International Immunopharmacology 36 (2016) 86–93

Contents lists available at ScienceDirect

International Immunopharmacology

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

Baicalin inhibits toll-like receptor 2/4 expression and downstream signaling in rat experimental periodontitis

Jun-Yi Sun [a,b], Dong-Ling Li [c], Yan Dong [d], Chun-Hui Zhu [a,b], Jin Liu [a,b], Jue-Dan Li [b], Tao Zhou [e], Jian-Zhong Gou [a,b], Ang Li [a,b,*], Wei-Jin Zang [c,**]

[a] Key Laboratory of Shaanxi Province for Craniofacial Precision Medicine Resarech, College of Stomatology, Xi'an Jiaotong University; Xi'an City, Shaanxi Province, China
[b] Department of Periodontology, College of Stomatology, Xi'an Jiaotong University; Xi'an City, Shaanxi Province, China
[c] Department of Pharmacology, School of Basic Medical Sciences, Xi'an Jiaotong University Health Science Center; Xi'an City, Shaanxi Province, China
[d] The Second Affiliated Hospital of Xi'an Jiaotong University; Xi'an City, Shaanxi Province, China
[e] State Key Laboratory for Manufacturing Systems Engineering, Xi'an Jiaotong University; Xi'an City, Shaanxi Province, China

ARTICLE INFO

Article history:
Received 24 February 2016
Received in revised form 26 March 2016
Accepted 7 April 2016
Available online xxxx

Keywords:
Periodontitis
Toll-like receptor
Baicalin
Porphyromonas gingivalis
MyD88
NF-kappaB

ABSTRACT

Periodontitis is a severe inflammatory response, leading to characteristic periodontal soft tissue destruction and alveolar bone resorption. Baicalin possesses potent anti-inflammatory activity; however, it is still unclear whether baicalin regulates toll-like receptor (TLR) 2/4 expression and downstream signaling during the process of periodontitis. In this study, the cervical area of the maxillary second molars of rats was ligated and inoculated with *Porphyromonas gingivalis* (*P. gingivalis*) for 4 weeks to induce periodontitis. Some rats with periodontitis were treated intragastrically with baicalin (50, 100 or 200 mg/kg/day) or vehicle for 4 weeks. Compared with the sham group, the levels of TLR2, TLR4 and MyD88 expression and the p38 MAPK and NF-κB activation were up-regulated in the experimental periodontitis group (EPG), accompanied by marked alveolar bone loss and severe inflammation. Treatment with 100 or 200 mg/kg/day baicalin dramatically reduced the alveolar bone loss, the levels of HMGB1, TNF-α, IL-1β, and MPO expression, and the numbers of inflammatory infiltrates in the gingival tissues. Importantly, treatment with 100 or 200 mg/kg/day baicalin mitigated the periodontitis-up-regulated TLR2, TLR4 and MyD88 expression, and the p38 MAPK and NF-κB activation. Hence, the blockage of the TLR2 and TLR4/MyD88/p38 MAPK/NF-κB signaling by baicalin may contribute to its anti-inflammatory effects in rat model of periodontitis. In conclusion, these novel findings indicate that baicalin inhibits the TLR2 and TLR4 expression and the downstream signaling and mitigates inflammatory responses and the alveolar bone loss in rat experimental periodontitis. Therefore, baicalin may be a potential therapeutic agent for treatment of periodontitis.

1. Introduction

Periodontitis is characterized by chronic inflammation induced by infection with oral bacteria, such as *Porphyromonas gingivalis* (*P. gingivalis*), and can lead to periodontal degradation and tooth loss [1–3]. Oral bacteria interact with host immune system and enable persistent infection in the local inflammatory environment of periodontitis [4]. Toll-like receptors (TLRs), a family of pattern recognition receptors (PRRs), play an important role in the activation of the host immune response and maintenance of periodontal health via the recognition of microbes [5]. However, chronic stimulation of TLRs can promote pro-inflammatory cytokine over-production, eventually leading to destruction of periodontal tissue [6,7].

TLRs are expressed in periodontal tissues, such as gingival epithelial cells, gingival fibroblasts, and osteoclasts [6]. Previous studies have shown that TLR2 is activated by *P. gingivalis*, one of the major periodontopathic pathogens, in mouse model of oral *P. gingivalis* infection [7,8]. Activation of TLR2 blocks phagocytosis and thereby suppresses antimicrobial responses, exacerbating bacterial infection while inhibition of TLR2 activation promotes the clearance of *P. gingivalis* and mitigates periodontal inflammation [9]. Besides TLR2, microbial lipopolysaccharide (LPS) can bind to TLR4 to activate the p38 mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK) and nuclear factor-kappaB (NF-κB) pathways, leading to the production of pro-inflammatory cytokines [5]. Blocking the activation of TLR4 and NF-κB induced by LPS reduces the alveolar bone loss in rat model of periodontitis [8]. More importantly, up-regulated TLR2 and TLR4 expression in salivary epithelial cells are detected patients with periodontitis, which modulates innate immune responses [9,10]. Hence, targeting TLR2 and

* Correspondence to: A. Li, Key Laboratory of Shaanxi Province for Craniofacial Precision Medicine Resarech, College of Stomatology, Xi'an Jiaotong University; Department of Periodontology, College of Stomatology, Xi'an Jiaotong University, Xi'an City, Shaanxi Province 710004, People's Republic of China.
** Correspondence to: W.J. Zang, Department of Pharmacology, School of Basic Medical Sciences, Xi'an Jiaotong University Health Science Center; Xi'an City, Shaanxi Province 710061, People's Republic of China.
E-mail addresses: drliang@mail.xjtu.edu.cn (A. Li), zwj@mail.xjtu.edu.cn (W.-J. Zang).

http://dx.doi.org/10.1016/j.intimp.2016.04.012
1567-5769/

TLR4 expression and their downstream signaling may be valuable for treatment of periodontitis.

Baicalin, 7-glucuronic acid, 5,6-dihydroxy-flavone, is an extracted flavonoid compound from *Scutellaria baicalensis* Georgi and has potent anti-inflammatory activity [11–13]. Baicalin has been used for the treatment of ulcerative colitis [14], endotoxemia [15], periodontitis [16] and various inflammatory diseases [17,18]. Other studies have shown that baicalin promotes human periodontal ligament cell (HPLC) proliferation, reduces the ratio of receptor activator of nuclear factor-kappaB ligand (RANKL) to osteoprotegerin (OPG) expression [19], cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) activity [16], interleukin-6 (IL-6) and IL-8 expression [20], and protects against tissue damage in animal models of periodontitis [16]. Although baicalin inhibits TLR4 activation in human oral keratinocytes [20], however, it is still unclear whether baicalin can regulate TLR2 and TLR4 expression and relevant signaling in a rat model of experimental periodontitis. In this study, the effects and anti-inflammatory mechanisms of baicalin treatment on the alveolar bone loss, connective tissue damage, TLR2, TLR4 and MyD88 expression as well as the p38MAPK and NF-κB activation were examined in a rat model of periodontitis.

2. Materials and methods

2.1. Animals

Male adult Sprague-Dawley rats (200–220 g) were obtained from the Experimental Animal Center of Xi'an Jiaotong University, China. The animals were housed in individual wire cages in a temperature- and humidity-controlled room with a cycle of 12-h light/dark in a specific pathogen-free facility, according to the Guidelines of the Care and Use of Laboratory Animals of the Chinese Council on Animal Research and Care. The experimental protocols were approved by the Medical Ethics Committee of Xi'an Jiaotong University.

2.2. Rat experimental periodontitis

The rats were randomly divided into five groups: the sham group, periodontitis group, and periodontitis + baicalin groups (50 mg/kg/day, 100 mg/kg/day or 200 mg/kg/day, n = 16 per group). The rats in the model and treatment groups were induced for periodontitis by ligature and *P. gingivalis* infection, as previously described with minor modification [21]. Briefly, individual rats were anesthetized with 10% chloral hydrate (0.3 ml/100 g, i.p.; Sigma-Aldrich, St. Louis, USA). The cervical area of the maxillary second molars in the model and treatment groups of rats was bilaterally ligated with a No. 5-0 nylon thread (Natsume, Tokyo, Japan) and the buccal and lingual gingival sulcus of the second molars in individual rats were inoculated with 20 μl of *P. gingivalis* (ATCC 33277, 10^{10} cfu/ml; Sichuan University, China) every other days for 4 times. The Sham group of animals were experienced the same procedure with nylon thread-ligature of the cervical area of the maxillary second molar and immediate removal of the thread and injection with 20 μl of 0.01 M phosphate buffered saline (PBS). After fed with soft diet for 4 weeks, the animals were anesthetized and their ligature threads were removed. Subsequently, the rats in the sham group and periodontitis group received PBS by gavage and rats in the treatment groups were administered by gavage with 50 mg/kg/day baicalin (low dose group), 100 mg/kg/day (middle dose group) or 200 mg/kg/day (high dose group) (National Institutes for Food and Drug Control, Beijing, China) for 4 weeks. All rats were sacrificed and their gingival tissues of the one-side maxillary second molar in individual rats were collected and stored at −80 °C for real-time PCR, western blot and ELISA. The maxillary tissues without gingiva were fixed in 4% paraformaldehyde and evaluated for the alveolar bone loss using micro-computed tomography (micro-CT). The opposite-side maxillary tissues were fixed in 4% paraformaldehyde and prepared for the histological and immunohistochemical analyses.

2.3. Micro-computed tomography (micro-CT) analysis

To assess the alveolar bone loss, the maxillary samples were scanned with the micro-CT system (Y.Cheetah, YXLON Ltd, Hamburg, German). The X-ray tube was operated at 90 Kv, 38.9 μA for 900 ms and the three-dimensional image was reconstructed with an isotropic voxel size of 11.7 μm using software. Linear measurements of the distance from the cemento-enamel junction (CEJ) to the coronal level of the alveolar bone crest (ABC) and root lengths (RLs) from the CEJ to the root apex (RA) were obtained (in millimeters) to assess the percentage of alveolar bone loss (Fig. 1A). The percent loss of bone was expressed as CEJ-ABC/RLs. For volumetric analysis of the alveolar bone, the region of interest (ROI) was identified as a cylindrical area below the roofs of the furcations (ROFs) and above the RAs and included the four roots at the maxillary second molar (Fig. 1 B–E). The ratios bone volume to total volume (BV/TV) in the ROI were the volumetric alveolar bone parameters.

2.4. Histologic and immunohistochemical analysis

The right maxillary specimens were decalcified with 10% EDTA disodium salt for 6 weeks and embedded in paraffin. Serial sections (5 μm) were stained with hematoxylin-eosin (HE) or anti-myeloperoxidase (MPO) for immunohistochemistry. The sections were stained with anti-MPO primary antibody (1:150, 4 °C overnight; Santa Cruz, CA, USA) and visualized with horseradish peroxidase (HRP) conjugated anti-mouse IgG streptavidin biotin complex (SABC) kit and 3, 3-diaminobenzidine (DAB) chromogenic reagent kit (Boster, Wuhan, China) according to the manufacturer's instructions. A total of five sections for each experiment were examined in five randomly selected fields at 400× magnification.

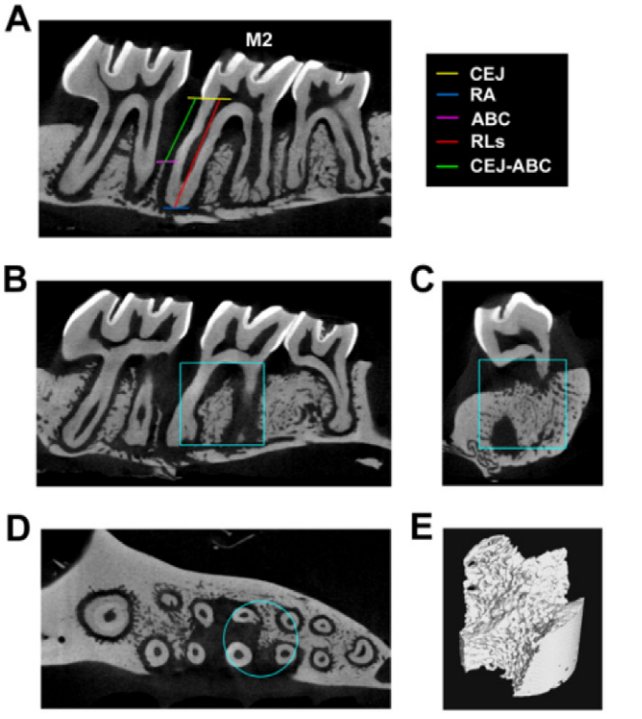

Fig. 1. Determination of the alveolar bone loss using micro-computed tomography (micro-CT). A: The linear measurement of CEJ-ABC and RLs. The cylindrical ROI on the mesio-distal (B), bucco-lingual (C) and coronal (D) sections. E: ROI removing the roots. CEJ: cemento-enamel junction; RA: root apex; ABC: alveolar bone crest; RLs: root lengths; ROI: region of interest.