Anti-inflammatory effects of bamboo salt and sodium fluoride in human gingival fibroblasts—An in vitro study

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Received 24 November 2014; accepted 4 March 2015
Available online 27 April 2015

KEYWORDS
Bamboo salt; Gingival fibroblast cells; Sodium fluoride

Abstract Dental caries preventive agents, such as sodium fluoride (NaF) and bamboo salt (BS), are known to cause cellular growth that is characterized by morphological and gene expression changes. This study was designed to investigate the dual effect of NaF and BS on interleukin (IL)-1β-induced gingival inflammation. Under in vitro experimental conditions, exposure to a subcytotoxic dose of IL-1β enhanced human gingival fibroblast inflammation, as characterized by increased levels of inflammation-associated proteins. A combination of NaF and BS significantly protected fibroblasts from IL-1β-induced cellular deterioration. Exposure to NaF and BS induced the cell growth and no changes in viability were found with the Lactate Dehydrogenase Assay (LDH) assay at the NaF and BS concentration analyzed. Molecular analysis demonstrated that NaF and BS increased resistance to inflammation by reduction of IL-1β, IL-8, and tumor necrosis factor (TNF)-α production. In addition, NaF and BS decreased the expression of IL-1β, IL-8, and TNF-α mRNA in IL-1β-induced human gingival fibroblast cells. The study identifies a new role for NaF and BS in the IL-1β-induced inflammation of gingival fibroblasts and provides a potential target for gingival protection.

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Introduction

Sodium fluoride (NaF) has long been recognized as one of the best public health measures in the prevention of dental caries [1]. It also has a potential role in the prevention of osteoporosis [2]. Fluoride is present in natural fresh water and a concentration of 1.6–1.8 ppm in drinking water is the threshold for the risk of dental fluorosis in the population [3]. The concentration of fluoride that cells are exposed to appears to be a critical factor in determining any changes in behavior that may occur. Li et al used organ culture to show that 2 mM fluoride affected the Rho/Rho-associated protein kinase (ROCK) signal transduction pathway, resulting in elevated F-actin in ameloblasts [4]. Millimolar levels of fluoride were also found to induce endoplasmic reticulum stress, apoptosis, and caspase-mediated DNA fragmentation in enamel organ epithelial-derived cell lines [5]. It was reported that the toxicity on a Smulow-Glickman human gingival epithelial cell line was first observed at an exposure to 1 mM NaF for 24 hours [6]. Thaweboon et al [7] reported a biphasic effect of fluoride at mM fluoride levels in dental pulp cells. Lower concentrations of fluoride promoted the proliferation of cells of the epithelial ameloblast-lineage, with the peak ~16 μM NaF [8]. Although the sensitivity to fluoride differs with the kind of cell and donor’s age, it is true that higher mM fluoride levels are toxic to human cells.

In Korea, bamboo salts (BS) are mainly ingested for health reasons because they are produced using processes that lead to decreased toxicity and the conversion of acidity to strong alkalinity when compared to sun-dried salts. BS is known to have therapeutic effects for diseases such as viral diseases, dental plaque, and inflammatory disorders [9–11]. The remineralization effects of bamboo salt on incipient artificial enamel caries were also reported [12].

Gingiva is covered by stratified squamous epithelium with architectural characteristics unique to dental areas. Several cell types have been identified within gingival connective tissue and gingival fibroblasts, which account for most connective tissue cells, and are likely to be responsible for the constant functional adaptation of gingival connective tissue [13]. Gingival fibroblasts play a major role in normal connective tissue turnover, as well as in wound healing repair and regeneration [14]. Gingival inflammation is a chronic disorder characterized by the breakdown of tooth-supporting tissues, and producing a loss of dentition. The cause is an ecological imbalance between the microbial biofilm on teeth and an impaired host inflammatory response. The disease involves the breakdown of the gingival connective tissue, namely gingival fibroblast dysfunction. Inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-8, are rapidly induced and expressed in inflammation in an antigen-independent manner. TNF-α is a pleiotropic cytokine capable of altering physiological and immunological sequelae as well as mediating the pathophysiological responses of various disease conditions [15]. IL-1β is a potent multifunctional proinflammatory polypeptide produced by monocytes and tissue macrophages [16]. IL-1β attracts and activates immune cells and controls the expression of most immunomodulatory genes [17]. It is well established that IL-1β is a major cytokine involved in the inflammatory process in periodontitis [18]. IL-1β acts directly on local fibroblasts in inflammatory conditions, inducing a variety of genes and helping to create an activated phenotype characterized by hyperplasia and invasiveness [19]. Evidence has been found indicating the role of low concentration NaF in proliferation of human gingival cells. However, to date no studies have been carried out to determine its status and mechanism of action during inflammation. Thus, it is important to examine the role of BS and NaF in the pathophysiology of gingival inflammation. The purpose of the present study was to investigate the effect of a combination of BS and NaF on inflammatory cytokine production and expression in human recombinant IL-1β-stimulated gingival inflammation.

Materials and methods

Cell culture

Human gingival fibroblast (HGF) was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS). They were seeded in 60 mm plastic tissue culture dishes and incubated in 5% CO2 at 37°C. When the cells reached subconfluence, they were harvested and subcultured.

Cell proliferation

Cultured cells were harvested from 80% confluent monolayer cultures by a brief trypsinization with 0.1% trypsin and 0.1% EDTA. The cells were seeded at a density of 5000 cells per well of 96-well tissue culture plates and cultured for 24 hours in DMEM. The medium was replaced with serum-free medium for 18 hours and then treated with DMEM supplemented with BS and NaF. Cell proliferation was assessed using a MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay at 24 hours after treatment. CellTiter 96 AQueous One Solution reagent (Promega, Madison, WI, USA) was added directly to each well and incubated for 2 hours, the absorbance at 490 nm was then measured using a microplate reader (iMark, Bio-Rad, Hercules, CA, USA).

Cytotoxicity assay

To determine the cytotoxic activity of BS and NaF, the Cytotox 96 nonradioactive cytotoxicity assay (Promega Corporation, Madison, WI, USA) was used. Briefly, the cells were incubated with BS and NaF for 24 hours, and then 50 μL of supernatant was assayed for LDH activity following the manufacturer’s protocol. All of the conditions were assayed in triplicate. Controls for spontaneous LDH release in experimental cells, as well as maximum release, were prepared. The calculation of cytotoxicity percentage was carried out as follows:

cytotoxicity (%) = \left(\frac{\text{experimental LDH release}}{\text{maximum LDH release}}\right) \times 100 \tag{1}
RNA extraction and quantitative real-time polymerase chain reaction

For gingival fibroblast cells (2 × 10^5 cells/well, 6-well plates), total RNA was isolated using QIAzol Reagent (Qia- gen KK, Tokyo, Japan) according to the manufacturer’s instructions, and purified using miRNeasy Mini Kit (Qiagen KK). A TURBO DNA-free Kit (Applied Biosystems, Foster City, CA, USA) was used to remove contaminating DNA from RNA preparations. First-strand cDNA synthesis was performed using a Superscript VILO cDNA Synthesis Kit (Applied Biosys- tems), according to the manufacturers protocol. The primer sequences are provided in Table 1. Real-time poly- merase chain reaction (PCR) amplification reaction was performed with Rotor-Gene Q (Qiagen KK). The reaction mixture (20 μL) contained QuantiTect SYBR Green PCR Kit (Qia- gen KK), 1 μL of diluted cDNA sample, and 10 μM of each pair of oligonucleotide primers. PCR conditions included an initial denaturation at 95°C for 15 minutes, followed by a 40-cycle amplification consisting of dena- turation at 94°C for 15 seconds and annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds. Gel elec- trophoresis and melting curve analysis at 0.2°C increments from 60°C to 99°C were used to confirm reaction specificity. Human β-actin mRNA was used as an internal control. The mRNA expression of target or reference samples was normalized to the mRNA expression of the corresponding human β-actin. The relative expression of the studied mRNA molecules was determined by relating the normalized expression of each target, in duplicate, to the normalized expression of a reference sample to calculate a fold-change value in term of mRNA ratio.

Enzyme-linked immunosorbent assay

Cell culture supernatants were centrifuged at 1000 rpm for 10 minutes at 4°C and then aliquoted and stored at −80°C until further use. The ProcartaPlex Human Cytokine Panel (Affymetrix, Inc., Santa Clara, CA, USA), a high-sensitivity Lumixx-based magnetic bead assay, was used to quantify IL-1β, IL-8, and TNF-α in culture supernatants according to the manufacturer’s instructions. In brief, samples were diluted 1:4 in the sample diluent provided with the kit and incubated with magnetic beads coupled to specific antibodies. Cytokines were detected using a premixed detection antibody. The data were analyzed using the Luminex 200 system. Absolute concentrations were measured from a standard curve generated from eight serially diluted standards pro- vided with the kit. Each sample was analyzed in triplicate. Values are presented in pg/mL. Each run included a blank and controls of known concentration for each cytokine.

Western blot analysis

Samples containing 20 μg of protein were loaded onto 15% polyacrylamide gels. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose mem- branes (Bio-Rad, Hercules, CA, USA). The membranes were then placed in the blocking solution at room temperature for 1 hour, washed with 0.01% Tween/PBS, and incubated then placed in the blocking solution at room temperature for 1 hour, washed with 0.01% Tween/PBS, and incubated with mouse monoclonal antibodies against cleaved caspase-3 (1:500; Cell Signaling Technology), anti-GAPDH rabbit polyclonal antibody (1:1000; Cell Signaling Technology) and anti-Glyceraldehyde 3-phos- phate dehydrogenase (GAPDH) rabbit polyclonal antibody (1:2000; Cell Signaling Technology). The blots were then incubated with horseradish peroxidase–conjugated second- ary antibody (Cell Signaling Technology). Protein bands were visualized using the ECL prime detection system (Amersham Biosciences, Buckinghamshire, UK).

Statistical analysis

All experiments were repeated at least three times, and results were expressed as mean ± standard deviation (SD) of triplicate independent samples. Data analysis was performed using SPSS version 18.0 (SPSS Inc., Chicago, IL, USA). One tail Student t test was used to calculate the significance of the difference between the mean expressions of given experimental samples and the control samples. A p value < 0.05 was considered significant.

Results

BS and NaF inhibits the secretion of inflammatory cytokines in HGF cells

The molecular mechanisms underlying the action of BS and NaF were investigated using HGF cells. The effects of BS (0.01%), and NaF (0.002%) on the proliferation of HGF cells were investigated using HGF cells. The effects of BS (0.01%), and NaF (0.002%) on the proliferation of HGF cells were investigated using HGF cells. The effects of BS (0.01%), and NaF (0.002%) on the proliferation of HGF cells were investigated using HGF cells. The effects of BS (0.01%), and NaF (0.002%) on the proliferation of HGF cells were investigated using HGF cells. The effects of BS (0.01%), and NaF (0.002%) on the proliferation of HGF cells were investigated using HGF cells. The effects of BS (0.01%), and NaF (0.002%) on the proliferation of HGF cells were investigated using HGF cells. The effects of BS (0.01%), and NaF (0.002%) on the proliferation of HGF cells were investigated using HGF cells. The effects of BS (0.01%), and NaF (0.002%) on the proliferation of HGF cells were investigated using HGF cells.

<table>
<thead>
<tr>
<th>Gene description</th>
<th>Direction</th>
<th>Nucleotide sequence (5’–3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>Forward</td>
<td>TCCAGGGACAGGTATGGAG</td>
</tr>
<tr>
<td>IL-8</td>
<td>Forward</td>
<td>ATG ACT TCC AAG CTG GCC GTG GCTT</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Forward</td>
<td>TCT CAG CCC TCT TCA AAA ACT TCT C</td>
</tr>
<tr>
<td>β-actin</td>
<td>Forward</td>
<td>GTGGGGGCGCCCCAGGCCACCA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CTCCTTAATGTCCACGCAGATTTC</td>
</tr>
</tbody>
</table>

HGF = human gingival fibroblast; IL = interleukin; PCR = polymerase chain reaction; TNF = tumor necrosis factor.
effect on cell proliferation. An LDH assay also showed that BS and NaF did not affect cell viability of HGF cells (Fig. 1B). Western blot analysis confirmed no significant effect on apoptosis in response to 0.01% BS + 0.002% NaF (Fig. 1C).

Levels of IL-1β, TNF-α, and IL-8 in cell culture media by HGF cells

IL-1β protein was expressed in a decreasing manner in BS and NaF groups with respect to human recombinant IL-1β-treated cells (Fig. 2A). Declining expression levels of TNF-α and IL-8 were also determined (Fig. 2B).

BS and NaF inhibit the expression of TNF-α, IL-1β, and IL-8 mRNA in HGF cells

This study examined the inhibitory effect of BS and NaF on the expression of IL-1β, TNF-α, and IL-8 mRNA in IL-1β-stimulated HGF cells through quantitative real-time (RT)-PCR. IL-1β-treatment in HGF cells increased in IL-1β,
BS and NaF showed the best protective effect against inflammation inhibition.

Thus, lower levels of IL-8, TNF-α, and IL-8 mRNA expression were found while BS and NaF stimulated HGF cells expressed very low levels of these cytokines (Fig. 3).

Discussion

The current study demonstrated that treatment of a human primary gingival fibroblast cell with 0.01% BS + 0.002% NaF resulted in the induction of cell growth and produced no inhibition on cell viability, in addition to no changes in cellular morphology and apoptosis (Fig. 1). To further confirm that the anti-inflammatory effects induced by 0.01% BS + 0.002% NaF were correlated, the reduction protein and mRNA levels of the proinflammatory cytokines were assessed (Figs. 2 and 3).

Chronic inflammation, as shown by increased levels of proinflammatory cytokines, is a common feature in patients with gingivitis and periodontitis and is associated with increased chronic gingival inflammation. Elevated levels of inflammatory cytokines play a central part in the vicious circle of chronic inflammation. Indeed, elevated levels of proinflammatory cytokines, such as IL-1β, TNF-α, and IL-8, cause progressive inflammatory diseases by several pathogenic mechanisms [20]. Serum cytokine levels in patients with inflammatory diseases are higher than those in healthy people [21]. This study showed that 0.01% BS + 0.002% NaF was nontoxic to the fibroblast cells and reduced IL-1β, TNF-α, and IL-8 production in IL-1β-treated fibroblast cells. Thus, lower levels of IL-8, TNF-α, and IL-1β are indicative of improved anti-inflammatory effects, and the mixture of BS and NaF showed the best protective effect against gingival inflammation. The results suggest a possible use of a mixture of BS and NaF in managing gingival inflammation.

Compounds with antioxidant properties may have anti-inflammatory effects and can actually prevent activation of inflammatory signals [22]. BS is known to contain higher concentrations of minerals and other antioxidant compounds than both solar and purified salt. In particular, BS was found to contain more potassium, calcium, magnesium, and manganese than purified and solar salts. The high mineral content and antioxidative property of BS in combination with NaF could show a superior anti-inflammatory action. The unique combination of minerals and antioxidant activity may contribute to the efficacy of BS in prevention of gingival inflammation. The hypothesis considered in the current study is the existence of an inhibitory effect of BS and NaF on the interaction between cytokines and genes associated with inflammation. In support of this hypothesis, Zhao et al [23] have previously shown that BS exerted an anti-inflammatory effect downregulating nuclear factor-kB, inducible nitric oxide synthase, and COX-2 expression. The findings that the inhibition of bone resorption by NaF in a variety of model systems [24,25] suggest that intake of NaF might protect against alveolar bone loss in periodontal disease also supported this hypothesis and raised the possibility that the combination of BS-NaF may have more potent anti-inflammatory effect that is responsible for inflammation inhibition.

In the inflammatory process, activated inflammatory cells (neutrophils, eosinophils, mononuclear phagocytes, and macrophages) secrete increased amounts of nitric oxide, prostaglandin EZ, and cytokines such as IL-1β, IL-6, and TNF-α [26]. These substances not only induce cell and tissue damage but also activate macrophages in chronic inflammation [27]. Minerals such as potassium, calcium, and magnesium, which are abundant in bamboo salts, appear to have anti-inflammatory effects. Levels of potassium, calcium, and magnesium in blood have shown correlation with severity of disease. Potassium has potent anti-inflammatory effects, low calcium levels are associated with hepatic disease, and supplementation with magnesium in the appropriate context can be used for treatment of hepatitis [28]. Zinc ions are an important anti-inflammatory factor [29]. Maintenance of an ion concentration gradient is essential for the function of many organs. Various mineral ions also play a crucial role in many cell functions such as cell proliferation, energy metabolism, protein and DNA syntheses, cytoskeleton activation, and Reactive Oxygen Species (ROS) scavenging activity [30]. BS also exhibits a higher reduction potential; this might be due to the fact that this type of salt contains more OH− groups than Purified Salt (PS) [31]. Together, these characteristics of BS contribute to its anti-inflammatory activity in gingival inflammation.

The effects of fluoride on gingival epithelial cells depend on fluoride concentration. Dose-dependent effects of NaF treatment on proliferation of caprine osteoblasts have been reported [32]. Fluoride causes proliferation and differentiation of cultured osteoblasts associated with increases in

![Figure 3. BS and NaF reduced IL-1β-induced proinflammatory cytokine expression. Treatment with BS and NaF prevented IL-1β-induced TNF-α, IL-1β and IL-8 mRNA expression. ( * = difference between control and rhIL-1β groups; ** = difference between rhIL-1β and other experimental groups). BS = bamboo salt; IL = interleukin; NaF = sodium fluoride.](image-url)
alkaline phosphatase and collagen synthesis [33]. The effect of fluoride on cellular activity may be biphasic in that low concentrations provide increased osteoprogenitor cell number, whereas high concentrations are associated with histological findings of osteoblast inactivity [34]. In the present study, the use of BS and NaF to treat inflammation exhibited favorable results in agreement with previous studies [35,36]. The inhibitory effect of BS and NaF on IL-1β, TNF-α, and IL-8 cytokine production was observed in IL-1β-treated fibroblast cells in vitro. The relative importance of fibroblast cells as a source of cytokines during inflammatory and immune responses are important areas for further studies.

Collectively, a mixture of BS and NaF effectively inhibited the production of inflammatory cytokines in gingival inflammation. Additional studies are currently underway to determine the roles and mechanisms of the differentially expressed genes in the pathogenesis of inflammation and to elucidate the molecular basis for the signaling pathways involved in BS-NaF-fibroblast communication under both physiological and pathophysiological conditions.

References