Molecular Immunology 63 (2015) 489–496

Contents lists available at ScienceDirect

Molecular Immunology

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

Porphyromonas gingivalis HSP60 peptides have distinct roles in the development of atherosclerosis

Euikyong Jeong[a], Koanhoi Kim[b], June Hong Kim[c], Gil Sun Cha[d], Sung-Jo Kim[d], Ho Sung Kang[a], Jeomil Choi[d,*]

[a] Department of Molecular Biology, College of Natural Sciences, Pusan National University, South Korea
[b] Department of Pharmacology, School of Medicine, Pusan National University, South Korea
[c] Department of Internal Medicine, School of Medicine Pusan National University, South Korea
[d] Department of Periodontology, School of Dentistry, Pusan National University, Dental Research Institute, South Korea

ARTICLE INFO

Article history:
Received 30 August 2014
Accepted 5 October 2014
Available online 23 October 2014

Keywords:
Atherosclerosis
Periodontitis
Autoimmunity
Porphyromonas gingivalis
Regulatory T cell

ABSTRACT

Different epitope peptides of bacterial heat shock proteins may function as effector or regulatory molecules in autoimmune responses in infection-triggered atherosclerosis. We investigated the mechanisms for the distinct roles of two epitope peptides from *Porphyromonas gingivalis* heat shock protein 60 (HSP60) in atherogenesis with regard to peptide-specific T cell polarization relevant to (1) phenotype and cytokine profiles, (2) expression of transcription factors, and (3) role of antigen presenting dendritic cell subsets.

Apolipoprotein E-knockout (ApoE KO) mice were immunized with peptide 14 or peptide 19 from *P. gingivalis* HSP60 prior to induction of atherosclerosis by infection with *P. gingivalis* plus a Western diet. Significant reductions in plaque/lipid droplet area and plasma cholesterol levels were observed in mice immunized with peptide 14, whereas the opposite phenomenon was evident in mice immunized with peptide 19. $CD4^+$ T-cells polarized to the regulatory T-cell type in peptide 14-immunized group, whereas they polarized to the Th1 cells in peptide 19-immunized group; this observation was supported by the cytokine profiles characteristic to each T-cell phenotype.

Significantly higher expression of Nr4a1 and Nr4a2 mRNA, transcriptional factors for regulatory T-cell type, were observed in peptide 14-immunized group. In contrast, the expression level of IFN-γ and T-bet mRNA, signaling molecules for Th1 cells, was higher in peptide 19-immunized group than in PBS-immunized group.

In non-immunized wild mice, BMDC-derived $CD11c^+$ dendritic cells have shown to stimulate Tregs significantly in antigen-nonspecific manner. However, each peptide antigen demonstrated a unique mode of preferential adoption of dendritic cell subsets.

In conclusion, peptide 14 or peptide 19 from *P. gingivalis* HSP60, respectively, may play either an anti- or pro-atherogenic role in the ApoE KO mouse model of infection-triggered atherosclerosis through distinct mechanisms operating in the polarization of T cells.

1. Introduction

Periodontitis, an oral infectious disease predominantly by *Porphyromonas gingivalis* (*P. gingivalis*) (Darveau, 2010), has been one of the risk factors for cardiovascular diseases (Friedewald et al., 2009), by triggering the initial pathogenesis of atherosclerosis, inflammation of the vasculature, through activating monocytes/macrophages (Miyajima et al., 2014). *P. gingivalis* was detected in atheromatous plaques and reported to perpetuate systemic inflammation (Darveau, 2010; Kozarov et al., 2005).

On the basis of the concept of molecular mimicry, bacterial heat shock protein (HSP) 60 has been claimed to be either triggering

* Corresponding author at: Department of Periodontology, School of Dentistry, Pusan National University, Beomeo-Ri, Mulgeum-Eup, Yangsan City 626-870, South Korea. Tel.: +82 55 360 5200; fax: +82 55 360 5194.
E-mail addresses: eui_kyong@pusan.ac.kr (E. Jeong), Koanhoi@pusan.ac.kr (K. Kim), junehongk@gmail.com (J.H. Kim), bunh12@pusan.ac.kr (G.S. Cha), sungjokim@pusan.ac.kr (S.-J. Kim), hspkang@pusan.ac.kr (H.S. Kang), jrapa@pusan.ac.kr (J. Choi).

http://dx.doi.org/10.1016/j.molimm.2014.10.004
0161-5890/

or suppressing molecules involved in autoimmunity (Rajaiah and Moudgil, 2009; Van Eden et al., 2007). Furthermore, HSP-derived peptides can promote the production of anti-inflammatory cytokines, indicative of their immunoregulatory potential (van Eden et al., 2005), while *P. gingivalis* HSP60 (PgHSP60) was reported to accelerate the development of experimental atherosclerosis (Ford et al., 2005).

T cells may play a pivotal role in the progression of atherosclerosis through their response to major autoantigens including human HSPs and oxidized low-density lipoprotein (oxLDL) (Gotsman et al., 2007). In atherosclerotic lesions, most HSP60-specific T cells polarize primarily into effector T cells (Teffs), particularly into interferon γ (IFN-γ)-producing T helper 1 (Th1) subsets (Hansson and Libby, 2006). IFN-γ is a proatherogenic cytokine that can activate macrophages and endothelial cells (Gotsman et al., 2007; Hallenbeck et al., 2005).

Nevertheless, mucosal administration of HSP60 or an HSP60-peptide was reported to induce regulatory T cells (Tregs), which play an anti-atherogenic role through the production of transforming growth factor β (TGF-β) and interleukin-10 (IL-10) (Maron et al., 2002; van Puijvelde et al., 2007). Hence, molecular mimicry-based tailored antigen from the HSP peptide might mobilize antigen-specific Tregs to suppress the periodontitis-triggered autoimmune response in atherosclerosis (Van Eden et al., 2007; Choi and Seymour, 2010). PgHSP60-specific T cells secreted cytokines characteristic of both Th1 and Th2 subsets identified in periodontal tissues and atherosclerotic lesions (Choi et al., 2002; Chung et al., 2003).

Cross-reactivity between anti-*P. gingivalis* and anti-human HSP60 immunoglobulin G (IgG) antibodies was demonstrated in patients with atherosclerosis (Ford et al., 2005; Choi et al., 2004). Depending on the mapping patterns of T- and/or cross-reactive B-cell epitopes in human periodontitis and atherosclerosis (Choi et al., 2004), we have selected two different immunodominant peptides, peptide 14 (Pg14) and peptide 19 (Pg19) from PgHSP60, to evaluate their capacity to induce Tregs or Teffs. It is of particular interest to note that Pg19 was found to be an immunodominant T- and cross-reactive B-cell epitope in the periodontitis-atherosclerosis axis (Choi et al., 2011; Jeong et al., 2012) that might serve as autoimmune target (Perschinka et al., 2003). However, the immune-modulating effects of a specific PgHSP60 peptide in atherosclerosis have not been fully elucidated.

This paper reports the distinct roles of Pg14 and Pg19, as tailored target antigens through molecular mimicry, in the development of atherosclerosis with regard to peptide-specific T cell polarization relevant to (1) phenotype and cytokine profiles, (2) expression of transcription factors, and (3) role of antigen presenting DC subsets.

2. Materials and methods

2.1. Synthetic peptides

Of a total of 37 overlapping peptides spanning the entire HSP60 protein sequence, Pg14 (TVEVVEGMQFDRGYISPYFV), Pg19 (TLVVNRLRGSLKICAVKAPG), mouse HSP60 peptide 14 (Mo14, ELEIIEGMKFDRGYISPYFI), and Mo19 (TLVLNRLKVGLQVVAVKAPG) were synthesized by 9-fluorenylmethoxycarbonyl solid-phase peptide synthesis (Peptron Inc., Daejeon, South Korea).

2.2. Growth and maintenance of the bacterial strains

P. gingivalis ATCC 33277 (American Type Culture Collection, Manassas, VA) was grown anaerobically in tryptic soy broth (Difco Laboratories, Detroit, MI) supplemented with hemin and menadione. The number of bacterial colony-forming units (CFUs) was standardized by measuring the optical density at 600 nm.

2.3. Mice and oral infection with P. gingivalis

The Animal Care and Use Committee of Pusan National University approved all animal protocols. ApoE KO homozygote mice (stock#2052) were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were fed a Western diet (0.2% of cholesterol, 21.2% of fat, 13.7% saturated fatty acid, and 7.3% total unsaturated fatty acid; Research Diets, D12079B, New Brunswick, NJ, USA) throughout the experiment. Seven-week-old male ApoE KO mice were divided into four groups (five mice per group).

Mice in groups I and II were injected subcutaneously with phosphate-buffered saline (PBS) in alum and served as Control group or PBS-immunized group, respectively. Mice in groups III and IV were immunized subcutaneously with 50 μg of either Pg14 or Pg19 peptide in alum and served as Pg14-immunized group or Pg19-immunized group, respectively. Mice in groups II, III, and IV were then challenged orally with *P. gingivalis* ($\sim 5 \times 10^8$ CFU/mouse) in 2% carboxymethyl cellulose three times per week for a period of three weeks, whereas mice in group I were challenged orally with PBS only in 2% carboxymethyl cellulose. At 14 weeks after the first infection, all mice were killed to collect their aortas, blood, and spleens. We have confirmed that oral infection of *P. gingivalis* have successfully led to an intraoral colonization by the bacteria by RT-PCR, induction of antigen-specific IgG antibody responses, and alveolar bone loss (data not shown).

2.4. Histometric analysis of aortic lesions

Briefly, cryosections were stained with hematoxylin and eosin. The percentage of plaque area was calculated as area of the plaque relative to the total aortic area observed. The same lesion was stained with Oil red O to evaluate the lipid droplet in the atherosclerotic lesion. Total plasma cholesterol levels were determined by an enzyme-linked immunosorbent assay by using a commercially available enzymatic kit (Shin Yang Chemical, Seoul, Korea).

2.5. Dot-immunoblot analysis

To identify the intensity of the dot blot as a measure of serum reactivity to peptide, each synthetic HSP60 peptide was spotted onto a nitrocellulose membrane. The membranes were separately blocked and incubated with individual serum samples from each group of mice. The membranes were washed and horseradish peroxidase-conjugated goat anti-mouse IgG was then added. The membranes were again washed followed by addition of tetramethylbenzidine for color development. The same procedure was repeated for PgHSP60 and oxLDL (a kind gift from Dr. Witztum, UCSD, La Jolla, CA).

2.6. Establishment of antigen-specific T cell lines

2×10^5 splenic $CD4^+$ T cells from groups I (Control), II (PBS-immunized), III (Pg14-immunized) or IV (Pg19-immunized) were negatively separated by MACS beads (>90%) (Miltenyi Biotec, Auburn, CA). T cells were then cocultured with 2×10^6 mitomycin C-treated splenic stimulator cells and each antigen (PBS, Pg14 or Pg19, 10 μg/mL) to evaluate the patterns of antigen-specific T cell polarization.

2.7. Evaluation of the role of $CD103^+$ DC in inducing antigen-specific Tregs

2.7.1. Generation of bone marrow-derived dendritic cells (BMDC)

To evaluate the specific role of BMDC as antigen presenting cells (APC's) in inducing antigen-specific Tregs, bone marrow cells were removed from the femurs and tibiae of 7–10-week-old C57BL/6