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ABSTRACT

Originally found in stomach mucosa, ghrelin is a peptide appetite hormone that has been implicated as an immuno-modulatory factor. Ghrelin has also been found in salivary glands and saliva; however, its expression patterns and biological properties in the oral cavity remain unclear. Therefore, we investigated the expression patterns of ghrelin in saliva, gingival crevicular fluid (GCF), and gingival tissue, as well as its *in vitro* effects on IL-8 production by TNF- α or LPS-stimulated oral epithelial cells. In the clinical samples obtained from 12 healthy volunteers, the concentration of ghrelin in GCF remarkably exceeded that detected in saliva. The expression of ghrelin mRNAs and growth hormone secretagogue (GHS) receptors could be detected in human oral epithelial cells. Immunohistochemical analysis revealed the expression of ghrelin in gingival epithelium, as well as in fibroblasts in the lamina propria. Ghrelin increased intracellular calcium mobilization and cAMP levels in oral epithelial cells, suggesting that ghrelin acts on epithelial cells to induce cell signaling. Furthermore, synthetic ghrelin inhibited the production of IL-8 from TNF- α or LPS-stimulated oral epithelial cells. These results indicate that ghrelin produced in the oral cavity appears to play a regulatory role in innate immune responses to inflammatory infection.

KEY WORDS: ghrelin, gingival crevicular fluid, saliva, gingival epithelium, IL-8, lipopolysaccharide.

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Expression and Possible Immune-regulatory Function of Ghrelin in Oral Epithelium

INTRODUCTION

Ghrelin is a 28-amino-acid acylated peptide hormone mainly secreted by X/A-like cells in the oxyntic mucosa of the stomach, and it is characterized by the presence of an n-octanoyl modification on the serine third position (Kojima *et al.*, 1999). In addition to inducing the production of growth hormone, ghrelin stimulates food intake, and it is involved in the regulation of energy homeostasis (Gualillo *et al.*, 2003). Recent evidence indicated a potential regulatory role of ghrelin in the immune system. For instance, it was reported that ghrelin could down-modulate the proliferation of T-cells, as well as the production of cytokines, from activated T-cells (Xia *et al.*, 2004). Furthermore, analysis of both *in vitro* and *in vivo* data showed that ghrelin can suppress pro-inflammatory cytokines produced from monocytes in response to stimulation with lipopolysaccharide (LPS) (Dixit *et al.*, 2004; Waseem *et al.*, 2008). Therefore, ghrelin may function as an important modulator not only for the endocrine and nervous systems, but also for the immune system.

As noted above, ghrelin was initially isolated from gastric mucosa. In addition, ghrelin was found to be widely expressed in different tissues, such as pancreas, kidney, and both small and large intestines (Ghelardoni *et al.*, 2006). In the oral cavity, ghrelin was detected in human saliva, and ghrelin-positive cells were also found in salivary glands and oral squamous cell carcinoma lesions (Gröschl *et al.*, 2005; Alnema *et al.*, 2010). However, it remains unclear if oral epithelial cells, or other cells in the lamina propria, contribute to the production of ghrelin.

Oral epithelial cells are key players in the oral innate immune system. Upon stimulation with the soluble mediators of inflammation, such as TNF- α as well as pathogen-associated molecular patterns (PAMPs), they secrete pro-inflammatory cytokines, including IL-8 (Li *et al.*, 1996; Sugiyama *et al.*, 2002). We know that ghrelin can reduce the LPS-induced pro-inflammatory response in murine macrophages (Waseem *et al.*, 2008). However, its influence on cytokine production by oral epithelial cells is unknown. Therefore, in the present study, we investigated the expressions of ghrelin in saliva, gingival crevicular fluid (GCF), gingival epithelium, and lamina propria. To evaluate the functional role of ghrelin expressed in the oral cavity, we also examined if ghrelin could affect IL-8 production from oral epithelial cell lines in response to LPS or TNF- α .

MATERIALS & METHODS

Participants

In total, 12 periodontally healthy individuals aged 19-58 yrs participated in this study and provided saliva and GCF specimens ($n = 12$; two men and 10 women; 34 ± 11.5 yrs, mean age $\pm$ SD). We determined periodontal health after establishing the absence of bleeding on probing, probing depths ≤ 3 mm, and attachment loss ≤ 2 mm. For immunohistochemical analysis, cervical gingival tissues were collected at the crown-lengthening procedure from another three individuals (one male, aged 46 yrs, and two females, both aged 35 yrs). Buccal mucosal cells were collected from healthy individuals for RT-PCR analysis (Spivack *et al.*, 2004). The study protocol was reviewed and approved by the Institutional Review Board at the Forsyth Institute, Harvard School of Dental Medicine, and Harvard Medical School, and all participants signed an informed consent statement.

Saliva and GCF Samples

Unstimulated saliva and GCF samples were collected as previously described (Figueredo *et al.*, 2008; Gursoy *et al.*, 2010). Saliva samples were centrifuged at $20,000 \times g$ for 10 min, and the resulting supernatants were kept frozen at -80°C . After the complete removal of dental plaque, GCF was collected from randomly selected quadrants at the mesiobuccal site of every tooth, by means of paperpoints (IDE, Amityville, NY, USA), which were inserted into the pockets and kept in place for 30 sec. The amount of GCF sampled into the paperpoint was measured with a Periotron (OraFlow Inc., Smithtown, NY, USA). The protein levels of IL-8 and ghrelin in saliva and GCF were determined with an IL-8 Duo-ELISA kit (R&D Systems, Minneapolis, MN, USA) and a Ghrelin Enzyme Immuno-Assay kit (Bachem, Torrance, CA, USA), respectively, to detect acylated ghrelin, according to the protocol provided by the manufacturers.

Cell Lines and Synthetic Ghrelin Peptides

Immortalized human oral epithelial cell lines RT7 and OBA9 were cultured in Keratinocytes-SFM (Invitrogen, Carlsbad, CA, USA), as described previously (Hosokawa *et al.*, 2006; Ohta *et al.*, 2008). Immortalized human oral epithelial cell line OKF6/TERT-2 cells were kindly provided by James G. Rheinwald (Brigham and Women's Hospital, Harvard Institutes of Medicine, Boston, MA, USA) (Dickson *et al.*, 2000). Human gingival fibroblast cell line GT1 cells (Kamata *et al.*, 2004) and human leukemic cell lines HL-60 and THP-1 (De Vriese and Delporte, 2007) were grown in appropriate medium supplemented with 10% fetal calf serum. A full sequence of n-octanoyl ghrelin peptide (28 amino acids) was purchased from ProspeC (Rehovot, Israel). Scrambled ghrelin peptide (consisting of the same amino acid sequence as ghrelin but in a different order) was generated by a custom synthesis service (United Peptide, Bethesda, MD, USA).

RNA Extraction and RT-PCR

Total RNA was prepared from cell lines by means of an RNeasy total RNA isolation Kit (Qiagen, Hilden, Germany). Single-stranded

cDNA for a polymerase chain-reaction (PCR) template was synthesized from superscript 3 reverse transcriptase (Invitrogen). cDNA was amplified by PCR with Hot Start *Taq* DNA polymerase (Qiagen). Specific primers were used: human ghrelin sense 5'-GCCACCTGTCTGCAACC-3' and antisense 5'-CTGCTTGACCTCCATCTTCC-3'; human GHS-R1a and GHS-R1b sense 3'-TCTTCCTTCCTGTCTTCTATC-5'; human GHS-R1a antisense 3'-AGTCTGAACACTGCCACC-5'; and human GHS-R1b antisense 3'-TCAGAGAGAAGGGAGAAG G-5'. PCR conditions were 1x (95°C , 15 min), 40x (95°C , 2 min; 55°C , 30 sec; 72°C , 1 min) and 1x (72°C , 7 min). The products were analyzed on 2% agarose gels containing SYBR Green (Invitrogen). β -actin was included as an internal control.

Immunohistochemistry

Frozen sections were fixed with 4% paraformaldehyde followed by incubation with 1.0% H_2O_2 to neutralize endogenous peroxidase activity. After blocking with 1.5% horse serum in PBS, each section was reacted with polyclonal rabbit IgG specific to n-octanoyl ghrelin (Abcam, Cambridge, MA, USA) overnight at 4°C . Biotin-labeled horse anti-rabbit IgG (Jackson Immuno Research, West Grove, PA, USA) with 1.5% horse serum was reacted for 30 min at room temperature, followed by incubation with avidin/biotin complex with horseradish peroxidase (Vector Laboratories, Burlingame, CA, USA). Specific staining was performed by color development with diaminobenzidine substrate in the presence of H_2O_2 , and counterstaining was done with hematoxylin.

Calcium Flux and cAMP Assays

OBA9 cells were exposed to ghrelin or scrambled ghrelin for 1-2 hrs. Intracellular calcium mobilization and cAMP were measured with the Fluo-8-no-wash calcium assay kit (AAT Bioquest, Sunnyvale, CA, USA) and cAMP assay kit (BioVision, Inc., Mountain View, CA, USA), respectively (SD).

Cytokine Determination

RT7 and OBA9 cells were stimulated with TNF- α (Peprotech, Rocky Hill, NJ, USA), *Escherichia coli* (*E. coli*) LPS, *Porphyromonas gingivalis* (*P. gingivalis*) LPS (Invivogen, San Diego, CA, USA), and ghrelin for 48 hrs. Supernatants from the cells were collected, and the concentration of IL-8 was measured by IL-8 DuoSet ELISA (R&D Systems).

Statistical Analysis

The data were analyzed by Student's *t* test or one-way analysis of variance (ANOVA), and the results were presented as the mean $\pm$ standard deviation (SD).

RESULTS

Detection of Ghrelin in Saliva and GCF

We examined the concentration of ghrelin in saliva and GCF from 12 healthy volunteers. The concentration of ghrelin

Figure 1. Concentrations of ghrelin and IL-8 in saliva and GCF. **(A)** Concentrations of ghrelin protein in GCF and saliva. **(B)** Concentrations of IL-8 protein in GCF and saliva. The protein levels of IL-8 and the levels of ghrelin in saliva and GCF from 12 healthy volunteers were determined by ELISA. *The concentrations of ghrelin in GCF are significantly different from those in saliva (Student's *t* test: $p < 0.05$).

measured in GCF was approximately 500-fold greater than in saliva samples (GCF, $19,129 \pm 13,280$ pg/mL; saliva, 35.7 ± 39.8 pg/mL) (Fig. 1). Like ghrelin, the concentrations of IL-8, as measured in both GCF and saliva samples, showed a trend of prominent elevation in GCF compared with saliva (GCF, $1,349,275 \pm 779,733$ pg/mL; saliva, 4992 ± 4574 pg/mL). No significant correlation was observed between ghrelin and IL-8 concentrations in either saliva or GCF. The concentration of ghrelin in serum samples from four healthy volunteers in this study was 46.99 ± 9.14 pg/mL, which was lower than that detected in GCF or saliva.

Ghrelin mRNA and GHS Receptors in Oral Epithelial Cells and Gingival Fibroblasts

It is plausible that the secretion of ghrelin by oral epithelial cells may contribute to the elevated levels of ghrelin in GCF. Therefore, we first examined whether oral epithelial cells express ghrelin. RT-PCR assay showed that these oral epithelial cells and fibroblasts constitutively expressed ghrelin mRNA (Fig. 2A). The biological activities of ghrelin are mainly mediated by its ligation to growth hormone secretagogue (GHS) receptors (Howard *et al.*, 1996). As a result of alternative splicing of a single gene, 2 GHS receptor subtypes have been cloned: GHS receptor type 1a (GHS-R1a) and GHS receptor type 1b (GHS-R1b) (Howard *et al.*, 1996). Both of them were detected in oral epithelial cells and fibroblasts (Fig. 2A). The mRNA of ghrelin and these 2 GHS receptors were detected in human buccal epithelial cells from three healthy volunteers (Fig. 2B).

Ghrelin Expression in Oral Epithelium

To examine if ghrelin is expressed *in situ*, we carried out immunohistochemical evaluation on healthy human epithelial tissues. The results displayed ghrelin-positive staining in gingival epithelium (Fig. 2C). However, fibroblasts present in the lamina

propria showed more prominent expression of ghrelin than those in gingival epithelium (Fig. 2C), indicating that fibroblasts, as well as gingival epithelium, albeit to a lesser extent, may contribute to the higher level of ghrelin found in GCF compared with saliva.

Effects of Ghrelin on Intracellular Calcium and cAMP Levels

GHS-R1a binds to ghrelin and leads to intracellular calcium concentration (Kojima *et al.*, 1999). Further, GHS-R1a increases intracellular cAMP, which is a second messenger that activates the subsequent signaling cascade (Kojima and Kangawa, 2005). Therefore, we examined intracellular calcium mobilization and cAMP levels in ghrelin-stimulated oral epithelial cells. A full sequence of

active ghrelin, but not control scrambled ghrelin, increased the intracellular calcium and cAMP levels in OBA9 (Fig. 3). These results indicate that oral epithelial cells can elicit intracellular signals in response to ghrelin, possibly *via* ligation to GHS-R1a.

Effect of Ghrelin on IL-8 Production by Oral Epithelial Cell Line Stimulated with TNF- α or LPS

Finally, because an increase of cAMP in cytoplasm is reported to elicit intracellular signals that down-modulate cytokine production in immune responses (Zidek, 1999), the finding that ghrelin increased cAMP in OBA9 cells (Fig. 3) led us to examine ghrelin's possible immune-regulatory effect on IL-8 production from oral epithelial cells. The stimulation of oral epithelial cell lines with TNF- α up-regulated the production of IL-8. However, the addition of ghrelin into the culture of the oral epithelial cell line significantly suppressed the production of IL-8 from TNF- α -stimulated oral epithelial cells (Fig. 4). Furthermore, ghrelin also suppressed IL-8 production induced by *E. coli* LPS or *P. gingivalis* LPS (Fig. 4). It is noteworthy that control scrambled ghrelin did not affect the production of IL-8 from TNF- α - or LPS-stimulated OBA9 cells.

DISCUSSION

The present study demonstrated that the oral epithelium and fibroblasts produce ghrelin, and that a much higher concentration of ghrelin is present in GCF compared with saliva. Furthermore, ghrelin demonstrated its ability to suppress IL-8 production by TNF- α - or LPS-stimulated oral epithelial cells, suggesting the modulatory role of ghrelin in the innate immune response mediated by IL-8 in the context of the oral cavity.

Ghrelin plays a major role in the regulation of gastrointestinal tract function, stimulating gastric contractility and acid secretion (Date *et al.*, 2001). Ghrelin is also responsible for a

Figure 2. Expression of ghrelin in oral epithelium. **(A)** Ghrelin mRNA expression in RT7, OBA9, and OKF6/TERT-2 epithelial cell lines. Total RNA was isolated from each cell line at a confluent culture, after which RT-PCR was performed for ghrelin and β -actin. Human leukemic cell lines HL-60 and THP-1, which were reported to express ghrelin mRNA, were used as positive controls (De Vriese and Delpote, 2007). **(B)** Ghrelin mRNA expression in human buccal mucosal cells from three healthy volunteers (one man, aged 40 yrs, and two women, ages 35 and 31 yrs). The samples were obtained from healthy individuals after they provided informed consent (Spivack *et al.*, 2004). Total RNA was isolated from each participant, after which RT-PCR was performed for ghrelin and β -actin. **(C)** Ghrelin immunostaining in gingival epithelium. Periodontal tissues collected from healthy human participants were stained with polyclonal rabbit IgG anti-ghrelin antibodies (a) and control non-immunized rabbit IgG antibodies (b). The gingival epithelium shown in (C) was isolated from the cervical epithelium that faces the inner side of gingival crevice. Original magnification, 200x.

metabolic response to starvation by modulating insulin and glucose levels *via* its ligation to the receptor GHS-R1a expressed in the pituitary gland (Papotti *et al.*, 2000). In addition, ghrelin was recently found to function as a negative regulator of lymphocyte proliferation (Xia *et al.*, 2004). In the oral cavity, it has been reported that ghrelin is produced by salivary glands and released into saliva (Gröschl *et al.*, 2005). However, the present study revealed that the concentration of ghrelin found in GCF was approximately 500-fold higher than that detected in saliva. Furthermore, ghrelin measured in serum was lower than the concentration found in either GCF or saliva. This result is supported by a study reporting higher levels of ghrelin in the saliva, as compared with the plasma, of healthy individuals (Aydin *et al.*, 2005). Although ghrelin-positive staining was detected in the outer layer of epithelium, little or no ghrelin staining was found in the basal layer. Therefore, autocrine signaling through

GHS receptors may occur prominently in the outer layer of epithelial cells. The high level of ghrelin found in GCF is thought to have a greater impact on the regulation of IL-8 production from gingival and oral epithelium than epithelial autocrine ghrelin. Therefore, we hypothesize that the high level of ghrelin detected in GCF produced prominently by fibroblasts in lamina propria and, to a lesser extent, by gingival epithelial cells, but not from the serum exudates, plays a role in IL-8 regulation.

Multiple species of bacteria colonize tooth and root surfaces and are present in the gingival crevice, and some of them are considered to be opportunistic pathogens for gingivitis as well as periodontitis. GCF plays a pivotal role in inhibiting the overgrowth of these bacteria by providing a mechanical washout flow as well as by delivering innate immune leukocytes (Delima and Van Dyke, 2003). However, it has been reported that IL-8-activated neutrophils produce superoxide, which might, in turn,

Figure 3. The effects of ghrelin on intracellular calcium and cAMP levels. **(A)** Confluent OBA9 cells in 96-well plates were exposed to various concentrations of ghrelin or scrambled ghrelin for 1 hr. Intracellular calcium mobilization was measured by means of a Fluo-8 no-wash calcium assay kit. Relative fluorescence intensity ratios are presented as the mean $\pm$ SD of 3 independent experiments relative to that of buffer alone. The ratios among the various concentrations of ghrelin showed statistical differences (ANOVA, $p < 0.01$). *Significantly different from non-treated cells (Student's t test; $p < 0.05$). N.D.: no significant difference. **(B)** Confluent OBA9 in 6-well plates were exposed to various concentrations of ghrelin or scrambled ghrelin for 2 hrs. Medium was removed, and cells underwent lysis in 0.1 M HCl. Intracellular cAMP levels were determined by means of a cAMP assay kit. The cAMP levels among the various concentrations of ghrelin showed statistical differences (ANOVA, $p < 0.01$). Data are shown as the mean $\pm$ standard deviation of 3 independent experiments. *Significantly different from non-treated cells (Student's t test; $p < 0.05$). N.D.: no significant difference.

injure oral epithelial cells (Altman *et al.*, 1992). Furthermore, analysis of clinical data indicates that the concentration of pro-inflammatory cytokines, including IL-8, in GCF is associated with periodontal status (Gamonal *et al.*, 2000). These findings imply that overactivation of neutrophils can cause periodontal tissue injury and resulting inflammation based on the production of reactive oxygen species produced from neutrophils in the context of chronic periodontal disease (Kantarci *et al.*, 2003; Iwata *et al.*, 2009). However, since we have established that ghrelin can suppress the production of neutrophil chemoattractant factor IL-8 from oral epithelial cells, it is plausible that ghrelin secreted in GCF may play an important role in regulating neutrophil-mediated innate immune responses, which may, in turn, protect periodontal tissue from the oxidative damage caused by the overaccumulation of neutrophils.

The concentration of ghrelin showing *in vitro* inhibitory effects on IL-8 produced from oral epithelial cells was higher than that measured in the GCF. However, in the physiological condition, the constant flow of GCF reaches a cumulative level of ghrelin that is sufficient to suppress the overexpression of IL-8. A previous study suggested that ghrelin secreted from saliva may promote the proliferation of oral keratinocytes (Gröschl *et al.*, 2005). Furthermore, treatment of human microvascular endothelial cells with ghrelin increased their proliferation, migration, and angiogenesis (Li *et al.*, 2007). Based on the findings in this study, along with previous reports, as noted above, it is suggested that ghrelin may play a role in the maintenance of gingival mucosal tissue turnover, as well as down-regulating the overreaction of innate immune response that involves IL-8-mediated neutrophil recruitment.

Figure 4. Effects of ghrelin on IL-8 expression from TNF- α or LPS-stimulated oral epithelial cells. RT7 and OBA9 cells were cultured for 48 hrs in the presence or absence of (A) TNF- α (10 ng/mL), (B) *E. coli* LPS (50 μ g/mL), or (C) *P. gingivalis* LPS (50 μ g/mL) with different concentrations of ghrelin, after which the level of IL-8 in the culture supernatant was measured by ELISA. Data are shown as the mean $\pm$ SD of 3 independent experiments. *Significantly different from TNF- α or LPS alone (Student's *t* test: *p* < 0.05). (D) OBA9 cells were stimulated with TNF- α (10 ng/mL), *E. coli* LPS (50 μ g/mL), or *P. gingivalis* LPS (50 μ g/mL) for 48 hrs in the presence or absence of scrambled ghrelin (10 μ g/mL). The level of IL-8 in culture supernatant was measured by ELISA. Data are shown as the mean $\pm$ SD of 3 independent experiments. N.D.: no significant difference.

In conclusion, this is the first demonstration that ghrelin (1) is produced from oral epithelium, (2) is present in GCF, and (3) can inhibit TNF- α - or LPS-induced IL-8 production in oral epithelial cells, suggesting that ghrelin may function as an immune-modulation factor in the context of oral mucosa.

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