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Inhibition of the receptor for advanced glycation promotes proliferation and repair of human periodontal ligament fibroblasts in response to high glucose via the NF-κB signaling pathway

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ABSTRACT

Objective: To observe if inhibition of the receptor for advanced glycation endproducts (RAGE) promotes proliferation and repair of human periodontal ligament fibroblasts (hPDLFs) stimulated by high glucose. In addition, we also discuss the effects of the NF-κB signaling pathway in relation to this process.

Methods: Primary cultured hPDLFs were exposed to either low glucose (5.5 mmol/L) or high glucose (25 mmol/L), and RAGE expression was measured by Western blot analysis. Cells were cultured in high glucose with different concentrations of the RAGE inhibitor, FPS-ZM1. We measured cell proliferation using the Cell Counting Kit-8 and expression of collagen type 1 and fibronectin by real-time PCR and ELISA, respectively. The relative protein expression levels of NF-κB p65 and phosphorylated p65 were measured by Western blot analysis.

Results: High glucose enhanced RAGE expression and suppressed cell growth. While FPS-ZM1 increased proliferation and expression of repair-related factors in high glucose, there was a concurrent decline in the phosphorylation level of NF-κB p65.

Conclusion: FPS-ZM1 rescued the proliferative capacity and repair capability of hPDLFs via the RAGE-NF-κB signaling pathway in response to high glucose.

1. Introduction

Periodontal health can be affected by multiple factors, including poor oral hygiene, malnutrition, genetic factors, and systemic disorders (Genco, 1996; Najeeb, Zafar, Khurshid, Zohaib, & Almas, 2016). Inflammation of periodontal tissues (periodontitis) is caused by specific microorganisms, which leads to progressive destruction of soft tissues and alveolar bone (Newman, Takei, Klokkevold, & Carranza, 2012). Progression of periodontitis and bone loss results in loss of teeth (Armitage & Robertson, 2009), requiring dental prostheses, such as implants. It has been widely known that periodontitis is the dominant risk factor for the clinical success of dental implants (Chrcanovic, 2015; Lee, 2014; Sgolastra, Petrucci, Severino, Gatto, & Monaco, 2015). For instance, periodontitis of adjacent teeth could affect implant prognosis. Therefore, it is particularly important to have a favorable implant-tissue interface. Although osteoblasts and gingival fibroblast cells directly affect the success of implants, healthy periodontal conditions are equally necessary. Human periodontal ligament fibroblasts (hPLDFs) are the most important cells in periodontal tissues. hPDLF proliferation, migration, and secretion influence tissue repair, wound healing, and implant placement. In addition, hPDLFs are involved in glucose metabolism (Liu, Liu, Wang, Feng, & Gao, 2011; Rath-Deschner, Deschner, Reimann, Jager, & Gotz, 2009) and are thus more sensitive to fluctuations in blood glucose levels. Hyperglycemia may increase inflammatory factors, further causing gingival inflammation, alveolar resorption, and even tooth loss. Hence, diabetic patients receiving implant surgery have an increased risk of suffering *peri*-implantitis and related complications (Lalla, 2007; Wang, Yang, & Huang, 2015).

The Receptor for Advanced Glycation Endproducts (RAGE) is a multi-ligand receptor expressed at the surface of most cells, including hPDLFs. Under physiological conditions, RAGE is expressed at low-levels, but under diabetic conditions RAGE expression increases in the periodontium(Hudson & Lippman, 2017). RAGE has a variety of ligands, such as advanced glycosylation end products (AGEs), high mobility group box-1 protein (HMGB1), and S100, all of which facilitate a series of downstream reactions leading to various diabetes related

Abbreviations: hPDLFs, human periodontal ligament fibroblasts; RAGE, the receptor for advanced glycation endproducts; FN, fibronectin; COL-1, collagen type 1; OD, optical density; qPCR, quantitative polymerase chain reaction; cDNA, complementary DNA; mRNA, messenger RNA; ELISA, enzyme-linked immunosorbent assay; NF-κB, nuclear factor kappa-light-chain-enhancer of active B cell; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6

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complications (Katz et al., 2005; Ramasamy, Yan, & Schmidt, 2011). Moreover, RAGE can increase its own expression once activated. This signaling cascade occurs constantly via positive feedback. Upon ligand binding to RAGE, apoptosis is accelerated in fibroblasts and osteoblasts, which leads to tissue damage and compromised repair capacity (Li, Deng, Lv, & Ke, 2014). (Sun, Hong, & Hou, 2014; J. J. Taylor, Preshaw, & Lalla, 2013). NF-κB signaling is important in the body's defense against tissue damage, stress, and other signaling mechanisms. Previous research indicated that NF-κB is involved in a series of tissue damage and inflammatory reactions mediated by RAGE, and plays a role in the positive feedback regulation of RAGE expression (Luan et al., 2010).

As RAGE plays a crucial role in the development of diabetic complications, inhibiting RAGE activation may be a new strategy to prevent related complications. The currently available RAGE inhibitors include soluble RAGE (sRAGE), an anti-RAGE antibody, and small molecule RAGE inhibitors. The first two macro-molecular inhibitors have been studied fairly well. Aside from their high cost and low-productivity, these inhibitors can induce allergic reactions. Therefore, micro-molecular drugs with a low molecular weight and high specificity are the focus of current research. Micro-molecule drugs may not directly affect the implant site as they may also affect adjacent teeth via diffusion mechanism. FPS-ZM1 is a kind of micro-molecule RAGE inhibitor reported recently. Deane and colleagues (Deane et al., 2012) originally synthesized FPS-ZM1 via a multiple filter. FPS-ZM1 is widely used in Alzheimer's disease research, whereby it demonstrates strong Aβ-RAGE blocking ability. Moreover, FPS-ZM1 significantly reduces inflammatory factors and is favorable for organ protection (Yang et al., 2015). Research indicates that the inhibitor can also effectively block the combination of RAGE and other ligands (Deane et al., 2012), such as AGEs and HMGB1 (Lee et al., 2017; Ma et al., 2017). For these reasons, we investigate if FPS-ZM1 can block RAGE in fibroblasts. A number of studies have indicated that inhibiting RAGE protects against organ damage and promotes tissue repair in response to high glucose (Flyvbjerg et al., 2004; Zhang, Yao, Huang, & Yu, 2008). Application of FSP-ZM1 has not been investigated in the oral health field. The current study aimed to assess the role of RAGE inhibition on proliferation and repair capacity of hPDLFs in response to high glucose. In addition, we discuss the effects of the NF-κB signaling pathway in relation to this process.

2. Materials and methods

2.1. Cell culture and drug treatment

Healthy premolars indicated for extraction were obtained from 20 patients (12–15 years of age) who were receiving treatment in the Department of Orthodontics at The Oral Hospital of Southwest Medical University, Luzhou. Informed written consent was obtained from the parents of each patient. The research protocol was reviewed and approved by the Ethics Committee of Southwest Medical University, Luzhou. In order to remove debris and blood, the extracted teeth were rinsed repeatedly using phosphate-buffered solution (0.01 M, pH = 7.4, PBS) containing 5% penicillin-streptomycin (Beyotime, Shanghai, China). The periodontal tissues were harvested from the middle third of the root surface and cut into small pieces (1 × 1 × 1 mm) using a surgical blade (11#). The slices were transferred to 0.1% collagenase-I (Sigma, St Louis, USA) for 40 min in a CO_2 incubator (SANYO MCO-15AC, Tokyo, Japan), and were then cultured in DMEM/low glucose (Hyclone, Pittsburgh, USA) supplemented with 10% fetal bovine serum (FBS; Sijiqing, Hangzhou, China) containing 1% penicillin-streptomycin at 37 °C with 5% CO_2. The medium was refreshed every 3 days. The cells were passaged after reaching 80% confluence as determined by light microscopy (OLYMPUS IX71, Tokyo, Japan).

The cells were seeded in 6-well plates at a density of 3×10^5 cells/ml, cultured in DMEM/low glucose media with 10% FBS for 24 h. To investigate the effects of high glucose on RAGE expression, the culture medium was replaced by DMEM/low glucose (containing 1000 mg/L glucose) or DMEM/high glucose (containing 4500 mg/L glucose), and incubated continuously for 48 h. To insure the RAGE inhibitor, FPS-ZM1, did not affect cell proliferation, cells were cultured in different media (low glucose, high glucose, low glucose + 250 nM FPS-ZM1 and high glucose + 250 nM FPS-ZM1). In order to investigate the effects of FPS-ZM1 on hPDLFs cultured in high glucose, the cells were treated with DMEM/high glucose containing FPS-ZM1 (0 nM, 250 nM, 500 nM, or 750 nM; Sellck, Houston, USA) for 12 h or 24 h, followed by total RNA extraction, protein extraction, or supernatant collection.

2.2. Immunohistochemistry

hPDLFs at passage 4 were seeded in a 24-well plate at a density of 1×10^5 cells/well and cultured in DMEM/low glucose media with 10% FBS. Once the cells reached 70% confluence, the cell culture media was refreshed with serum-free DMEM/low glucose for 12 h, washed with PBS twice, and fixed in 4% paraformaldehyde solution for 10 min. Cells were soaked in 3% deionized H_2O_2 for 15 min, blocked with normal goat serum, incubated in primary antibodies at 4 °C overnight, and then at room temperature for 30 min. We used the rabbit anti-vimentin monoclonal antibody (Proteintech, Illinois, USA) at a dilution of 1:150, as well as the rabbit anti-keratin monoclonal antibody (Proteintech, Illinois, USA). Primary antibodies were detected using a biotinylated secondary antibody followed by horseradish peroxidase (HRP)-conjugated streptavidin. 3, 3′-diaminobenzidine (DAB; Bioss, Beijing, China) was used to visualize the target proteins. Nuclei were stained with hematoxylin. Stained sections were imaged using a light microscope (Olympus BX43, Tokyo, Japan).

2.3. Cell proliferation assay

We used the Cell Counting Kit-8 (CCK-8) to assay cell proliferation. hPDLFs at passage 4 were seeded in 96-well plates at a density of 1×10^4 cells/well. 10 μL of the CCK-8 solution (DOJINDO, Kyushu, Japan) was added to the media at 24 h, 48 h, and 72 h, followed by an additional 2 h incubation. Afterwards, we measured absorbance at 450 nm in each well using a micro-culture plate reader (Biotex SYNERGY HTX, Vermont, USA).

2.4. RNA isolation and real-time quantitative PCR

Total RNA was extracted from prepared cells using an RNA simple Total RNA Kit (TIANGEN, Beijing, China) according to the manufacturer's protocol. Total RNA was reverse transcribed into cDNA using ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan). Reverse transcription polymerase chain reaction (RT-PCR) was performed by SYBR Green Realtime PCR Master Mix (TOYOBO, Osaka, Japan) according the conditions provided in the instructions. The primers were synthesized as follows: 5′-AGGAGAATGGACCTGCAAGC-3′ (forward primer) and 5′-TCTACCATCATCCAGCCTTGG-3′ (reverse primer) for the human *Fn* gene, 5′-GGCGAGAGAGGTGAACAAGG-3′ (forward primer) and 5′-GCCAAGGTCTCCAGGAACAC-3′ (reverse primer) for the human *Col-1* gene. The internal reference control was glyceraldehyde-phosphate dehydrogenase (GAPDH). The experiments were performed in triplicate (Bio-Rad DNA Engine Opticon 2, California, USA), and the results were quantified using the 2-△△CT method.

2.5. Enzyme-linked immunosorbent assay (ELISA)

Supernatants were harvested at 12 h and 24 h. Protein concentration was measured with the BCA Protein Assay KIT (Beyotime, Beijing, China). The experiment was executed according to the manufacturer's instructions for the Human COL-1 ELISA Kit and the Human FN ELISA Kit (R&D systems, Minnesota, USA). The reference standard and sample (50 μL) were added to the 96-well plates and incubated for 2 h followed