyde.

2.4. Atherosclerotic plaque assessment

The extent of aortic atherosclerotic lesions was analyzed by *en face* staining with Oil red O, as previously described [39]. Briefly, the aortic tissue of each mouse (n = 5 mice per group) was harvested from the aortic valve to the iliac bifurcation. All adventitial fat and connective tissue were carefully removed. The aorta was then opened longitudinally, followed by fixation overnight in 4% paraformaldehyde. The next day, the aorta was rinsed with 5 mL of 60% isopropanol and pinned out to black wax simultaneously to reveal the entire luminal surface area. Then the aorta was stained in 5 mL of filtered 60% Oil Red O solution (Sigma, MO, USA) for 15 min at 57 °C, followed by rinsing with 60% isopropanol for 5 min. Finally, the aorta was submerged in PBS and photographed. Photographs were obtained using a digital camera (Canon EOS 650D) with a macro lens (EF 100 mm f/2.8L Macro IS USM). The lesion area was selected using Photoshop 8.0 (Adobe) software and quantified using Image-Pro Plus 6.0 (Media Cybernetics) software.

2.5. Histology and immunohistochemistry of the aortic sinus

After fixation in 4% phosphate-buffered paraformaldehyde phosphate for 48 h, five samples in each group were embedded in optimal cutting temperature compound (OTC; Sakura Finetek, Beijing, China). Serial cryosections (8 mm thick) of the aortic sinus were cut and 4–6 sections from each sample were selected for Oil Red O staining to detect lipids in the plaque. The other samples in each group were embedded in paraffin. Sections (5 μm thick) were prepared and stained with hematoxylin and eosin (HE) or Masson Trichrome or used for immunostaining.

Immunohistochemical staining was performed to determine the expression levels of several inflammatory markers in the aortic root. Briefly, sections were deparaffinized and rehydrated before antigen retrieval using EDTA buffer (pH 9.0). The sections were incubated with H_2O_2 (0.3%) for 25 min at room temperature and protected from light to block endogenous peroxidase activity. Then the sections were blocked with 3% bovine serum albumin for 30 min at room temperature to prevent non-specific binding. Subsequently, the sections were incubated