Short Communication

Reactive oxygen species-dependent release of damage-associated molecular patterns from human gingival epithelial Ca9-22 cells during butyrate or propionate exposure

Yui Fujiwara1, Takahisa Murofushi2, Ryosuke Koshi3, Yoshikazu Mikami3, and Hiromasa Tsuda3,4

1) Nihon University School of Dentistry, Tokyo, Japan
2) Department of Biochemistry, Nihon University School of Dentistry, Tokyo, Japan
3) Department of Oral Health Sciences, Nihon University School of Dentistry, Tokyo, Japan
4) Division of Microscopic Anatomy, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan

(Received August 4, 2020; Accepted November 18, 2020)

Abstract: Treating the gingival epithelial Ca9-22 cell with butyrate, a short-chain fatty acid (SCFA) produced by bacteria within mature dental plaque, induces necrotic cellular death. In this report, it was examined whether SCFA-mediated cellular death is accompanied by a release of damage-associated molecular patterns (DAMPs). In addition, the role of reactive oxygen species (ROS) in the release of DAMPs was evaluated. Human gingival epithelial Ca9-22 cells were treated with butyrate or propionate. The amounts of dead cells were then measured using SYTOX-green dye. Released DAMPs were detected by western blot. The role of ROS scavengers, ascorbic acid and N-acetylcysteine, on DAMP-release was evaluated. Dose and time-dependent induction of Ca9-22 cell death was observed during butyrate and propionate treatments. This was accompanied by the release of DAMPs. Ascorbic acid or N-acetylcysteine reduced cellular death and inhibited DAMP-release induced by exposure to butyrate or propionate. These data collectively suggest that SCFA-induced death of gingival epithelial Ca9-22 cells and accompanying release of DAMPs are dependent on ROS.

Keywords: cellular death, DAMPs, gingival epithelial cells, ROS, SCFAs

Introduction

Accumulated dental plaque surrounding marginal gingival grooves is comprised of numerous anaerobic bacteria, which produce high doses of short-chain fatty acids (SCFAs) as metabolic byproducts [1]. Tsuda et al. demonstrated that butyrate treatment induces necrotic gingival epithelial cellular death [2,3]. However, a lack of clarity persists concerning both the molecular mechanisms of how butyrate induces cellular death and the role of butyrate-induced gingival epithelial cellular death in the onset of periodontal disease. Butyrate treatment of gingival fibroblasts induces reactive oxygen species (ROS) production [4], and ROS production can induce several types of cellular death [5]. These indicate that butyrate-induced ROS may trigger SCFA-induced gingival epithelial cellular death.

Damage-associated molecular pattern molecules (DAMPs) are intracellular molecules that play important physiological roles [6]. They also stimulate inflammatory responses once they are passively released from necrotic dead cells into interstitial fluids [6]. Binding of high mobility group box 1 (HMGB1), which functions as DAMPs, to its receptor results in cytokine/chemokine-release [6]. Histones also play a role as DAMPs. Released histones bind to toll-like receptor 2 (TLR2) and TLR4, which results in immune-mediated inflammation [6]. This study investigated whether SCFA-treatment induces the release of DAMPs from dying gingival epithelial cells and also whether the release of DAMPs is ROS-dependent. These findings contribute to understanding early mechanisms that may lead to the onset of periodontal disease.

Materials and Methods

Antibodies

Anti-anti-HMGGB1 rabbit polyclonal and anti-histone H4 rabbit monoclonal antibodies were purchased from Santa Cruz Biotechnology (#sc-74085, Santa Cruz, CA, USA) and Upstate (#05-858, Temecula, CA, USA), respectively. A secondary antibody, WestVision peroxidase polymer anti-rabbit IgG, was obtained from Vector Laboratories (#WB-1000, Burlingame, CA, USA).

Cell culture

The human gingival epithelial Ca9-22 cell line (Riken Bio Resource Center, Tsukuba, Japan) was maintained in MEMs (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS, Biofill, Elsternwick, Australia) and 1% penicillin/streptomycin (Wako) at 37°C in a 5% CO2 atmospheric condition. During treatment with sodium butyrate (Wako) or sodium propionate (Wako), S7-supplemented Epi-Life medium (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used. Ascorbic acid or N-acetylcysteine (Wako) was added to the medium containing S7-Supp (S7-supplemented Epi-Life medium; Thermo Fisher Scientific Inc., Waltham, MA, USA) to maintain appropriate conditions for experiments involving oxidative stress.

SYTOX-green dye cell death assay

Necrotic cellular death was measured as described in previous reports [2,3]. Briefly, Ca9-22 cells (2 × 104 cells/well) were treated with 100 μL of several doses of butyrate or propionate-containing Epi-Life supplemented with S7 in the presence or absence of N-acetylcysteine (Wako) or ascorbic acid (Wako). After incubation at 37°C, 100 μL of 400 nM SYTOX-green dye (Thermo) in RPMI1640 (Wako) were added and emitted fluorescent intensity (Ex/Em = 485/535 nm), which reflected the number of dead cells, was measured using a Wallac ArvoSX1420 spectrophuorometer (PerkiElmer, Waltham, MA, USA). Equal volumes of RPMI1640 were added instead of SYTOX-green dye to establish background.

Western blot

Ca9-22 cells (2 × 105 cells/well) were treated for 48 h with 400 μL of S7-supplemented Epi-Life medium containing several concentrations of butyrate or propionate in the presence or absence of N-acetylcysteine or ascorbic acid. Supernatants (20 μL) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins in the gels were transferred to polyvinylidene difluoride membranes (Wako). After blocking with EzBlockChemi (Atto, Tokyo, Japan), membranes were treated with a series of primary and secondary antibodies labeled with horseradish peroxidase. The band images were developed with ChemiDoc XRS (Bio-Rad).

Results

Butyrate or propionate-treatment induces Ca9-22 cellular death and subsequent DAMP-release

The gingival epithelium is exposed to high concentrations (mM order) of butyrate and/or propionate at the attachment site of mature dental plaque...
Therefore, the effects of butyrate or propionate-treatment on the gingival epithelial Ca9-22 cell line were examined. Treatment with 4 mM butyrate or 16 mM propionate exhibited a stronger increase of dead cells at 48 h than those of control treatment (Fig. 1A). In addition, exposure to butyrate or propionate induced cellular death in a dose-dependent manner (Fig. 1B). The maximum responses were observed at 4 or 16 mM of butyrate or propionate-treatment, respectively (Fig. 1B). During necrotic cellular death, DAMPs are released and play important roles in the initiation and promotion of inflammation [6]. Therefore, it was then examined whether butyrate or propionate-exposure induces the release of HMGB1 and histone H4, which can function as DAMPs. As Fig. 2 shows, exposure to 4 mM butyrate or 16 mM propionate induced release of HMGB1 and histone H4 after 36 h or 24 h, respectively (Fig. 2). In contrast, only faint bands of HMGB1 and histone H4 were observed at 48 h in the control (Fig. 2).

Antioxidants suppress butyrate or propionate-induced cellular death and accompanying release of DAMPs

Butyrate exposure reportedly induces ROS production from cells [4]. Therefore, it was then examined whether the effects of ROS scavengers such as ascorbic acid and N-acetylcysteine upon Ca9-22 cellular death were induced by exposure to butyrate or propionate. Ascorbic acid (5 or 10 mM) reduced cellular death induced by exposure to butyrate or propionate in a dose-dependent manner (Fig. 3A). In the presence of 10 or 20 mM of N-acetylcysteine, the amount of cellular death induced by butyrate or propionate reduced and this decline was dose-dependent (Fig. 3B). Finally, the effects of antioxidants on the release of DAMPs during exposure to butyrate or propionate were examined. As Fig. 4A demonstrates, ascorbic acid worked in a dose-dependent manner to reduce the release of HMGB1 and histone H4 extracellularly (Fig. 4A). A concentration of 10 mM ascorbic acid almost completely inhibited the release of DAMPs (Fig. 4A). Similarly, N-acetylcysteine also inhibited the release of DAMPs in a dose-dependent manner, though its strongest inhibitory effects were observed at a higher concentration (20 mM), when compared with ascorbic acid (Fig. 4B).

Discussion

In this study, it was demonstrated that exposure of butyrate or propionate to Ca9-22 gingival epithelial cells increased the amount of cellular death that accompanies release of HMGB1 and histone H4, which are also known as DAMPs. The induction of these protein releases was suppressed by ROS quench, indicating that the oxidizing environment established by SCFA-treatment may participate in the mechanisms underlying the onset of death and the subsequent release of DAMPs. It has been reported that butyrate treatment on gingival fibroblast induces ROS production [4]. In addition, Pant et al. demonstrated that oxidative stress induced by butyrate treatment induces autophagy in hepatoma cells [7]. These reports validate their connection to previous reports that butyrate exposure induced an autophagy-dependent death of gingival epithelial Ca9-22 cells [2,3]. Therefore,
treatment of butyrate or propionate may induce ROS and subsequent autophagy, resulting in cellular death and DAMP-release. Further research is required to better understand the mechanisms and pathways involved.

Necrotic cellular death induces a passive release of DAMPs. In this study, it was demonstrated that butyrate or propionate treatment (with concentration ranges matching what mature dental plaque reportedly contains) induces release of HMGB1 and histone H4. As the members of DAMPs, HMGB1 and histones play important roles in inflammatory response [8]. Therefore, the release of DAMPs from gingival epithelial cells exposed to SCFAs, which are contained in mature dental plaque, may play an important role in inducing gingivitis. Since many other intracellular molecules are released during the death of gingival epithelial cells, studying SCFA-induced death of gingival/periodontal cells and the release of DAMPs may be a key to understanding early mechanisms that lead to the onset of periodontal disease.

Ascorbate and N-acetylcysteine are characterized by reducing agents which can quench ROS. As shown in Fig. 4, these agents inhibited DAMP-release. Tada and Miura systematically reviewed the effects of reducing agents on periodontal disease and concluded that ascorbic acid contributes to a reduced risk of periodontal disease [9]. Thus, these antioxidants may be useful in the prevention of gingivitis and periodontitis. The addition of antioxidants to toothpaste formulas may contribute to the healthy maintenance of periodontal tissues.

Acknowledgments
This work was supported by the JSPS KAKENHI (Grant-in aid for Scientific Research (C) #17K11686); a grant from the Dental Research Center Nihon University School of Dentistry; the Sato Fund, Nihon University School of Dentistry; the Uemura Fund, Nihon University School of Dentistry.

Conflict of interest
The authors declare no conflict of interest associated with this manuscript.

References
1. Pöllänen MT, Salonen JI, Uitto VJ (2003) Structure and function of the tooth-epithelial interface in health and disease. Periodontol 2000 31, 12-31.
2. Tsuda H, Ochiai K, Suzuki N, Otsuka K (2010) Butyrate, a bacterial metabolite, induces apoptosis and autophagic cell death in gingival epithelial cells. J Periodontal Res 45, 626-634.
3. Evans M, Murofushi T, Tsuda H, Mikami Y, Zhao N, Ochiai K et al. (2017) Combined effects of starvation and butyrate on autophagy-dependent gingival epithelial cell death. J Periodontal Res 52, 522-531.
4. Chang MC, Tsai YL, Chen YW, Chan CP, Huang CF, Lan WC et al. (2013) Butyrate induces reactive oxygen species production and affects cell cycle progression in human gingival fibroblasts. J Periodontal Res 48, 66-73.
5. Zou Z, Chang H, Li H, Wang S (2017) Induction of reactive oxygen species: an emerging approach for cancer therapy. Apoptosis 22, 1321-1335.
6. Gong T, Liu L, Jiang W, Zhou R (2020) DAMP-sensing receptors in sterile inflammation and inflammatory diseases. Nat Rev Immunol 20, 95-112.
7. Pant K, Saraya A, Venugopal SK (2017) Oxidative stress plays a key role in butyrate-mediated autophagy via Akt/mTOR pathway in hepatoma cells. Chem Biol Interact 273, 99-106.
8. Roh JS, Sohn DH (2018) Damage-associated molecular patterns in inflammatory diseases. Immune Netw 18, e27.
9. Tada A, Miura H (2019) The relationship between vitamin C and periodontal diseases: a systematic review. Int J Environ Res Public Health 16, 2472.