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Molecules and Cells

S100A2 Level Changes Are Related to Human Periodontitis

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Periodontitis is an inflammatory disease, which, when severe, can result in tooth loss, that affects the quality of life. S100A2 was previously identified as a component of gingival crevicular fluid (GCF) *via* proteome analysis, but it has not been investigated whether S100A2 plays a role in periodontitis. In this study, we analyzed mRNA expression of S100A2 in gingival tissues from normal and classified periodontal disease patients and compared it to that of S100A8 and S100A9. Quantitative real time-PCR revealed that the mRNA expression levels of S100A2, S100A8, and S100A9 were significantly upregulated in gingival tissues with gingivitis, moderate periodontitis, and severe periodontitis compared to normal tissues. In addition, S100A2 proteins in GCF and the conditioned media of lipopolysaccharide (LPS)-treated Jurkat cells were confirmed by ELISA. S100A2 protein levels were significantly higher in GCF in gingivitis and moderate periodontitis groups than in normal groups. S100A2 mRNA expression and protein secretion were also increased by LPS stimulation. Based on the up-regulation of S100A2 in LPS-stimulated immune cells, gingival tissues and GCF from periodontal disease groups, we conclude that S100A2 is a functional component in the immune response during periodontitis and may serve as a potential biomarker for periodontitis.

INTRODUCTION

Periodontitis is a chronic inflammatory disease that results in destruction of the periodontium, which is the most common cause of tooth loss in the world. Periodontitis is also related to systemic diseases, such as cardiovascular disease (Suzuki et al., 2010) and diabetes (Nagasawa et al., 2010). Periodontitis is affected by genetic and environmental factors. Genetic factors include gene polymorphisms of interleukin-1, interleukin-10 and Fc-gamma receptor, human leukocyte antigen traits and familial aggregation. Environmental factors include smoking, oral hygiene, stress and systemic diseases (Stabholz et al., 2010). In addition, periodontitis is characterized by a change in the oral bacteria from mainly Gram-positive to mainly Gram-negative species, which include *Porphyromonas gingivalis, Tannerella forsythia* and *Treponema denticola* (Darveau, 2010).

S100 protein was identified as a brain protein by Moore in 1965, and closely related molecules, S100A1 and S100B, were isolated in the same fraction of the brain (Donato, 1999; Zimmer et al., 1995). Twenty-one human S100 proteins are currently known, with similarity ranging from 22% to 57%. The S100A1 to S100A16 cluster is located on chromosomal region 1q21, S100B is found on chromosome 21, and S100P and S100Z are on chromosomes 4 and 5, respectively (Fritz et al., 2010). S100 protein is a low molecular weight protein (9-13 kDa) that is modulated by Ca^{2+} binding through EF-hand motifs (Donato, 2003; Han et al., 2011; Schafer and Heizmann, 1996). The expression of S100 family members is restricted in cell and/or tissue type. For example, S100A2 is expressed in lung, kidney and smooth and heart muscle cells; S100A4 is expressed in fibroblasts, myoepithelial cells, smooth and heart muscle cells, and tumor cells; S100A6 is expressed in fibroblasts, tumor cells, and smooth and heart muscle cells; S100A8 and S100A9 are expressed in epithelial cells, granulocytes and monocytes; and S100P is expressed in the placenta (Donato, 1999). Each member of the S100 family is associated with a specific disease based on its expression pattern. For example, S100A1 is associated with cardiomyopathies; S100A2 to S100A6, S100A10, S100B and S100P are associated with cancer; S100A7 is associated with psoriasis; S100A8, S100A9 and S100A12 with are associated with inflammatory disorders; and S100B is associated with neurodegeneration (Donato, 2003; Marenholz et al., 2004). Knockout mouse models of various S100 proteins have revealed that S100A1 functions in cardiac contractility, S100A4 is involved in tumorigenesis and tumor metastasis, and S100B plays a role in the nervous system. However, knockout models have been less useful in determining the function of other S100 proteins. For example, an S100A8 knockout model is embryonic lethal, and knockout of either S100A9 or S100A11 have no obvious abnormalities (Marenholz et al., 2004).

Various members of the S100 family are related to periodontitis. Several studies have suggested that a heterodimer of S100A8 or S100A9, named Calprotectin/Myeloid-related Protein (MRP), could be a marker for periodontitis. Calprotectin significantly correlates with both clinical indicators of periodontitis, gingival index and probing depth, and current biomarkers of periodontitis, such as collagenase, interleukin-1β and prostaglandin E2 (Kido et al., 1999; Nakamura et al., 2000). In addi-

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tion, S100A8 was identified as one of the main responsive proteins in chronic periodontitis (Lundy et al., 2000) and the levels of S100A8 were significantly higher in inflammatory gingival tissue than in normal tissue (Lundy et al., 2001). S100A8 and S100A9 were identified in GCF from a periodontitis patient compared to serum proteins from the same patient (Kojima et al., 2000). We recently analyzed the gingival crevicular fluid (GCF) proteome data to identify periodontitis biomarkers. Among the proteins identified in this study were several S100 proteins including S100A2, S100A6, S100A8, S100A9, S100A11, S100A12 and S100P (manuscript submitted). Interestingly, unlike the other proteins listed, S100A2 had not previously been detected in saliva or the GCF proteome under normal or diseased conditions.

The aim of this study was to compare the expression and secretion levels of S100A2 in gingival tissues and GCF from periodontitis patients and healthy individuals. S100A2 mRNA was detected in gingival tissues at different stages of periodontal disease and compared to the mRNA levels of S100A8 and S1009 which were previously suggested as candidate periodontitis biomarkers. The expression and secretion levels of S100A2 were higher in gingival tissue and GCF of gingivitis and moderate periodontitis patients than in normal patients. The mRNA expression and secreted proteins of S100A2 were increased in lipopolysaccharide (LPS)-induced immune cells. Our results suggest that S100A2 could be a potential biomarker for periodontal disease.

MATERIALS AND METHODS

Gingival tissues and gingival crevicular fluid

Gingival tissue samples were obtained from patients undergoing periodontal surgery. Gingival crevicular fluid (GCF) samples were collected from periodontitis patients and healthy individuals at the Department of Periodontology, School of Dentistry, Kyungpook National University. The standard protocol was approved by the IRB of the Kyungpook National University Hospital (IRB NO. 74005-830). Informed consent was obtained from all donors. Gingival tissues were classified as normal, gingivitis, moderate periodontitis, and severe periodontitis according to criteria such as gingival index considered immune reaction, probing depth, clinical attached loss and bone loss on radiograph. For analysis using quantitative real time-PCR, gingival tissues were isolated from 14 healthy individuals (normal), 13 patients with chronic gingivitis, 48 patients with moderate periodontitis, and 27 patients with severe periodontitis. S100A2 protein levels in the GCF were determined by ELISA in GCF samples collected from 19 healthy individuals, 39 patients with chronic gingivitis, 48 patients with moderate periodontitis and 33 patients with severe periodontitis.

Cell culture

Jurkat cells (human T cell lymphoblast-like cell line), THP1 (Human acute monocytic leukemia cell line) and HL-60 cells (Human promyelocytic leukemia cells) were cultured in RPMI media supplemented with 10% FBS in the presence of 5% CO_2 at 37°C. For lipopolysaccharide (LPS) stimulation, cells were incubated in RPMI without FBS for 1 h, and the media was replaced with or without LPS at the indicated times and concentrations. For *Streptococcus mutans* (*S. mutans*) and *Streptococcus mitis* (*S. mitis*) stimulation, 5×10^5 cells were incubated in RPMI with 1% FBS and *S. mutans* or *S. mitis* for 3 h.

Extraction of RNA and real-time PCR

Total cellular RNA was isolated from gingival tissue or Jurkat cells using TRIzol Reagent (Invitrogen) following the manufacturer's instructions. Total RNA (2 μg) was used to synthesize single-stranded cDNA using oligo$(dT)_{18}$ as a primer and the Omniscript Reverse Transcriptase (Qiagen, Germany). Gene specific primers were designed using Primer 3, a web-based primer design tool. The mRNA expression levels of 3 S100 genes and a reference gene (GAPDH) were determined by real time PCR using the following primers: S100A2 sense 5′-GGCTGTGCTGGTCACTACCT-3′, S100A2 antisense 5′-CCT GCTGGTCACTGTTCTCA-3′, S100A8 sense 5′-ATGCCGTCT ACAGGGATGAC-3′, S100A8 antisense 5′-ACGCCCATCTT TATCACCAG-3′, S100A9 sense 5′-CAGCTGGAACGCAACA TAGA-3′, S100A9 antisense 5′-TCAGCTGCTTGTCTGCATTT-3′, GAPDH sense 5′-GAGTCAACGGATTTGGTCGT-3′, and GAPDH antisense 5′-GACAA GCTTCCCGTTCTCAG-3′. Florescence-based real time PCR was conducted on a DNA Engine OPTICON®2 system (MJ Research, USA) using SYBR Green I (Molecular Probes, USA). The reaction consisted of 40 cycles of 95°C for 30 s, 50°C for 40 s and 72°C for 60 s. Human GAPDH was amplified as an internal control and was used a reference to normalize each sample.

Protein isolation

Total protein was collected from GCF, Jurkat cell-cultured media and HL-60 cell-cultured media. GCF samples were diluted into 20 μl of 0.9% NaCl and centrifuged to remove contaminants such as tissue debris, and the supernatant was transferred to a new tube. Cultured media were collected from the supernatant by centrifugation for 3 min at 100 × *g*. The media were concentrated by trichloroacetic acid (TCA)/acetone precipitation. Protein concentrations in the GCF and conditioned media were measured using the Bradford method.

ELISA

S100A2 protein levels were quantified by ELISA (Cusabio) according to the manufacturer's instructions. Briefly, each sample was added to a well and incubated for 2 h at 37°C. The liquid was removed from each well, 100 μl a working solution of Detection Reagent A was added, and the samples were incubated for 1 h at 37°C. The mixture was removed, and the wells were washed with Wash Buffer (400 μl) using a multi-channel pipette. After washing, a working solution of Detection Reagent B was added to each well, and the samples were incubated for 1 h at 37°C. The wells were washed, the substrate solution was added, and the samples were incubated for 30 min at 37°C. The reaction was stopped by adding 50 μl of Stop solution to each well. The optical density at 450 nm of each well was measured using a microplate reader.

Statistical analysis

The data are presented as the mean ± S.E.. Statistical significance was performed using one-way ANOVA followed by the Student's *t*-test for unpaired samples. Probability (p-value) of less than 0.05 was considered to be significant.

RESULTS

mRNA expression of S100A2, S100A8, and S100A9 in gingival tissues

Gingival tissue samples were collected from 42.4 ± 16.4-year-old healthy individuals (normal), 50.6 ± 14.7-year-old gingivitis patients, 47.2 ± 7.7-year-old moderate periodontitis patients and 47.5 ± 7.2-year-old severe periodontitis patients (Fig. 1A). The normal group consisted of 9 female and 5 male individuals, the gingivitis group consisted of 7 female and 6 male patients;

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	Normal	Gingivitis	Moderate periodontitis	Severe periodontitis
Sample # (female:male)	14 (9:5)	13 (7:6)	48 (21:27)	27 (10:17)
Age	42.4 ± 16.4	50.6 ± 14.7	47.2 ± 7.7	47.5 ± 7.2

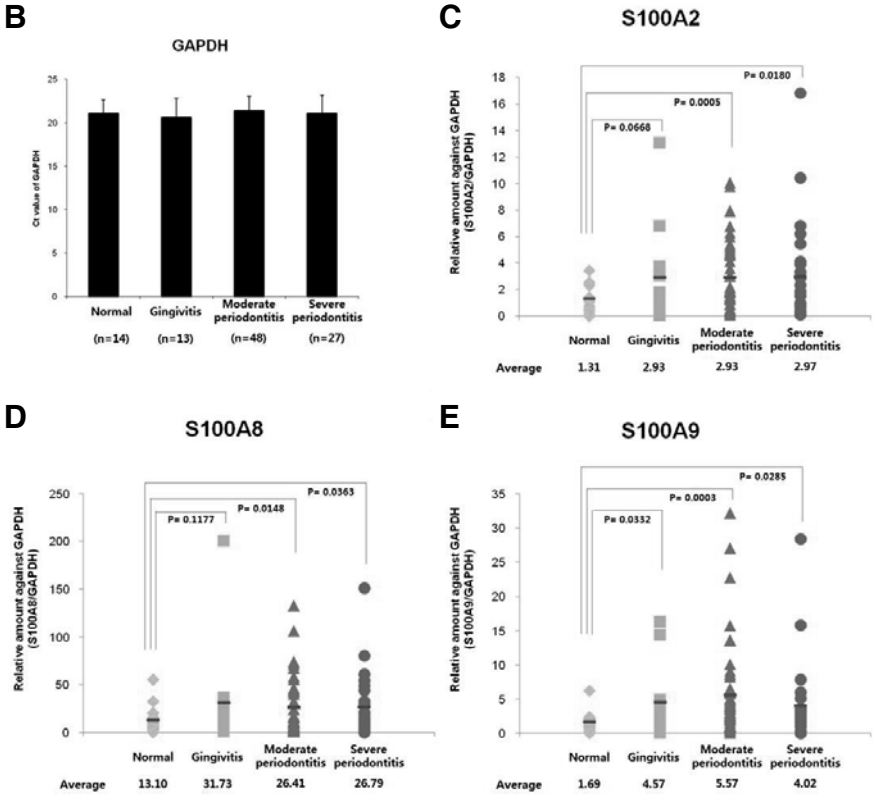

Fig. 1. The expression of S100A2, S100A8 and S100A9 mRNAs in the gingival tissues of normal, gingivitis, moderate periodontitis, and severe periodontitis patients. (A) Tissue sample information. For quantification of mRNA expression of 3 S100 genes, the gingival tissues were collected from normal (n = 14), gingivitis (n = 13), moderate periodontitis (n = 48), and severe periodontitis patients (n = 27). (B) Threshold cycles (Ct) values of GAPDH for the samples in real-time PCR. For normalization of each sample the internal PCR control, GAPDH, was used. The Ct value of GAPDH in samples did not differ among groups. (C, D, and E) The mRNA expressions of S100A2, S100A8, and S100A9 in gingival tissues using real-time PCR. Total RNA was extracted from gingival tissues in normal, gingivitis, moderate periodontitis, and severe periodontitis patients. For quantification, the internal PCR control, GAPDH, was used as a reference to normalize each sample. The horizontal bar indicates the means of relative amount against GAPDH in each group.

the moderate periodontitis group consisted of 21 female and 27 male patients, and the severe periodontitis group consisted of 10 female and 17 male patients (Fig. 1A). The expression levels of GAPDH mRNA were used as a reference and did not differ between the 4 groups (Fig. 1B).

The mRNA expression levels of S100A2, S100A8 and S100A9 genes were higher in the gingival tissues of gingivitis and periodontitis groups than in the normal group; however, the 3 S100 genes displayed different mRNA expression profiles between the gingivitis, moderate periodontitis and severe periodontitis groups (Figs. 1C, 1D, and 1E). The expression of S100A2 did not differ between the gingivitis, moderate periodontitis and severe periodontitis groups (Fig. 1C) whereas S100A8 expression was slightly higher in the gingivitis group than in the two periodontitis groups (Fig. 1D), and S100A9 expression was higher in the moderate periodontitis group than in the gingivitis and severe periodontitis groups (Fig. 1E).

S100A2 protein expression in gingival crevicular fluid (GCF)

S100A2 protein expressions in GCF were evaluated to determine whether S100A2 could be an indicator or biomarker of gingivitis or/and periodontitis, similar to S100A8. GCF samples were obtained from 34.7 ± 11.1-year-old healthy individuals (9 females and 10 males, normal); gingivitis samples were collected from 44.3 ± 13.6-year-old patients (19 females and 20 males); moderate periodontitis samples were collected from 51.4 ± 9.3-year-old patients (23 females and 25 males); and severe periodontitis samples were collected from 51.5 ± 9.3-year-old patients (10 females and 23 males) (Fig. 2A). The GCF samples were tested to determine the amount of GCF required for the detection of S100A2 (data not shown). For current ELISA analysis, GCF samples need to be combined to contain 40 μg of proteins. Average S100A2 levels in the GCF were 130.3, 173.5, 201.0 and 125.4 pg/ml in normal, gingivitis, moderate periodontitis and severe periodontitis samples, respectively (Fig. 2B). S100A2 levels in GCF were higher in gingivitis and moderate periodontitis samples than in normal samples with the large variation among all of the groups tested. S100A2 levels did not correlate with gender or age (data not shown).

LPS increases mRNA expression and secretion of S100A2 in immune cells

The mRNA expression of S100A2 in Jurkat cells, THP1 and HL-60 cells was measured using real-time PCR after treatment with 30 to 1000 ng/ml lipopolysaccharide (LPS) for 6 h. S100A2 mRNA expression was increased in a dose-dependent manner following LPS stimulation except at 300 ng/ml in Jurkat cells and 200 ng/ml in THP1 cells, although S100A2 mRNA was also

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	Normal	Gingivitis	Moderate periodontitis	Severe periodontitis
Age (Avg. ± S.E.)	34.7 ± 11.1	44.3 ± 13.6	51.4 ± 9.3	51.5 ± 9.3
Sample number (Female:Male)	19 (9 : 10)	39 (19 : 20)	48 (23 : 25)	33 (10 : 23)

B

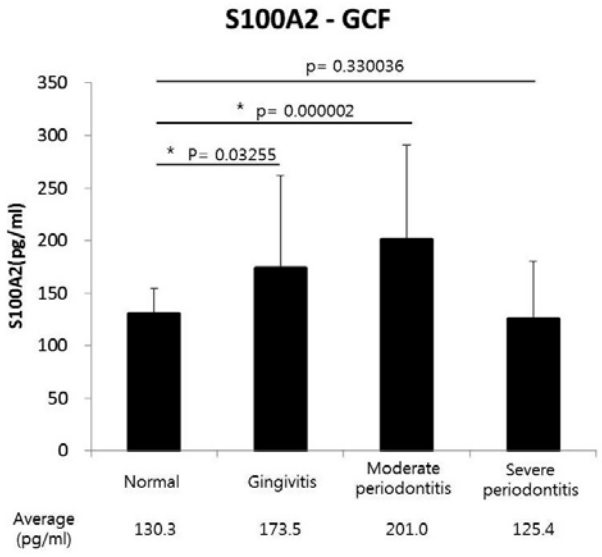

Fig. 2. Protein expressions of S100A2 in gingival crevicular fluid (GCF) of normal, gingivitis, moderate periodontitis, and severe periodontitis patients. (A) Information on gingival crevicular fluid samples. (B) S100A2 protein expressions in gingival crevicular fluid analyzed by a S100A2 ELISA assay. Proteins were isolated from gingival crevicular fluid in normal (n = 19), gingivitis (n = 39), moderate periodontitis (n = 48), and severe periodontitis patients (n = 33). Four to eight samples were pooled to detect S100A2 in gingival crevicular fluid by ELISA. A total 40 μg of proteins was used to quantify S100A2 expression by the ELISA. The horizontal bars indicate the mean of S100A2 levels in each group.

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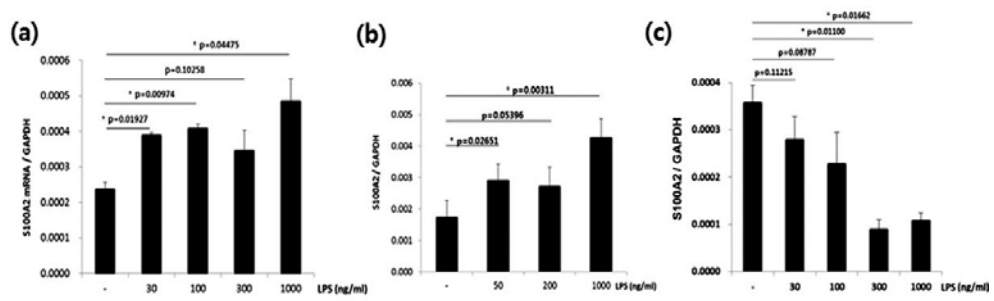

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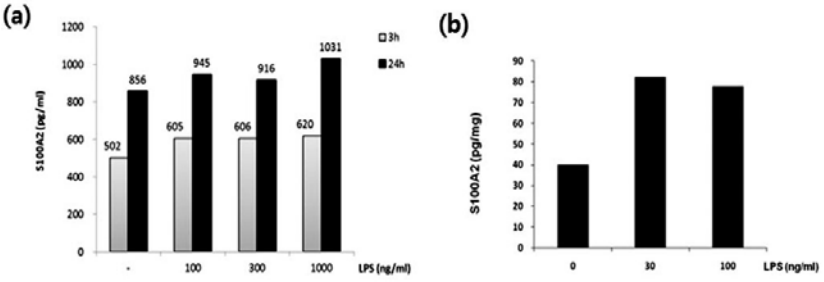

Fig. 3. S100A2 levels increased by LPS stimulation in immune cells. (A) The mRNA expression of S100A2 in LPS-stimulated immune cells. mRNAs were extracted from Jurkat cells after LPS treatment at the indicated concentration for 6 h. The expression levels of S100A2 mRNA were evaluated by the Ct value of S100A2 over the Ct value of GAPDH as an internal standard. S100A2 expression was upregulated by LPS treatment (30 to 1000 ng/ml) in Jurkat cells (a), THP1 cells (b) and HL-60 cells (c). (B) Secreted protein levels of S100A2 in Jurkat cells and HL-60 cells after LPS treatment. Jurkat cells were incubated with or without LPS (100, 300, or 1000 ng/ml) for 3 h or 24 h (a) and HL-60 cells were incubated with or without LPS (30 or 100 ng/ml) for 24 h (b). A total 25 μg of proteins extracted from the media were used to measure S100A2 protein levels by an ELISA.

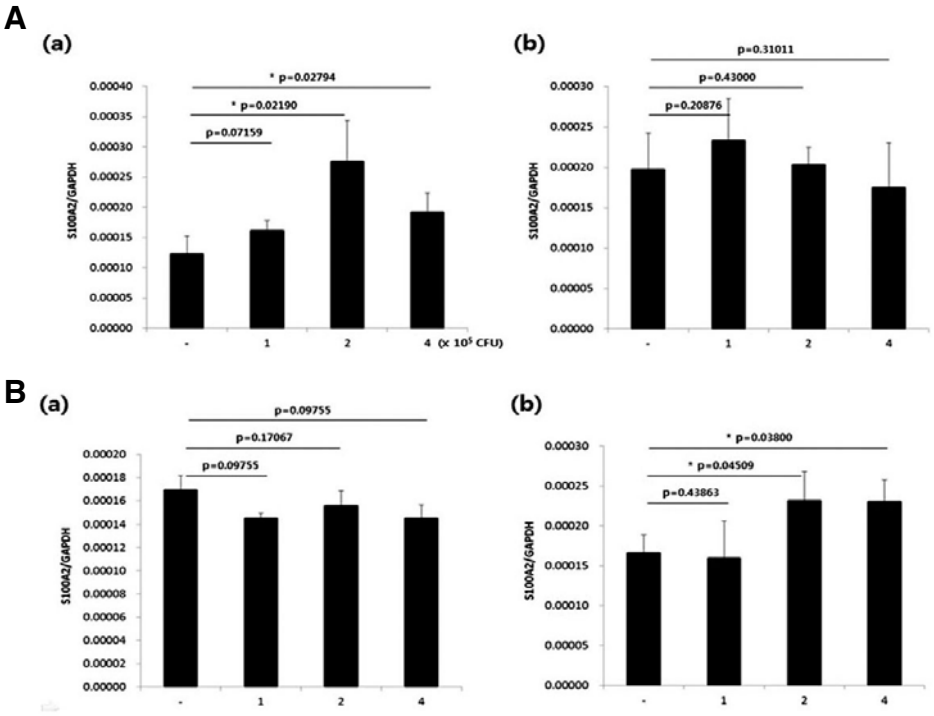

Fig. 4. S100A2 levels increased by *Streptococcus mutans* and *Streptococcus mitis* stimulation in HL-60 cells (A) and Jurkat cells (B). The mRNA expression of S100A2 in *Streptococcus mutans* (a) and *Streptococcus mitis* (b) stimulated cells. The mRNAs were extracted from immune cells after *S. mutans* treatment or *S. mitis* at the indicated concentrations (1, 2, 4×10^7 CFU) for 3 h. S100A2 mRNA expression levels were evaluated by the Ct value of S100A2 over the Ct value of GAPDH as an internal standard.

elevated in 300 ng/ml or 200 ng/ml LPS-treated cells compared with untreated cells (Figs. 3A(a) and (b)). However, S100A2 mRNA expression was decreased in a dose-depen-dent manner following LPS stimulation in HL-60 cells (Fig. 3A(c)).

In addition, S100A2 secretion levels were also evaluated in Jurkat cells and HL-60 cells after LPS stimulation. Our proteomics study identified the S100A2 in the GCF proteome, and its presence in GCF was confirmed in this study (Fig. 2B). Secretion of S100A2 protein was slightly higher in LPS-treated cells compared with untreated cells at 3 h and 24 h, as determined by ELISA (Fig. 3B). Secretion of S100A2 in two cell types was increased by LPS treatment.

S100A2 mRNA expression by *Streptococcus mutans* and *Streptococcus mitis* treatment

Since the S100A2 mRNA expression can be different depending on the stimulants and immune cell types, S100A2 mRNA expression was confirmed in other HL-60 immune cells by introduction of microorganism inhabiting in mouth. Although S100A2 mRNA expression was not affected by introduction of *S. mitis* in HL-60 cells, S100A2 mRNA expression was significantly increased by *S. mutans* at 2 and 4×10^7 CFU treatment (Fig. 4A). Conversely, in Jurkat cells, S100A2 mRNA expression was significantly higher at 2 and 4×10^7 CFU treatment of *S. mitis* than untreated group (Fig. 4B). These results showed that S100A2 mRNA expressions are different depending on cell types and microorganism types.

DISCUSSION

In the U.S., half of adults suffer from chronic periodontitis, and about 10% of the population is at risk of developing destructive severe periodontitis (Albandar, 2002). In Korea, the prevalence of periodontal disease is reported about 50% for people over 50 years old (2009 National health statistics, Ministry of Health & Welfare, Republic of Korea, ISSN 2005-6362). Although the prevalence of periodontal disease in Korea has gradually decreased from 2007 to 2009, it is still higher than in the UK (2009 Adult Dental Health) and U.S. (NHANES 2005-2008). There are several periodontitis biomarkers that could aid in diagnosing, treating and monitoring periodontitis. Our proteomics study identified the S100A2 in the GCF, which previously has not been linked to periodontitis. In this study, we showed the protein and mRNA levels of S100A2 in both GCF and gingival tissues related to classified periodontal diseases, and demonstrated that mRNA expression and secreted protein levels of S100A2 were elevated by LPS and microorganism.

S100A2 is designated as S100L and CaN19. S100A2 was originally isolated from bovine lung and has been known to be primarily expressed in bovine kidney and lung tissues (Glenney et al., 1989; Marenholz et al., 2004). S100A2 binds 2 Ca^{2+} and 2 Zn^{2+} ions; Zn^{2+} binding to S100A2 decreases its Ca^{2+} affinity. S100A2 also interacts with p53 in a Ca^{2+}-dependent manner, which affects stability of S100A2-p53 interaction (Mueller et al., 2005). In addition, the interaction of S100A2 with tropomyosin regulates the interaction between F-actin and tropomyosin in microvilli (Gimona et al., 1997; Mandinova et al., 1998), and S100A2 functions as a chemoattractant in eosinophils (Komada et al., 1996). Although S100A2 is upregulated in non-small cell lung carcinomas and esophageal squamous carcinoma, it is considered a tumor suppressor because it is down-regulated in many cancers, including malignant melanoma, prostate cancer, breast cancers, lung cancers and oral cancers (Salama et al., 2008). In this study, we showed that S100A2 was expressed in gingival tissues, gingival crevicular fluid (GCF) and immune cells. In addition, S100A2 was differentially expressed in classified periodontitis groups (Figs. 1 and 2), and S100A2 expression was upregulated in LPS-stimulated Jurkat cells and THP1 cells (Fig. 3) and in microorganism-stimulated HL-60 cells and Jurkat cells (Fig. 4). These results suggest that S100A2 plays a

role in the immune response related to periodontitis.

Unlike S100A1 and S100A4, which are predominantly located in the cytosol, S100A2 is primarily known to be located in the cell nucleus (Mandinova et al., 1998) and has also been shown to be secreted (Komada et al., 1996; Nagy et al., 2001). In this study, S100A2 secretion was detected in the GCF and in immune cells (Jurkat cells) too by ELISA (Figs. 2B and 3B). S100A2 mRNA expression was also confirmed in 14 normal and 88 classified periodontitis samples. The expression levels of S100A2 mRNA were significantly higher in the periodontal disease groups than in the normal group, which suggests that S100A2 could be a potential biomarker for periodontal disease, similar to S100A8 (Fig. 1). However, secretion levels of S100A2 in GCF should be further investigated with a larger sample size to validate this protein as a useful biomarker for periodontitis.

In conclusion, our results show the expression patterns of S100A2 in gingival tissues and GCF in classified periodontal disease and upregulation of S100A2 in LPS-stimulated Jurkat cells. The mRNA expression of S100A2 was significantly higher in periodontal disease groups than in the normal group. In addition, S100A2 expression was upregulated by LPS and microorganism inhabiting in mouth. These results suggest that S100A2 functions in the inflammatory response related to periodontitis, similar to S100A8 and S100A9, and can serve as a potential periodontitis biomarker.

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