Biochemical and Biophysical Research Communications 394 (2010) 928–933

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Involvement of the endocannabinoid system in periodontal healing

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ARTICLE INFO

Article history:
Received 8 March 2010
Available online 15 March 2010

Keywords:
Cannabinoid receptors
Gingival fibroblasts
Proliferation
Wound healing
Endocannabinoids
Mitogen-activated protein kinases

ABSTRACT

Endocannabinoids including anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are important lipid mediators for immunosuppressive effects and for appropriate homeostasis via their G-protein-coupled cannabinoid (CB) receptors in mammalian organs and tissues, and may be involved in wound healing in some organs. The physiological roles of endocannabinoids in periodontal healing remain unknown. We observed upregulation of the expression of CB1/CB2 receptors localized on fibroblasts and macrophage-like cells in granulation tissue during wound healing in a wound-healing model in rats, as well as an increase in AEA levels in gingival crevicular fluid after periodontal surgery in human patients with periodontitis. *In-vitro*, the proliferation of human gingival fibroblasts (HGFs) by AEA was significantly attenuated by AM251 and AM630, which are selective antagonists of CB1 and CB2, respectively. CP55940 (CB1/CB2 agonist) induced phosphorylation of the extracellular-regulated kinases (ERK) 1/2, p38 mitogen-activated protein kinase (p38MAPK), and Akt in HGFs. Wound closure by CP55940 in an *in-vitro* scratch assay was significantly suppressed by inhibitors of MAP kinase kinase (MEK), p38MAPK, and phosphoinositol 3-kinase (PI3-K). These findings suggest that endocannabinoid system may have an important role in periodontal healing.

1. Introduction

Wound-healing processes are largely regulated by various cytokines and growth factors [1]. These molecules manifest pro- and anti-inflammatory functions as well as pro- and anti-fibrotic functions, and direct the course of healing. Wound healing is a complex and dynamic process initiated by injury [2]. The endocannabinoid system (ECS) is a recently identified intercellular communication network mediated via lipid mediators. The ECS has been implicated in multiple regulatory functions in health and disease. The anti-inflammatory and immunological effects of the ECS in the skin have been observed, suggesting that alteration of the ECS may be important for the maintenance of skin health [3]. The ECS has also been shown to be involved in repairs in the spinal cord, colon, and liver [4–6]. It is therefore likely that the ECS is involved in wound healing in some organs.

Periodontal disease is a chronic inflammatory disease caused by bacterial dental plaque. It is characterized by gingival inflammation, periodontal pocket formation, and destruction of the supporting alveolar bone and connective tissue around the teeth. Recent studies have demonstrated that periodontal disease may be involved in systemic diseases including cardiovascular diseases and type-2 diabetes, suggesting that periodontal healing may lead to improvement of the systemic diseases [7]. A variety of growth factors and cytokines including platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) are signal inducers for periodontal healing, as well as wound-healing processes [8]. Previously, we have shown that the endocannabinoid anandamide (AEA) in inflamed gingival tissues may regulate periodontal inflammation through inhibition of the NF-κB pathway via the cannabinoid (CB) receptors CB1 and CB2 [9]. However, the physiological roles of the ECS in periodontal healing have not been elucidated yet.

The purpose of the present study was to elucidate the physiological roles of the ECS in periodontal healing. The effect of activation of CB receptors with or without CB antagonists or inhibitors of p38 mitogen-activated protein kinase (p38MAPK), MAP kinase kinase (MEK), and phosphoinositol 3-kinase (PI3-K) signal-transduction inhibitors in cell proliferation assays and *in-vitro* wound healing was also examined. Our data showed that the ECS has an important role in the expression of CB receptors in healing granulation tissue and enhancing the fibroblast proliferation and cell

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doi:10.1016/j.bbrc.2010.03.080

repopulation for wound filling via CB1/CB2 receptors through phosphorylation of ERK1/2 and p38 MAPK and PI3-K/Akt signal transduction pathways.

2. Materials and methods

2.1. Ethical approval of the study protocol

The study protocol (for experiments on humans and rats) was approved by the Ethical Committee of Kagoshima University Graduate School of Medical and Dental Sciences (Kagoshima, Japan). Informed consent was obtained from all patients to participate in the study.

2.2. Reagents

AEA and 2-AG were purchased from Calbiochem Novabiochem Company (La Jolla, CA). The CB1-specific antagonist AM251 and the CB2-specific antagonist AM630 were purchased from Tocris Cookson Limited (Bristol, UK). LY294002 (PI3-K inhibitor), U0126 (MEK inhibitor), and CP55940 (CB1/CB2 receptor agonist) were from Cayman Chemical Company (Ann Arbor, MI). SB203580 (p38MAPK inhibitor) was from Jena Bioscience (Jena, Germany). Rabbit polyclonal antibody against the CB1 and CB2 receptors were purchased from Affinity BioReagents, Incorporated (Golden, CO), Alpha Diagnostics International (San Antonio, TX), respectively. Akt, p38MAPK, ERK1/2, phosphor (p)-Akt, p-p38MAPK, and p-ERK1/2 were purchased from Cell Signaling Technology (Beverly, MA).

2.3. Sampling of gingival crevicular fluid (GCF)

Samples of human GCF were collected from periodontal pockets wounded by periodontal surgery from periodontitis patients. Briefly, paper strips (Periopaper®; ProFlow Inc., Amityville, NY) were inserted in gingival pockets for 30 s immediately before surgery (pre-surgery) and at day-3, -7, or -14 post-surgery. After removing the strips from the sites, they were transferred to 1 ml of phosphate-buffered saline (PBS). After 60 s, the procedure was repeated twice. Samples were stored at −80 °C until assay.

2.4. Experimental wounds in rats

Twelve-week-old Wistar rats ($n = 4$) were anesthetized by intraabdominal injection of nembutal (5 mg/kg; Dainippon Pharmaceutical Company, Limited, Osaka, Japan). After making crevicular incisions, wounds were made in the maxillary pre-molar regions using a round burr. Wound sites were sutured using a 4–0 silk suture. The opposite sides had no treatment (which served as the control). Postoperative infection was controlled by injection of 1 ml of penicillin G (500 IU, i.p.). Rats were killed 14 days after surgery.

2.5. Immunohistochemistry for detection of CB1/CB2 receptors in wounded tissues

The wounded tissue blocks and control tissue blocks were fixed in 4% paraformaldehyde/0.1 M phosphate buffer (pH 7.4) for 24 h. They were demineralized with 0.5 M ethylenediamine tetra-acetic acid (EDTA, pH 7.4) for 3 weeks at 4 °C. After dehydration through a graded series of ethanol solutions, they were embedded in paraffin. Deparaffinized sections (6-μm thick) were incubated with antibody against the CB1 receptor (1:1500) and CB2 receptor (1:500) or isotype control rabbit IgG for 1 h at room temperature. Sections were then incubated with Envision + Dual Link System-Horseradish Peroxidase (Dako Cytomation, Kyoto, Japan) for 30 min. Antibody localization was determined using 3,30-diaminobenzidine (DAB) and counterstaining with hematoxylin.

2.6. Measurement of endocannabinoid levels

Taking advantage of the ability of polymyxin B (PMB) to bind to AEA and 2-AG [10], we measured the levels of AEA and 2-AG in biological fluids after selective absorption onto PMB-immobilized beads. The elution from the beads was directly fractionated using reverse-phase high-performance liquid chromatography (HPLC). The fractions corresponding to authentic AEA and 2-AG were derivatized with a fluorogenic reagent and quantified by HPLC with fluorometric detection. The concentrations of AEA and 2-AG in biological fluids were quantified by comparison with calibration curves constructed using synthetic AEA and 2-AG [11].

2.7. Cell culture of human gingival fibroblasts (HGFs)

HGFs were isolated from gingival tissues obtained during periodontal surgery. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/ml of penicillin, and 125 μg/ml of streptomycin at 37 °C.

2.8. Cell proliferation assay

HGFs (6.0×10^3 cells/well) were seeded into 96-well microplates in DMEM with 10% FBS and incubated for 24 h. The medium was replaced with DMEM containing 0.5% FBS for a 24-h starvation period. After 24 h, cells were pretreated with or without CB antagonists or signal inhibitors for 1 h, and incubated with or without AEA, CP55940 (CB1/CB2 receptor agonist) for 24 h. Bromodeoxyuridine (BrdU) was added 18 h after the start of incubation. Cellular proliferation was assessed with the Cell Proliferation ELISA, BrdU kit (Roche Incorporated, Manheim, Germany). The absorbance at 450 nm was measured by a microplate reader (Thermo Electron Corporation, Waltham, MA).

2.9. In-vitro wound effect

HGFs (4.0×10^4 cells/well) were plated onto four-well chamber slides. HGFs were grown to confluence overnight in DMEM supplemented with 10% FBS. Confluent monolayers were scratched with P200 Gilson pipette tips as described [12]. Cells were pretreated with or without CB antagonists or signal inhibitors for 1 h, and incubated with or without CP55940 (CB1/CB2 receptor agonist) for 24 h in fresh FBS-free media. Images under phase-contrast microscopy were taken immediately after the scratch wound (0 h) and 24 h after stimulation. The width between the wound edges in each well at 0 h and after 24 h was measured at six fixed points using a standard template on the image. Data were expressed as closure rate (%) based on initial wound width.

2.10. Western blot analyses

HGFs (0.5×10^6 cells/well) were seeded onto 100-mm cell culture dishes in DMEM with 10% FBS and incubated for 24 h. After a 24-h starvation period with DMEM containing 0.5% FBS, they were treated with or without 100 nM CP55940. Cells were then analyzed for expression of ERK1/2, p38MAPK, Akt, p-ERK1/2, p-p38MAPK, and p-Akt. Briefly, after washing with PBS, they were lysed with lysis buffer (0.5 M Tris–HCl, 10% sodium dodecyl sulfate (SDS), 10% of 2-mercaptoethanol, and 20% glycerol). Cell lysates were subjected to 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) at 20 mA for 120 min and transferred to nitrocellulose membranes (Whatman, Cassel, Germany). After blocking with 5% non-fat dry milk in Tris-buffered saline (TBS, pH 7.4) containing 0.02% Tween-20 (TBST) for 1 h, samples were incubated overnight with polyclonal antibodies against Akt, p38 MAPK, ERK1/2, p-Akt, p-p38MAPK, and p-ERK1/2 in TBST containing 1% non-fat dry milk.