Original Article

Int J Oral Biol 44:182-190, 2019
pISSN: 1226-7155 • eISSN: 2287-6618
https://doi.org/10.11620/IJOB.2019.44.4.182

International Journal of Oral Biology
IJOB
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Apelin-APJ axis inhibits TNF-alpha-mediated expression of genes involved in the inflammatory response in periodontal ligament cells

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Periodontitis is an inflammatory disease of the supportive tissues surrounding the teeth, and is characterized by irreversible destruction of the gingiva, periodontal ligament (PDL), and alveolar bone, which results in the loss of teeth. In the present study, we elucidated the correlation between periodontitis and apelin (APLN), an adipokine and a regulatory peptide, respectively, which are involved in inflammation and bone remodeling. The expression of *APLN* is negatively correlated with periodontitis progression in gingival tissue. In addition, treatment with TNF-α downregulated the expression of *APLN* in PDL cells and gingival fibroblasts, indicating the protective role played by *APLN* against periodontitis progression. The overexpression of *APLN* or treatment with exogenous *APLN* suppressed the TNF-α-mediated catabolic gene expression of *MMP1*, *IL6*, and *PTGS2* in PDL cells. Moreover, the inhibition of the APLN-APJ axis by ML221, an APJ inhibitor, induced catabolic gene expression in PDL cells. Thus, the results of this study provided evidence to support APLN as a regulatory factor of the inflammatory response during periodontitis.

Keywords: Apelin, APLN, Periodontal ligament, Periodontitis, Inflammation

Introduction

Periodontitis is an inflammatory disease of the supportive tissues surrounding the teeth and is caused by infection of microorganisms such as *Porphyromonas gingivalis* and *Fusobacterium nucleatum* resulting in the loss of teeth [1]. Periodontitis affects 40–90% of the global population, making it one of the most common diseases in the world [2]. The representative symptom of periodontitis is irreversible destruction of the periodontium, which is composed of the gingiva, cementum, periodontal ligament, and the alveolar bone [3]. Periodontal pathogens such as lipopolysaccharides (LPS) are released from *P.gingivalis* and elicit an inflammatory response that is characterized by the production of pro-inflammatory factors in gingival fibroblasts (GFs) and periodontal ligament (PDL) cells, the resident cells of the periodontium. These factors include tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, IL-8, and chemokines [4]. Matrix metalloproteinase (MMP) family, matrix-degrading enzymes are also released and activated from inflamed GFs and PDL cells resulting in the degradation of the gingiva and PDL tissues [5-7]. LPS-induced inflammatory response also induces infiltration of various immune cells into the periodontium [8]. Alveolar bone destruction and teeth loss are also induced by accelerating osteoclastogenesis which is induced by pro-inflammatory cytokines and receptor activator of nuclear factor kappa-B ligand expressed from GFs, PDL

Received November 11, 2019; **Revised** December 17, 2019; **Accepted** December 18, 2019
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cells, and infiltrated immune cells [9,10]. Though decades of research, periodontitis is recognized as a systemic disease that affects other diseases such as diabetes [11], hypertension [12], cardiovascular disease [13], chronic kidney disease [14–16], and alzheimer's disease [17,18]. Recent studies have shown that metabolic syndromes including obesity and diabetes have been significantly related to periodontitis pathogenesis, indicating that adipokines could be a key factor in regulating pathogenesis [19]. Researchers are currently working to identify the role of adipokines in GF and PDL cells that act as essential catabolic regulators for the pathogenesis of periodontitis. There are several studies that report the regulatory role of adipokines in periodontitis pathogenesis [20–23].

Among the adipokines, apelin (APLN) is a small regulatory peptide, whose activity is mediated by a receptor named APJ, a G-protein-coupled receptor [24]. APLN-APJ axis is expressed in diverse tissues, including the gastrointestinal tract, brain, kidney, liver, lung, cardiovascular system, and adipose tissues [25]. Especially, several studies have reported the regulatory role of the APLN-APJ axis in bone homeostasis. According to these previous studies, APLN could regulate the apoptosis, proliferation, and differentiation of osteoblasts and skeletal phenotypes in mice [26–30]. In addition, many studies have indicated that the APLN-APJ axis has certain roles in inflammation-related diseases such as atherosclerosis, diabetic nephropathy, and ischemia reperfusion injury [31]. These previous reports have revealed the relevance of APLN in bone homeostasis and inflammation, the major events during periodontitis progression, allowing us to investigate the role of APLN in periodontitis. In this study, we demonstrate the decreased expression of APLN in the progression of the periodontitis, and its protective role in periodontitis by modulating the expression of catabolic factors in human PDL cells and partially in GFs.

Materials and Methods

1. Human gingival tissues

Human gingival tissues containing both epithelial and connective tissues were obtained from 16 patients (20–73 years; 40.80 ± 18.80) during tooth extraction comprising eight healthy patients for non-inflamed gingiva and eight chronic periodontitis patients for inflamed gingiva. The Institutional Review Board at the Chonnam National University Dental Hospital (Gwangju, Republic of Korea) approved this study (CNUDH-2018-003). After all procedures had been fully explained, written informed consent was obtained from each study subject. Gingival tissues were promptly maintained in liquid nitrogen and stored at −80℃ until further use.

2. Cell culture and stimulation

Human PDL cells and GFs were isolated from gingival papillary explants, obtained from clinically healthy donors with no systemic and/or periodontal disease who were informed of the purpose of this study. Briefly, after the dissection of gingival biopsies using dispase (Gibco BRL, Gaithersburg, MD, USA), the epithelial cell layer was microscopically dissected from the underlying connective tissue and GF was extracted from the subepithelial tissue as described in a previous study [32]. Pieces of PDL tissue were harvested from the middle of the tooth root and digested with 3 mg/mL of collagenase type 1 and 4 mg/mL of dispase [32]. Isolated primary human PDL cells and GFs were treated with the indicated amount of recombinant human APLN protein (MyBioSource, San Diego, CA, USA), human IL-1β (GenScript, Piscataway, NJ, USA), and human TNF-α (Merck-Millipore, Billerica, MA, USA). Primary cultured human PDL cells and GFs were infected with empty vector (Ad-C) and APLN-expressing adenovirus (Ad-APLN) for 2 hours at the indicated multiplicity of infection (MOI) and incubated for an additional 24 hours alone or in the presence of IL-1β or TNF-α. The APJ inhibitors, ML221 (Abmole BioScience, Houston, TX, USA) was added at the indicated concentration to the PDL cells. Dimethyl sulfoxide or phosphate-buffered saline was used as a vehicle.

3. RNA isolation, reverse transcription-polymerase chain reaction, and quantitative real time-PCR

Total RNA was isolated from human gingival tissues and primary cultures of human GFs and PDL cells using TRIzol reagent (Ambion, Carlsbad, CA, USA). Normal or periodontitis human gingival tissues were homogenized in TRIzol reagent using a glass tissue grinder. RNA was reverse-transcribed, and the complementary DNA was amplified by polymerase chain reaction (PCR) using *Taq* polymerase (GeneAll, Seoul, Korea). Quantitative real time (qRT)-PCR was performed using an iCycler (Bio-Rad Laboratories, Hercules, CA, USA) and the SYBR premix Ex *Taq* (Takara Bio, Shiga, Japan). All qRT-PCR was performed in duplicates, and the target gene amplification signal was normalized to that of glyceraldehyde-3-phosphate

dehydrogenase (*GAPDH*) in the same reaction. The relative levels of *APLN* (sense:5'-ATG AAT CTG CGG CTC TGC GTG-3', antisense:5'-GGA ATT TCC TCC GAC CTC CCT G-3'), *MMP1* (sense:5'-GGA GGG GAT GCT CAT TTT GAT G-3', antisense:5'-TAG GGA AGC CAA AGG AGC TGT-3'), *MMP3* (sense:5'-GAT GCG CAA GCC CAG GTG TG-3', antisense:5'-GCC AAT TTC ATG AGC AGC AAC GA-3'), *IL6* (sense:5'-GTA CAT CCT CGA CGG CAT CTC AG-3', antisense:5'-TGG CAT TTG TGG TTG GGT CAG G-3'), and *PTGS2* (sense:5'-AAT CCT AGC TGT TCC CAC CCA TG-3', antisense:5'-AAG GGA GTC GGG CCA TCA TCA GG-3') gene expression were analyzed using the comparative C_t (cycle threshold) method, as described in a previous study [22].

4. Statistical analysis

The data obtained with qRT-PCR were initially tested for confirmation of normal distribution using the Shapiro-Wilk test and subsequently analyzed with Student's t-test (pair-wise comparisons). The threshold for significance was set at $p <$

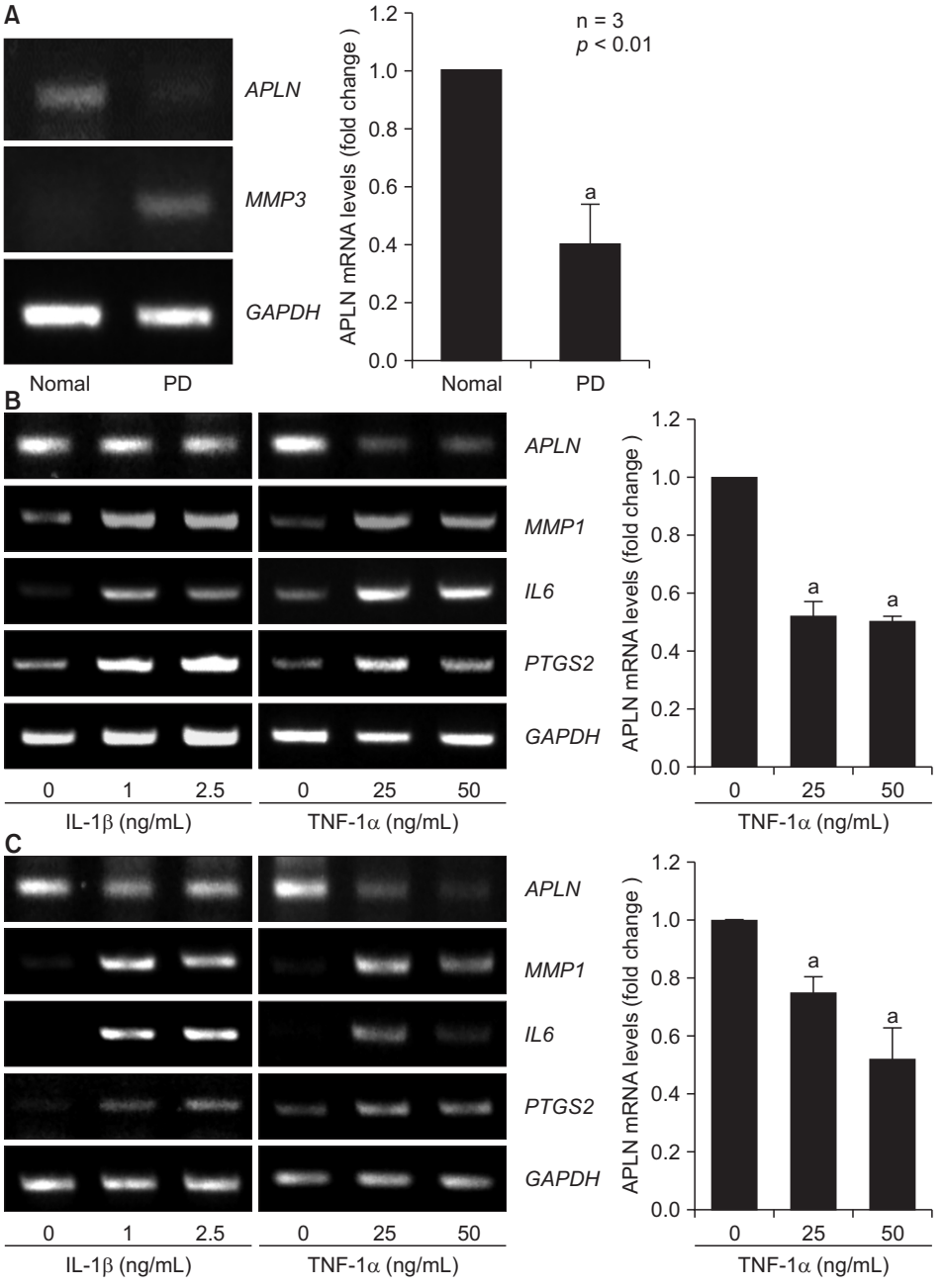

Fig. 1. Downregulated expression of APLN in human gingiva during periodontal disease progression. (A) Total RNA was extracted from whole gingival tissues of normal or periodontitis patients (PD). mRNA levels of indicated genes were determined by real time-polymerase chain reaction (RT-PCR) and relative APLN mRNA levels (compared to those of normal tissues) were quantified by quantitative real time (qRT)-PCR (n = 3). Primary cultures of human periodontal ligament (PDL) cells (B) and GFs (C) were treated with the indicated amounts of cytokines interleukin (IL)-1β and tumor necrosis factor-α (TNF-α) for 24 hours in serum-free condition. The mRNA levels of the indicated genes were detected by RT-PCR analysis. Relative APLN mRNA levels (compared to those of untreated cells) were quantified by qRT-PCR (n = 3). Values are presented as a mean ± standard error of the mean.
[a]$p < 0.05$ compared with the control.

0.05.

Results

1. Expression of *APLN* was downregulated in human gingiva affected with periodontitis

To investigate the correlation between *APLN* and periodontitis, we examined the expression levels of *APLN* in the gingiva of patients with periodontitis. In contrast to the upregulation of *MMP3* expression in inflamed gingival tissues, *APLN* expression was significantly downregulated (Fig. 1A). To confirm the downregulation of *APLN* expression in the periodontium during periodontitis, we detected the expression levels of *APLN* in human PDL cells and GFs that were treated with pro-inflammatory cytokines. Treatment with TNF-α dramatically suppressed the expression of *APLN* in both types of cells. However, IL-1β treatment could not regulate the expression of APLN in PDL cells (Fig. 1B) and GFs (Fig. 1C). The upregulation of *MMP1*, *IL6*, and *PTGS2* represents the severity of inflammation induced by treatment with pro-inflammatory cytokines. These data suggest a negative correlation between APLN and periodontal inflammation.

2. Adenoviral overexpression of *APLN* suppressed the expression of genes involved in inflammation and tissue degradation in human periodontal ligament cells

To explore the function of APLN in PDL tissue, we induced overexpression of *APLN* by adenovirus infection in PDL cells. Overexpression of *APLN* by infection of Ad-*APLN* in human PDL cells led to a marked reduction in the gene expression of *MMP1*, *IL6*, and *PTGS2* (Fig. 2A). Moreover, overexpression of *APLN* significantly suppressed the upregulation of *MMP-1*, *IL-6*, and *Cox2* mRNA levels in inflamed PDL cells treated with TNF-α (Fig. 2C). However, *APLN* overexpression only suppressed the *IL6* expression among the IL-1β-induced upregulated genes in inflamed PDL cells (Fig. 2B). These data suggest that APLN could suppress inflammation in periodontal

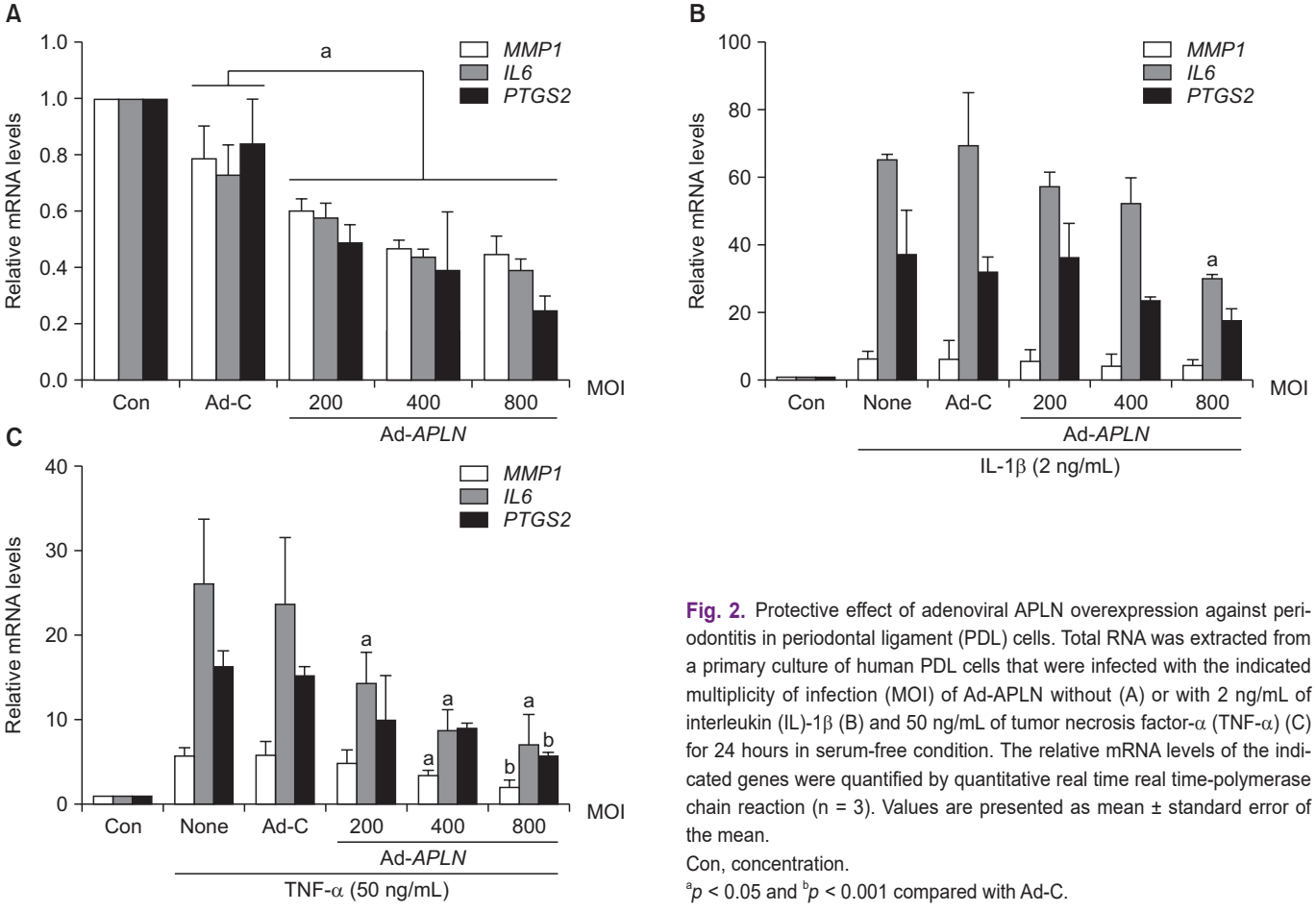

Fig. 2. Protective effect of adenoviral APLN overexpression against periodontitis in periodontal ligament (PDL) cells. Total RNA was extracted from a primary culture of human PDL cells that were infected with the indicated multiplicity of infection (MOI) of Ad-APLN without (A) or with 2 ng/mL of interleukin (IL)-1β (B) and 50 ng/mL of tumor necrosis factor-α (TNF-α) (C) for 24 hours in serum-free condition. The relative mRNA levels of the indicated genes were quantified by quantitative real time real time-polymerase chain reaction (n = 3). Values are presented as mean ± standard error of the mean.
Con, concentration.
[a]$p < 0.05$ and [b]$p < 0.001$ compared with Ad-C.

ligament tissues during periodontitis progression.

3. Adenoviral overexpression of *APLN* partially suppressed the expression of genes involved in inflammation in human gingival fibroblasts

To explore the function of APLN in gingiva tissue, we induced overexpression of *APLN* by adenovirus infection in human GFs. Contrary to the results in PDL cells, adenoviral overexpression of *APLN* in GFs reduced only the mRNA levels of *PTGS2* but not the mRNA levels of *MMP1* and *IL6* (Fig. 3A). Moreover, overexpression of *APLN* also suppressed the upregulation of *PTGS2* expression levels in inflamed GFs treated with IL-1β and TNF-α. However, the expression of *MMP1* and *IL6* induced by pro-inflammatory cytokines in GFs was not regulated by overexpression of *APLN* (Fig. 2B and 2C). These data suggest that APLN partially suppresses the inflammation of gum tissues during periodontitis progression.

4. APLN-APJ axis could regulate the expression of genes involved in inflammation and tissue degradation in human periodontal ligament cells

Based on the aforementioned results, we further validated the role of APLN-APJ axis in PDL tissues. We treated exogenous APLN or an APJ blocker, ML221, to human PDL cells. Similar to the results observed on using Ad-*APLN*, treatment with recombinant APLN led to a marked reduction in the gene expression of *MMP1*, *IL6*, and *PTGS2* in PDL cells (Fig. 4A). In addition, recombinant APLN inhibited the expression of *MMP1*, *IL6*, and *PTGS2* induced by TNF-α in inflamed PDL cells (Fig. 4C). However, IL-1β-induced expression of *MMP1*, *IL6*, and *PTGS2* was not regulated by the treatment with recombinant APLN in PDL cells (Fig. 4B). At the highest dose of recombinant APLN with TNF-α co-treatment, the expression of *MMP1* is rather increased (Fig. 4C). Although not statistically significant, this reversed expression pattern also appeared at the highest dose of recombinant APLN with IL-1β co-treatment (Fig. 4B). These data indicated the cytotoxicity or unexpected

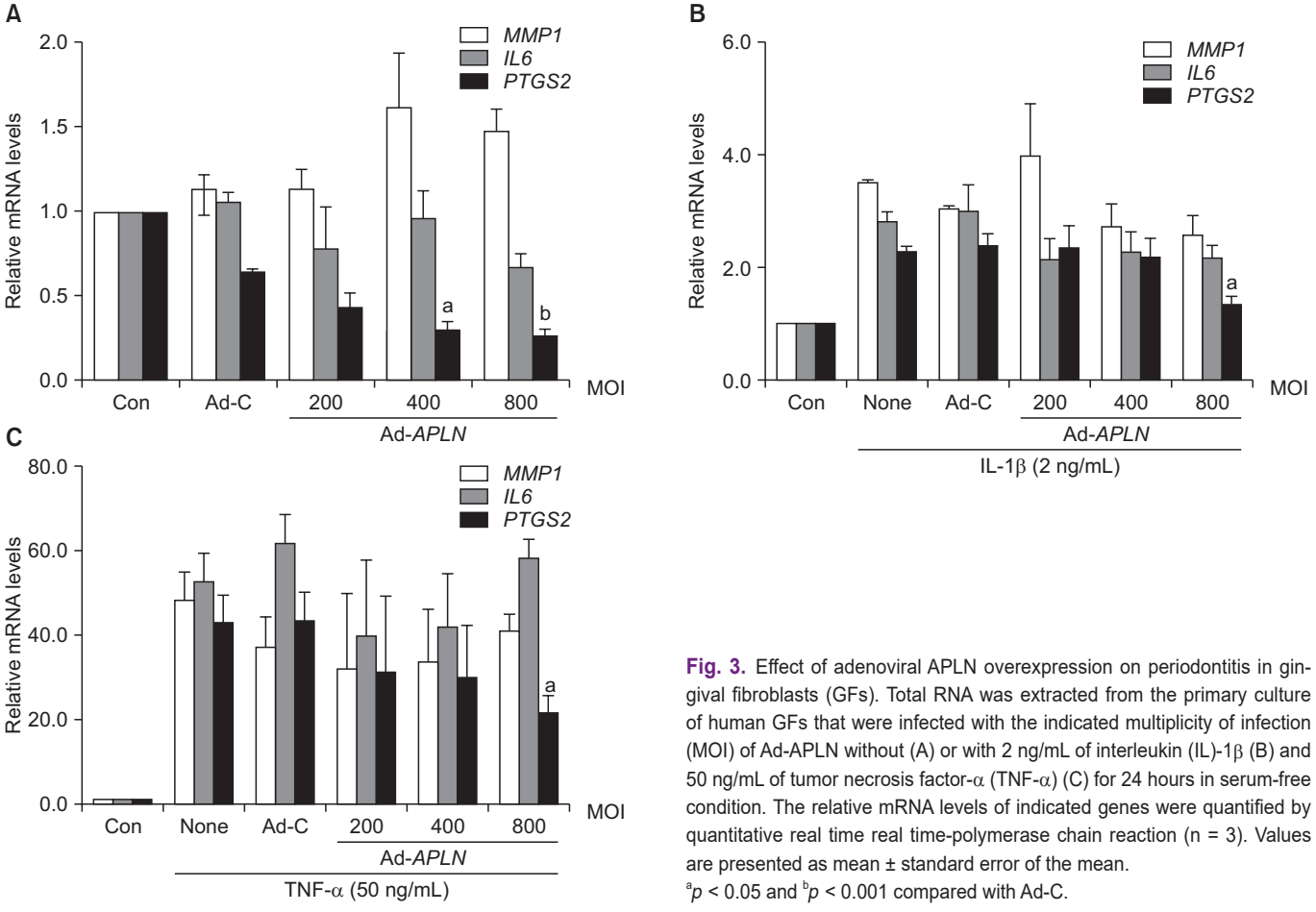

Fig. 3. Effect of adenoviral APLN overexpression on periodontitis in gingival fibroblasts (GFs). Total RNA was extracted from the primary culture of human GFs that were infected with the indicated multiplicity of infection (MOI) of Ad-APLN without (A) or with 2 ng/mL of interleukin (IL)-1β (B) and 50 ng/mL of tumor necrosis factor-α (TNF-α) (C) for 24 hours in serum-free condition. The relative mRNA levels of indicated genes were quantified by quantitative real time real time-polymerase chain reaction (n = 3). Values are presented as mean ± standard error of the mean.
[a]$p < 0.05$ and [b]$p < 0.001$ compared with Ad-C.

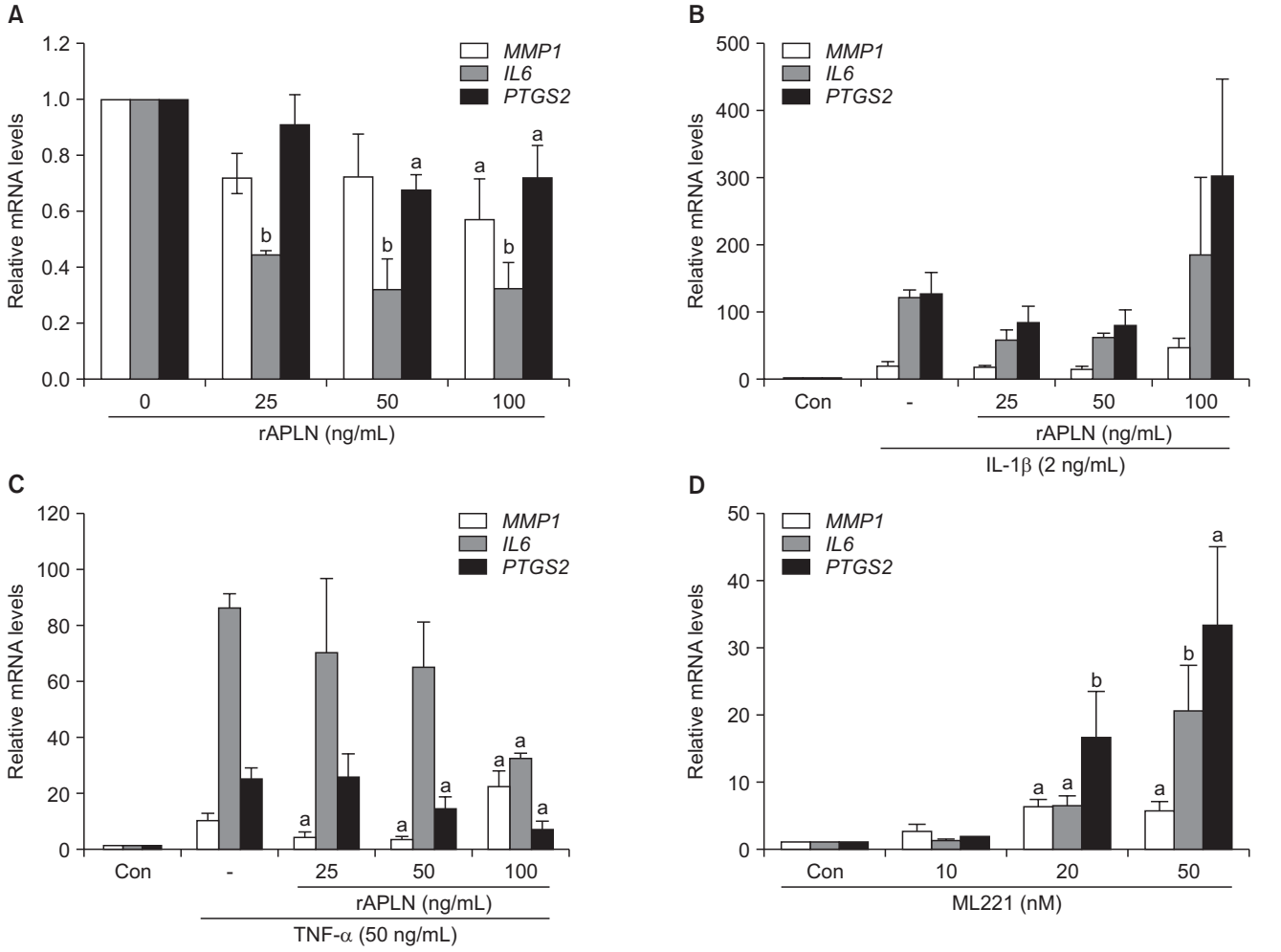

Fig. 4. Regulation of periodontitis by treatment with recombinant APLN or APLN receptor inhibitor (ML221) in periodontal ligament (PDL) cells. Total RNA was extracted from the primary culture of human PDL cells that were treated with the indicated amount of recombinant APLN without (A) or with 2 ng/ml of interleukin IL-1β (B) and 50 ng/mL of tumor necrosis factor-α (TNF-α) (C) for 24 hours in serum-free condition. (D) Total RNA was extracted from the primary culture of human PDL cells treated with the indicated amount of ML221 for 24 hours in serum-free condition. The relative mRNA levels of the indicated genes were quantified by quantitative real time real time-polymerase chain reaction (n = 3). Values are presented as mean ± standard error of the mean.
[a]$p < 0.05$ and [b]$p < 0.001$ compared with the control.

collateral effect of recombinant APLN at the high dose. In contrast to the result using recombinant APLN, APJ inhibition by ML221 rather upregulated the mRNA levels of *MMP1*, *IL6*, and Cox2 in PDL cells (Fig. 4D). Although the APLN–APJ axis could not block the IL-1β–induced inflammation and tissue degradation, these data suggest that the APLN–APJ axis partially suppresses the inflammation and tissue degradation and consequently serves as a new therapeutic target for periodontitis.

Discussion

In this study, our results demonstrated that *APLN* expression in gingival tissues is inversely correlated to periodontitis. In addition, *APLN* expression was dramatically downregulated in PDL cells and GFs that were stimulated with TNF-α. This result was unexpected because it was known that TNF-α upregulates *APLN* expression in human and mouse adipose tissue via phosphoinositide 3-kinase (PI3K), c-Jun N-terminal kinase (JNK), and mitogen-activated protein kinase (MAPK) pathways [33]. Although IL-1β could not significantly decrease the expression of *APLN* in PDL cells and GFs, we speculated that the expression of *APLN* was sufficiently inhibited by TNF-α produced by PDL cells, GFs, and other infiltrated immune cells. Based on these results, we postulated that APLN could have a protective role in the inflammatory response during periodontitis progression.

As expected, the adenoviral overexpression of *APLN* suppressed the expression of genes involved in inflammation and tissue degradation in PDL cells. In particular, *APLN* overexpression inhibited the upregulation of these genes induced by TNF-α more effectively than IL-1β. However, overexpression of *APLN* could not suppress these genes in GFs except for *PTGS2*. These results indicated that APLN may have different regulatory functions in PDL and gingiva tissues. Further research should be conducted to identify the regulatory functions of APLN in each tissue.

Since the regulatory function of APLN in inflammatory response was more pronounced in PDL cells, exogenous gain-of-function and loss-of-function experiments were performed only in PDL cells. In line with the previous results, recombinant APLN inhibited the gene expressions involved in inflammation and tissue degradation in PDL cells. Similar to the results obtained on using adenoviral overexpression of *APLN*, exogenous APLN inhibited the upregulation of these genes induced by TNF-α more effectively than when induced by IL-1β. Blocking the APLN-APJ axis by an APJ inhibitor, ML221, rather upregulated the gene expressions involved in inflammation and tissue degradation in PDL cells. These results showed that the suppressive effect of APLN on the inflammatory response is achieved by regulating the TNF-α signaling pathway rather than the IL-1β pathway.

In our current study, we demonstrated the inhibitory effect of APLN on inflammation during periodontitis progression. In agreement with our results, many other studies have reported the relevance of APLN and inflammation on various diseases [31]. It is known that APLN decreased the expression of inflammatory cytokines such as IL-1β, TNF-α, and intercellular Adhesion molecule 1 in ischemia/reperfusion injury rats [34]. In a chronic pancreatitis mice model, APLN inhibited the upregulation of pancreatic TNF-α, macrophage inflammatory protein (MIP)-1α/β, and IL-1β expression by blocking the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway [35]. However, certain contradictions exist about the role of APLN in inflammation and oxidative stress. It is known that exogenous recombinant APLN activates the expression of TNF-α, IL-1β, monocyte chemoattractant protein-1 (MCP-1), and MIP-1α in microglial BV2 cells via PI3K/Akt and MEK/Erk pathways [36]. In vascular smooth muscle cells, APLN promotes the increased expression of NOX4 and the generation of ROS [37]. The APLN-APJ axis also induces MCP-1 expression via NF-κB/JNK pathway in human umbilical vein endothelial cells [38].These conflicting reports indicate that there are more complex regulatory mechanisms of APLN in inflammation.

In this study, we focused on the effect of APLN on the regulation of the inflammatory response in PDL and gingiva tissues. However, along with the inflammatory response in PDL and gingiva tissues, the destruction of the alveolar bone is another major symptom of periodontitis. Several studies show that the adipose tissue-derived APLN has regulatory roles in bone homeostasis. It is known that the APLN-APJ axis could enhance the proliferation and also inhibit the apoptosis of osteoblasts via the PI3K/Akt pathway [26,27]. It is also known that APLN could regulate osteogenic differentiation of human mesenchymal stem cells indirectly via the Wnt/β-catenin pathway [29]. However, the role of APLN in bone homeostasis is still controversial. Despite many *in vitro* results indicating the osteogenic potential of APLN, the skeletal phenotypes of APLN knockout mice showed increased rates of bone formation and mineral apposition, with evidence of promoted osteoblast proliferation and differentiation [30]. Given these controversies, the role of APLN on alveolar bone remodeling during periodontitis should be elucidated.

Herein, we provide evidence suggesting that APLN is a regulatory factor of the inflammatory response during periodontitis. Given that the inflammatory response provokes immune cell infiltration and alveolar bone destruction during periodontitis, these results suggest that APLN could be a potential therapeutic target to treat or prevent periodontitis. However, this study also has several limitations. In this study, only the mRNA level of *APLN* was measured by RT-PCR and qRT-PCR. Since APLN is a small protein consisting of 77 amino acids and intracellular APLN is present as active forms of various lengths consisting of 36, 17, or 13 amino acids, therefore, more detailed protein level validation is needed. In order to further confirm the *in vitro* result in this study, *in vivo* result from a periodontitis animal model using APLN knockout mice is required. However, since there is no effective and reliable mouse periodontitis animal model to date, further *in vivo* verification through subsequent experiments is considered necessary.

Acknowledgements

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (No. 2019R1A5A2027521) and a grant (BCR18012) of Chonnam National University Hospital Biomedical Research Institute.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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