S100A4 and S100A8 are involved in the pathogenesis of periapical granulomas

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Contents

This thesis is composed by an article ahead of print and an additional data listed below.

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2. S100A8 mRNA expression in periapical granulomas suggesting the possibility of involvement in the pathogenesis of periapical granulomas.

Abstract

Purpose: Calcium-binding proteins (termed S100 proteins) are involved in the pathogenesis of various inflammatory diseases. Of the S100 proteins, the expression of S100A4 and S100A8 is associated with the pathology of chronic inflammatory diseases. Herein, the role of two S100 proteins (S100A4 and S100A8) and four inflammatory mediators (interleukin (IL)-1β, inhibitor of nuclear factor-kappa B (IκB), IL-10, and tumor necrosis factor (TNF)-α) in human periapical granulomas (PGs) was investigated.

Methods: S100A4 expression in PGs obtained by apicoectomy was examined using immunohistochemistry. The expression of S100A4, S100A8, and the four inflammatory mediators was compared between PGs and healthy gingival tissues (HGTs) using real-time polymerase chain reaction (PCR). Correlations in mRNA expression among S100A4, S100A8, and IL-1β, IκB, IL-10, and TNF-α were investigated using Pearson's correlation coefficients.

Results: In the PGs, immunohistochemical analysis demonstrated that S100A4 was expressed by endothelial cells and fibroblasts; none of the inflammatory cells in HGTs showed S100A4 expression. Real-time PCR detected the expression of S100A4, S100A8, IL-1β, IκB, IL-10, and TNF-α mRNA in PGs and the expression levels were significantly higher than in HGTs. A positive correlation occurred between S100A4 and S100A8. Positive correlations between the expression of two S100A proteins and IL-1β or TNF-α

were observed; correlations between the expression of two S100 proteins (S100A4 and S100A8) and IκB or IL-10 were not detected.

Conclusion: The expression of S100A4 and S100A8 may be correlated with the pathogenesis of PGs in association with IL-1β and TNF-α expression.

Introduction

Periapical granulomas (PGs), a type of refractory periapical periodontitis, arise in response to the presence of oral bacteria and their products [1,2]. Treatment of apical periodontitis eliminates the source of root canal infection that causes lesions of the apical periodontal tissue, and it has a high success rate [3]. However, despite proper treatment by the dentist, apical periodontitis sometimes may not heal, and tooth extraction may be required [3].

Cytokines and growth factors play an important role in the development of periapical lesions and are associated with tissue damage [4]. Notably, these inflammatory mediators increase lymphocyte and osteoclast activation and inflammatory cell migration in the lesions [5-7]. Although various studies have investigated the mechanisms underlying the development of periapical periodontitis [1-7], several details remain unclear. It is expected that the elimination of inflammatory mediators may help to heal inflammation. Thus, the elucidation of the pathology of apical periodontitis could be useful for endodontic treatment.

Calcium-binding protein S100 (termed S100 proteins) family has 25 subclasses and is expressed only in vertebrates [8,9]. A protein from this family is known to be secreted by tumor cells and stromal cells, wherein it supports tumorigenesis by inducing angiogenesis [9,10]. S100 proteins exhibit a cell-specific expression pattern and are

involved in regulating different processes, such as cell proliferation and differentiation, apoptosis, and inflammation [11]. Furthermore, recent studies have reported that S100 expression is associated with chronic inflammatory diseases, such as rheumatoid arthritis and Crohn's disease, and that S100 proteins play a role in determining the pathologies of diseases [9,12-15]. A prior study has shown that S100A4 is involved in metastasis and is a major player in the transition from benign growth to malignancy [16]. Additionally, it has been suggested that S100A4 plays an essential role in the induction of chronic inflammation [17]. Furthermore, S100A4 downregulation suppresses bone resorption in periodontal diseases [18-20].

In autoimmune diseases, the expression of interleukin (IL)-1β, tumor necrosis factor-α (TNF-α), and IL-10 in mononuclear cells is associated with S100A4 expression [21-24]. The binding of S100A4 to the receptor for advanced glycation end products activates nuclear factor-kappaB (NF-κB) and mitogen-activated protein kinase pathways, thereby promoting tumor cell invasion and survival [25]. S100A4 also impairs osteoblast-mediated calcification, thereby inhibiting new bone formation and causing an imbalance in bone homeostasis [26]. Via activation of NF-κB, S100A4 affects the calcification function of osteoblasts, inhibits the formation of new bone, and causes an imbalance in bone homeostasis [26]. Conversely, it has been reported that S100A8, which is also part of the S100 protein family, is associated with various types of inflammation [27].

Lipopolysaccharide (LPS) activates the caspase-4 and -5 inflammasomes to promote S100A8 secretion from macrophages. From this perspective, a marked increase in S100A8 and S100A9 levels was observed in typhoid fever patients [28]. S100A8 has also been reported to be expressed in inflammatory murine arthritis [29]. In addition, it has been reported that S100A8 is upregulated by LPS, TNF, and IL-1 and recruits leukocytes, such as neutrophils, during inflammation [30]. Nishimura et al. reported that S100A8 is expressed in cultured human periodontal ligament (PDL) cells and induces autocrine chemotactic activity in PDL cells [31]. Interestingly, S100A4 is an upstream regulator of S100A8 [15,32]. Thus, S100A4 and S100A8 could delicately regulate the pathophysiology of inflammation.

Inhibitor κB (IκB), bound to the NF-κB dimer, is phosphorylated and degraded by the IκB kinase (IKK) complex; as a result, NF-κB is activated and translocated to the nucleus [33-35]. Cilmiaty et al. previously reported that NF-κB is expressed in periapical granulomas and may be associated with its progression [36]. Thus, it was speculated that IκB phosphorylated by IKK separates NF-κB and promotes the translocation of NF-κB to the nuclei of inflammatory cells in PGs. Therefore, IκB levels could be indicative of NF-κB activity [37].

On the basis of these observations, it was hypothesized that S100A4 and S100A8 are expressed in inflamed periapical tissues and may be involved in the pathogenesis of

PGs. The aim of this study was to verify the role of S100A4 and S100A8 in PGs and in healthy gingival tissues (HGTs).

Materials and Methods

Sample Collection

The Ethics Committee of the Nihon University School of Dentistry approved the experimental protocol of this study (EP18D014), and all patients provided written informed consent for any discomfort during the treatment and for the publication of findings. The study was conducted in accordance with the tenets of the Declaration of Helsinki. Periapical periodontitis samples were obtained from patients (n = 44; 26 men and 18 women; age, 32-64 years) during endodontic surgery and tooth extraction upon ordinary dental treatments. No systemic disease was observed in the patients. Additionally, the patients did not report consuming any medications (for six months prior to the endodontic surgery) that could affect the inflammatory responses. HGTs were obtained when impacted wisdom teeth were extracted from patients at the Department of Oral Surgery (n = 5; 3 men and 2 women; age, 25-37 years).

Preparation of Tissue Sections

The samples were cut in two parts with similar dimensions immediately after the periapical lesions were excised. One half of the divided tissues was fixed with 10% neutral-buffered formalin, and paraffin sections (4 μm thick) were prepared. The other half of the tissue samples was fixed with 4% paraformaldehyde prepared in phosphate-

buffered saline (PBS), embedded in OCT compound (Tissue-Tek, Elkhart, IN, USA), and frozen in dry ice-acetone. These samples were then used for RNA preparation. HGT samples were prepared using the same method.

Histological Examination

Hematoxylin-eosin (HE) staining was performed to investigate tissue histopathology. After staining, three different fields in each tissue section were examined under a light microscope (Olympus BX60, Tokyo, Japan).

Immunohistochemistry

Paraffin sections were subjected to immunohistochemistry to identify S100A4-expressing inflammatory cells in human PGs. The paraffin sections were first treated for 30 min at room temperature (RT) with 0.3% H_2O_2 prepared in methanol to inactivate endogenous peroxidase, and non-specific binding was blocked by incubation in 1.5% normal horse serum (Vector Laboratories, Burlingame, CA, USA) for 90 min. The sections were probed with a rabbit anti-human S100A4 monoclonal antibody (1:100; Abcam, Cambridge, UK) in a humid atmosphere at RT for 2 h. Then, the sections were washed with PBS and incubated with biotinylated horse anti-rabbit IgG antibody (1:500; Vector Laboratories) at RT for 30 min, followed by incubation with the

avidin:biotinylated enzyme complex (Vector Laboratories) for 30 min. Optimum color development of positive cells was achieved by incubating the samples with 3,3′ -diaminobenzidine (DAB) substrates (Vector Laboratories) for 1 min. Immunohistochemistry on HGT sections (negative control) was performed using the same technique as that used for the PG tissues.

RNA Extraction and Real-time PCR

Total RNA was extracted from frozen PG tissues and HGT using 1 mL TRIzol (Invitrogen, Carlsbad, CA, USA) and was converted to complementary DNA (40 ng) using Takara Prime Script (Takara Bio, Shiga, Japan). Next, real-time PCR analysis was performed in 25 μL reaction mixtures containing TB Green Premix Ex *Taq* (Takara Bio) and the sense and antisense PCR primers (20 μmol/L, each; Table 1) against human S100A4, S100A8, IL-1β, IκB, IL-10, TNF-α, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Real-time PCR was performed on a Smart Cycler (Cepheid, Sunnyvale, CA, USA) in accordance with the manufacturer's protocols. The expression of target genes was normalized to that of GAPDH.

Statistical Analysis

BellCurve for Excel version 2.12 (Social Survey Research Information Co., Ltd.,

Tokyo, Japan) was used for statistical analysis. The expression of S100A4, S100A8, IL-1β, IκB, IL-10, and TNF-α in PGs and HGTs was statistically analyzed using Mann-Whitney *U* test. *P*-values <0.05 were considered significant. Pearson's correlation coefficient (R^2) was used to verify the correlation between S100A4 and S100A8 expression and IL-1β, IκB, IL-10, and TNF-α. *P*-values <0.05 were considered significant. R^2 >0.04 or R^2 <0.04, between the targets showed positive or negative correlation, respectively.

Results

Histological Evaluation of Periapical Lesions and HGTs

To define the pathological features of the periapical lesions (n = 44), the paraffin sections were evaluated using HE staining. Of the 44 periapical lesions examined by light microscopy, 32 contained granulomatous tissues characterized by infiltration of numerous inflammatory cells, such as lymphocytes, macrophages, and plasma cells (Figure 1A). No epithelial cells were detected in any fields of these samples, and the specimens were diagnosed as PGs. The remaining 12 lesion sections exhibited granulomatous tissues with complete epithelial lining and surrounding collagen fibers (Figure 1B). These specimens were diagnosed as radicular cysts (RCs) and were excluded from this study. HGTs contained more collagen fibers beneath the epithelial cell layers and fewer inflammatory cells than the periapical lesions (Figure 1C).

Immunohistochemistry

S100A4-positive endothelial cells, fibroblasts, and lymphocytes were observed in the human PG sections. Additionally, S100A4 was expressed in the lymphocytes present in the blood vessels of PGs (Figure 2A). S100A4 expression was not detected in HGTs (Figure 2B).

Real-time PCR Analysis

To detect S100A4, S100A8, IL-1β, IκB, IL-10, and TNF-α mRNA expression in PGs and HGTs, all frozen tissues (32 PGs and 5 HGTs) were examined using real-time PCR analysis. All specimens expressed the mRNAs of S100A4, S100A8, and the four inflammatory mediators (Figure 3). The HGT samples also expressed the mRNAs of S100A4, S100A8, and the four inflammatory mediators; however, the expression levels were significantly lower than those in PGs ($p < 0.01$).

The correlations between S100A4, S100A8, IL-1β, IκB, IL-10, and TNF-α mRNA expression were then analyzed using Pearson's correlation coefficient. Positive correlations between the expression of S100A4 and IL-1β ($p = 0.0055$, $R^2 = 0.2704$) and that of S100A4 and TNF-α ($p = 7.17E\text{-}5$, $R^2 = 0.409$) were observed (Figure 4 A, D). However, no correlations between the expression of S100A4 and IκB ($p = 0.8283$, $R^2 = 0.0016$) and that of S100A4 and IL-10 ($p = 0.2784$, $R^2 = 0.0389$) were observed (Figure 4 B, C). In addition, positive correlations between the expression of S100A8 and S100A4 ($p = 0.0436$, $R^2 = 0.1282$), IL-1β ($p = 0.0181$, $R^2 = 0.1957$), or TNF-α ($p = 0.0239$, $R^2 = 0.1578$) were found (Figure 5 A, B and E). However, no correlations between the expression of S100A8 and IκB ($p = 0.1057$, $R^2 = 0.0297$) and that of S100A8 and IL-10 ($p = 0.0308$, $R^2 = 0.2784$) were observed (Figure 5 C, D).

Discussion

The purpose of this study was to clarify the functions of S100A4 and S100A8 in periapical periodontitis, which is an inflammatory disease associated with bone resorption, similar to rheumatoid arthritis and periodontitis.

In this study, the expression of S100A4 in human PGs, but not in HGTs, was demonstrated via immunohistochemistry. S100A4 is known to contribute to cartilage destruction and the pathogenesis of chronic inflammations [26]. Moreover, stimulation by an inflammatory cytokine, IL-1β, results in the overexpression of S100A4 and promotes the progression of periodontitis [20]. These findings suggest that S100A4 expression may be involved in the pathogenesis of human PGs.

In PGs, the expression of all target genes tested in this study was significantly higher than that in HGTs. Various studies have shown that IL-1β and IL-10 induce the up-regulation of S100A8 expression *in vitro* [38,39]. Kyang et al. have described that NF-κB is activated by S100A8 in lung epithelial cells [40]. Furthermore, it has been demonstrated that S100A8 expression is strongly induced by TNF-α and IL-1 [41]. Thus, S100A8 may be involved in the pathogenesis of PGs, in association with these inflammatory mediators.

To clarify the interactions of S100A4, S100A8, and the four inflammatory mediators, the correlations among the expression of the 6 mRNAs in PGs were verified using

Pearson's correlation coefficient analysis. A positive correlation was observed between the expression of S100A4 and S100A8. In addition, positive correlations were observed between the expression of S100A4 or S100A8 and IL-1β or TNF-α. Recent studies have reported that the IL-1β-induced matrix metalloproteinase (MMP)-13 expression is upregulated by S100A4, and the absence of S100A4 expression inhibits bone metabolism by reducing MMP-3 and MMP-9 expression [18,42]. Furthermore, IL-1β stimulation induces S100A4 expression, resulting in the suppression of bone formation, and enhances matrix degradation in periodontal models [20]. Peripheral blood mononuclear cells stimulated by S100A4 induce the expression and synthesis of TNF-α and IL-1β [22]. Previous study reported that *Porphyromonas gingivalis*-derived LPS in combination with TNF-α, and IL-1β induces S100A8 production from human monocytes [43], suggesting that these inflammatory mediators induce S100A8 expression and contribute to inflammation in PGs. S100A4 is also an upstream regulator of the well-known inflammation-associated member of the S100 family protein, S100A8 [15,32], and S100A4 may upregulate S100A8 expression in PGs.

The results of this study indicate that S100A4 and S100A8 are expressed in PGs through stimulation of the inflammatory cytokine IL-1β. Furthermore, S100A4 may promote TNF-α expression, thereby exacerbating the inflammatory response in PGs. Additionally, IL-1β and TNF-α expressed by S100A4 may be associated with S100A8

expression in PGs. Thus, S100A4 and S100A8 may be intricately involved in the pathogenesis of PGs. It is proposed that the administration of S100A4 and S100A8 inhibitors, using local drug delivery systems, during root canal treatments may facilitate tissue healing and prevent disease progression.

Conclusion

Based on these results, it was concluded that S100A4 and S100A8 could be interrelated with and involved in the pathophysiology of PGs, in association with IL-1β and TNF-α expression.

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Table.1 PCR primers used to detect mRNA for S100A4, S100A8, IL-1β, IκB, IL-10, TNF-α, and GAPDH in PGs and HGTs.

Gene	Primer sepuence	GenBank accession number
S100A4	Forward 5'- GTGTCCACCTTCCACAAATACTCA - 3' Reverse 5'- ACTTCATTGTCCCTGTTGCTGTC -3'	NM_002961
S100A8	Forward 5'- GACCGAGCTGGAGAAAGCCTTGA - 3' Reverse 5'- CCAGACGTCTGCACCCTTTTTCC - 3'	NM_001319198
IL1-*β*	Forward 5'- CCAGGGACAGGATAGGAGCA - 3' Reverse 5'- TTCAACACGCAGGACAGGTACAG - 3'	NM_000576
IκB	Forward 5'- CCAGTTGCAGGTGGCCTATC - 3' Reverse 5'- CTGCTGGAGCTGCTGTTTGAG -3'	NM_1099857
IL-10	Forward 5'- GAGATGCCTTCAGCAGAGTGAAGA - 3' Reverse 5'- AGTTCACATGCGCCTTGATGTC - 3'	NM_000572
TNF-*α*	Forward 5'- CTGCCTGCTGCACTTTGGAG - 3' Reverse 5'- ACATGGGCTACAGGTTGTCACT -3'	NM_000594
GAPDH	Forward: 5' -GCACCGTCAAGGCTGAGAA - 3' Reverse: 5' - ATGGTGGTGAAGACGCCAGT - 3'	NR_152150

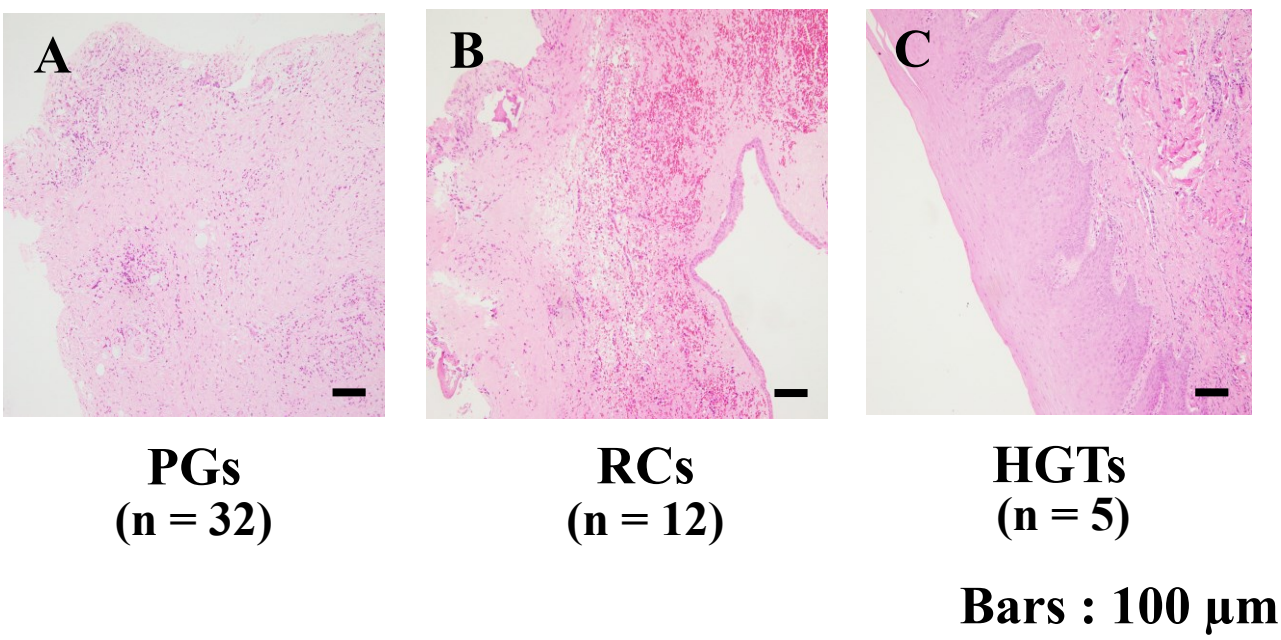

Figure 1 Histological examination of periapical lesions and HGTs stained by hematoxylin and eosin. (A) PGs showing rich blood vessels and inflammatory cell infiltration; (B) radicular cysts with epithelial cell lining; and (C) HGTs (bars = 100 µm, A-C).

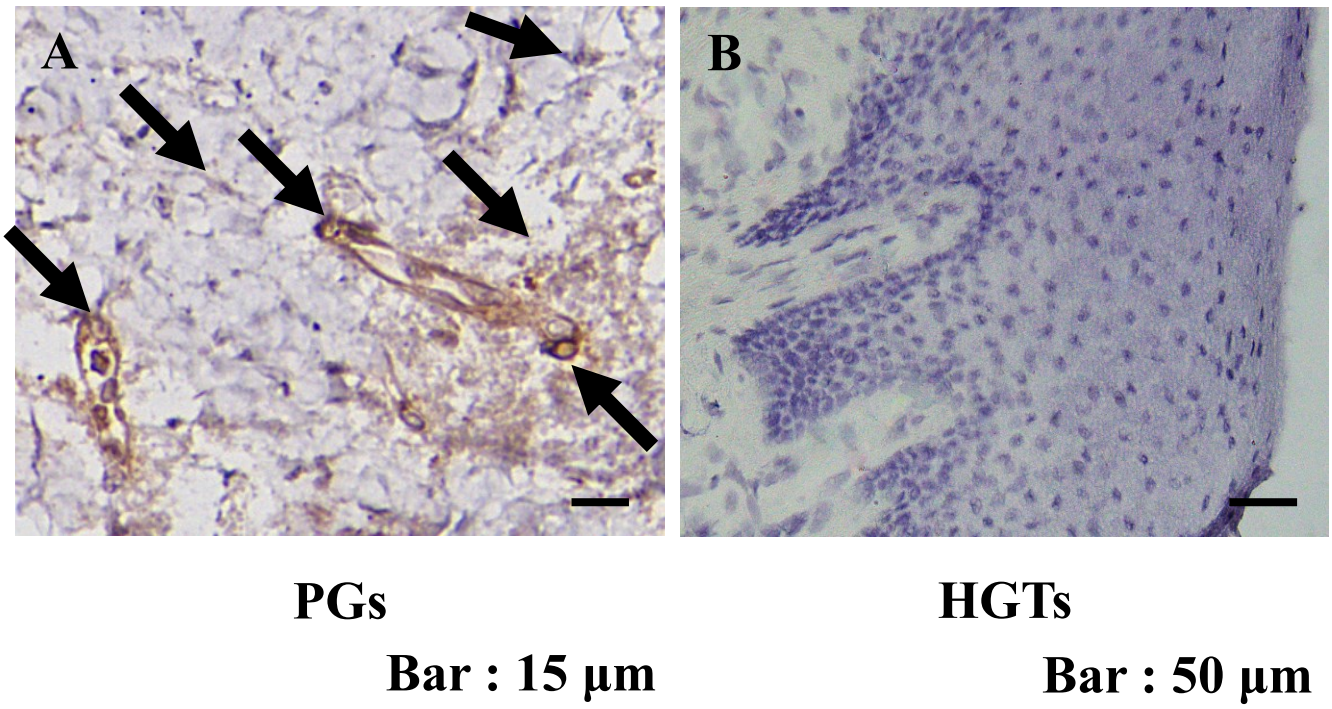

Figure 2 Immunohistochemical analysis using human S100A4 antibody. (A) S100A4-positive endothelial cells, fibroblasts and lymphocytes were seen in human PGs. The arrows indicate S100A4 expressing cells (bar = 15 μm). (B) S100A4 expression was not seen in HGTs (bar = 50 μm).

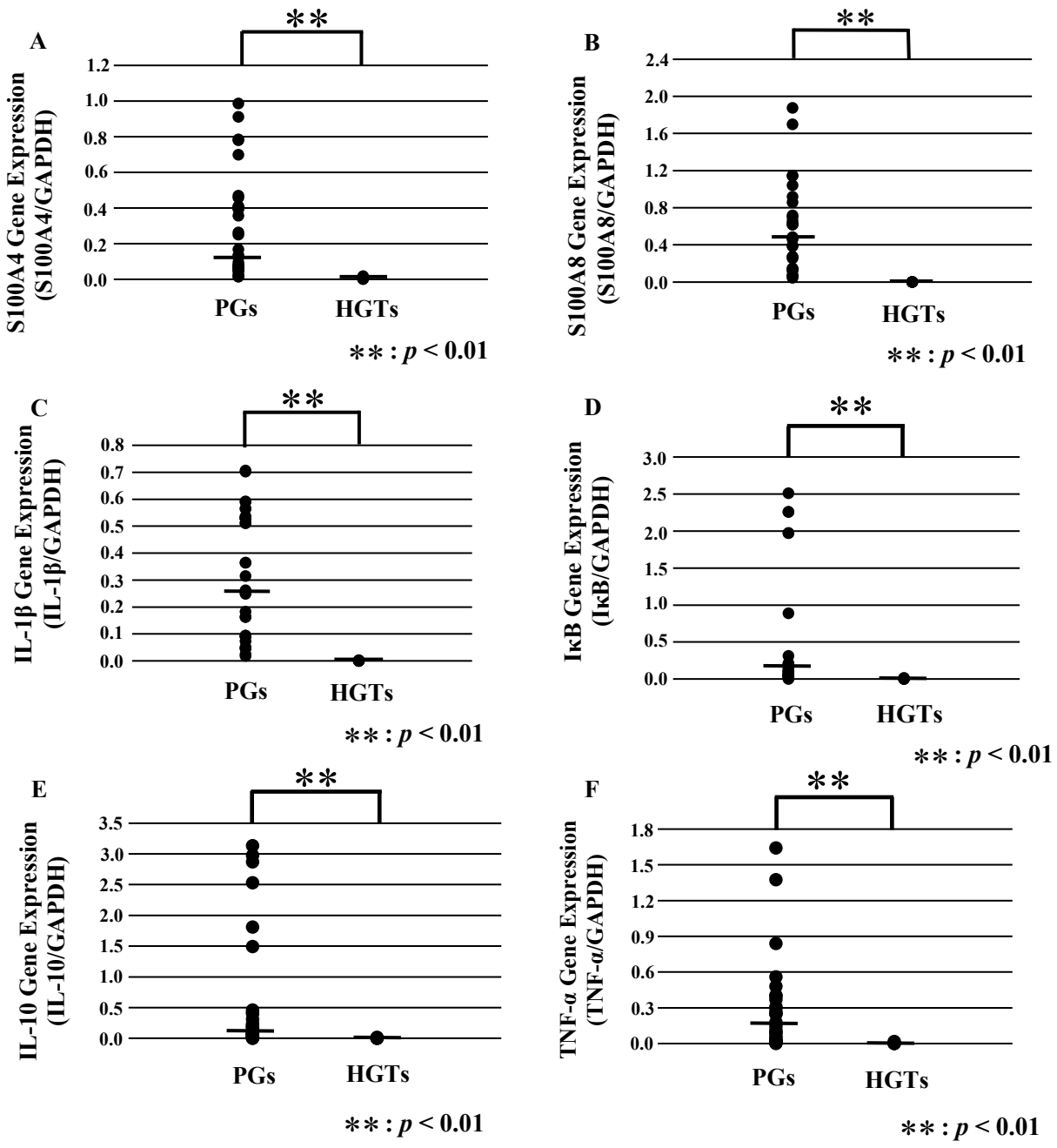

Figure 3 Real-time PCR analysis for detecting S100A4, S100A8, IL-1β, IκB, IL-10, and TNF-α mRNA expression in PGs and HGTs.
(A-F) Expression levels of S100A4, S100A8, IL-1β, IκB, IL-10, and TNF-α in PGs and HGTs. mRNA expression was normalized to GAPDH expression. Horizontal black bars indicate the median. The expression levels of all mRNAs in HGTs were significantly lower than those in PGs. **, $p < 0.01$ (Mann–Whitney *U* test).

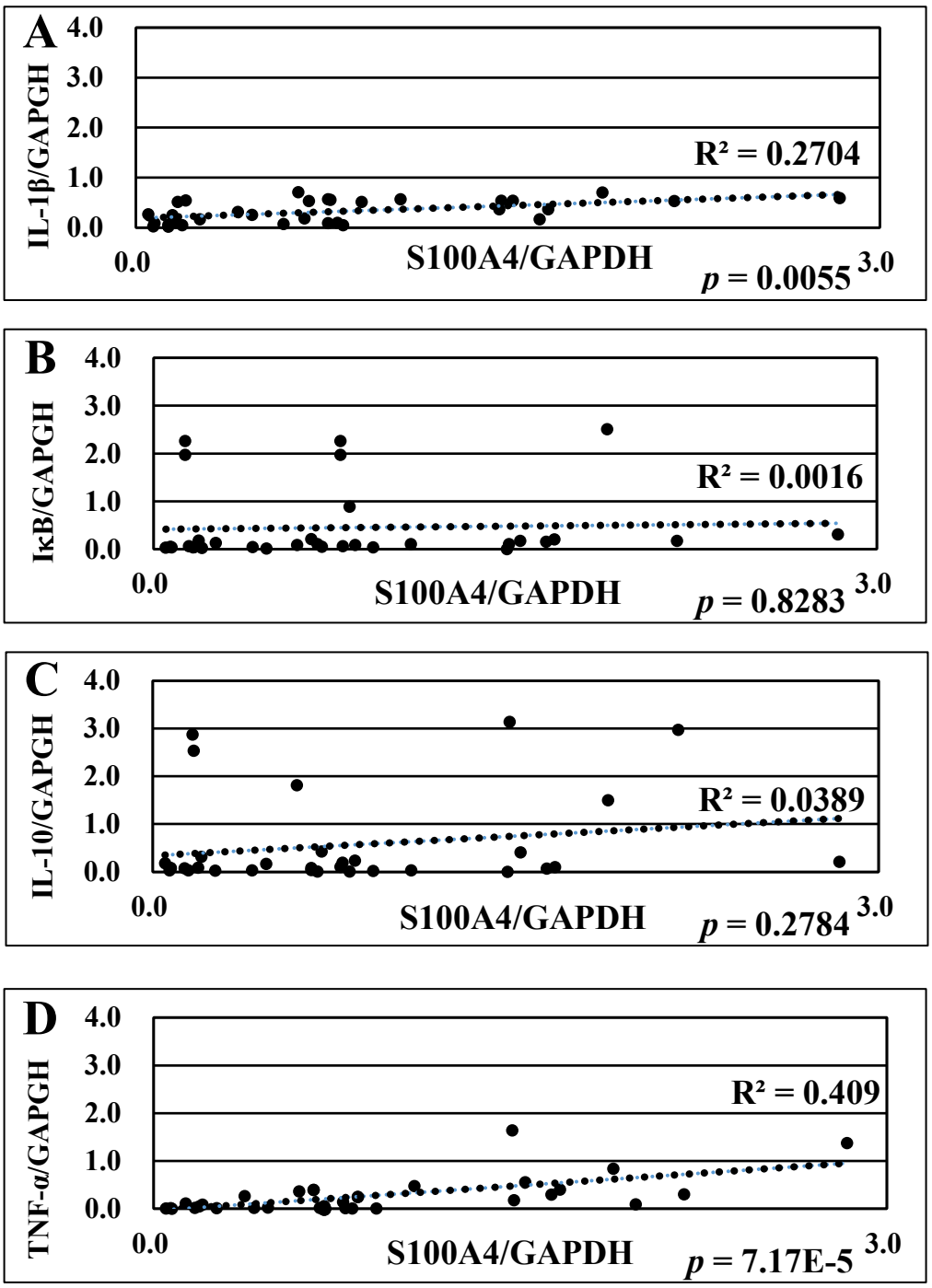

Figure 4 Pearson's correlation coefficient analysis.
Correlations of S100A4 mRNA expression with IL-1β, IκB, IL-10, and TNF-α expression in human PGs were analyzed. (A) Correlation between the expression of S100A4 and IL-1β, (B) S100A4 and IκB, (C) S100A4 and IL-10, (D) S100A4 and TNF-α .

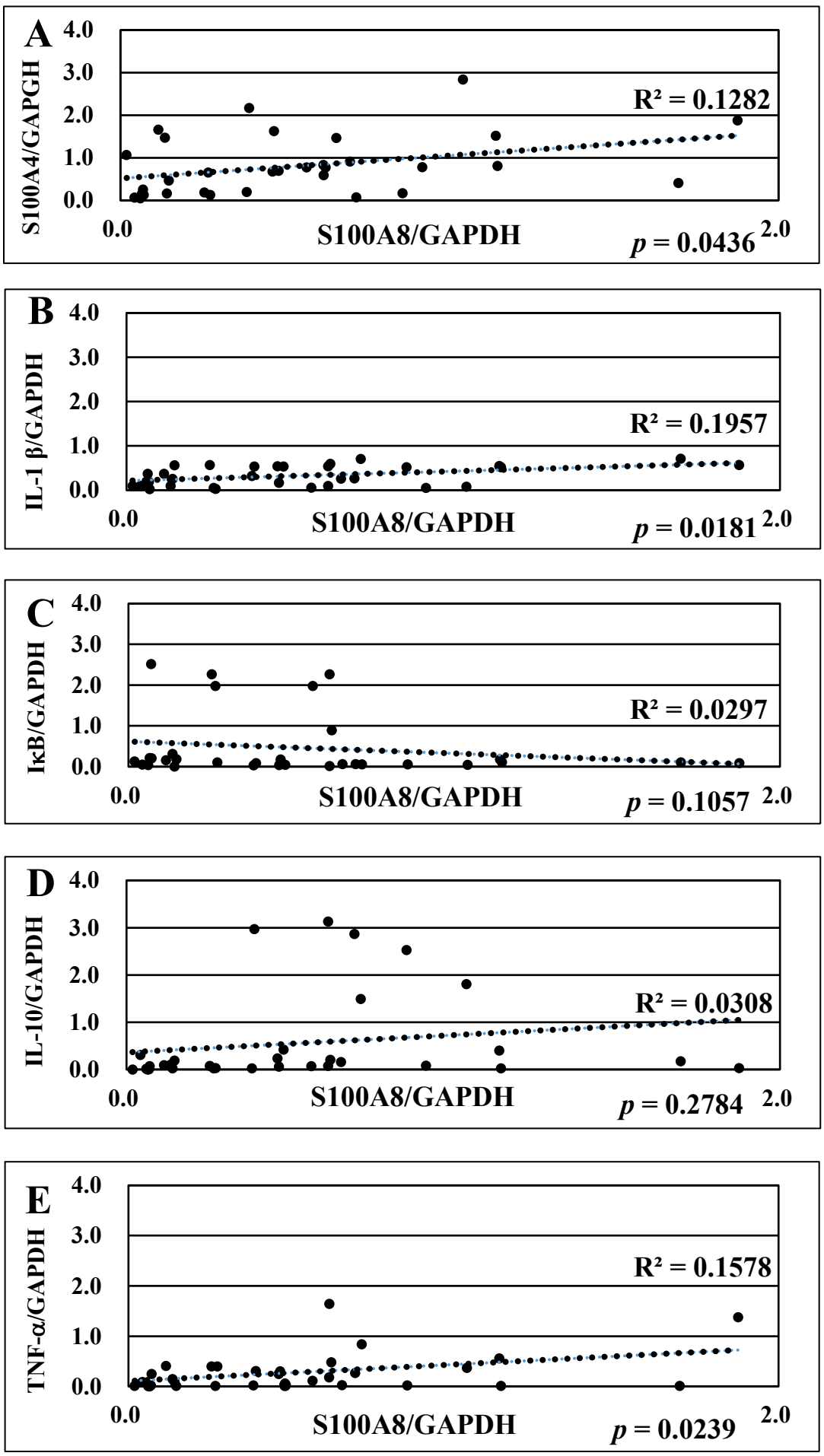

Figure 5 Pearson's correlation coefficient analysis of S100A8 to S100A4, IL-1β, IκB, IL-10, or TNF-α mRNA expression in human PGs.
(A) Correlation between the expression of S100A4 and S100A8, (B) S100A8 and IL-1β, (C) S100A8, and IκB, (D) S100A8 and IL-10, (E) S100A8 and TNF-α .