INTERRODUCTION

Periodontal diseases demonstrate various clinical symptoms such as gingival bleeding, swelling, formation of periodontal pocket along with alveolar bone destruction. Generally, incidence of periodontitis cause the broad extent of periodontium destruction by gingival inflammation resulting from the various local and systemic factors. During this process, many environmental and external conditions are also highly related [1].

Although bacteria that colonize the tooth surface and gingival sulcus are known to initiate the periodontal disease [2], it is believed that during the disease process, host response plays important role in the both connective tissue
and bone destruction which is the key features of the disease. Cytokine stimulates inflammatory events that activate effector mechanism, and as inflammatory mediators between bacterial stimulation and tissue destruction, it plays important role in various cells that promotes or inhibits the process of periodontitis [3]. Thus, there are many studies investigating the effect of cytokine on the target cell. Since osteoclast, one of target cells under the effect of cytokine, is known to cause the alveolar bone resorption and determines the severity of periodontitis, it is very important to figure out the factors regulating its activation.

Plasma membrane calcium ATPase (PMCA) which is recently known as a factor related to the regulation of osteoclast. It is one of P-type pump family, and during the reaction cycle it forms the high-energy phosphorylated intermediate [4]. Accurate regulation of Ca$^{2+}$ dynamics is critical for proper differentiation and function of osteoclasts. There are 4 major types of PMCAs isofrom and they are expressed in different parts of the human body [5,6]. Among these isoforms, PMCA-1 is known to play important role in osteoclastogenesis and bone homeostasis regulation by regulating Ca$^{2+}$ signaling of osteoclast. In vitro study, PMCAs affect the differentiation of osteoclast by inhibiting receptor activator of NF-kappa B ligand (RANKL)–induced Ca$^{2+}$ shift in immature/undifferentiated cell. Furthermore, increased expression of PMCAs in mature osteoclasts prevents osteoclast apoptosis both in vitro and in vivo study. Based on such study result, ‘dual role’ of PMCAs toward the osteoclastogenesis has been suggested [7]. Though it is expected to play significant role in the regulation of osteoclast, not many researches on this subject related to the periodontitis are available. Therefore, there is need for study investigating how the expression of PMCA-1 is correlated with periodontitis.

Interleukin (IL)-11 is a human protein which arises from the bone marrow–derived fibroblast or stromal cells and it first separated from the stromal cells. IL-11 is key regulator of multiple events in hematopoiesis, most notably the stimulation of megakaryocyte maturation [8]. At the same time, supporting the human osteoclast formation through RANKL-independent pathway [3]. Bekerik et al. [11] report lower LIF level in GCF with chronic periodontitis. In addition, it shows significant positive correlation with GCF IL-11 level observed in the same study. LIF which clearly has effect on the differentiation and growth of cells might have possible correlation with periodontitis since various types of cells are involved in the progression and severity of periodontitis.

Leukemia inhibitory factor (LIF) is mainly expressed from the trophectoderm of the developing embryo. LIF affects cell growth by inhibition of cell differentiation [12]. In addition, it has been clearly demonstrated that LIF is also associated with bone resorption through RANKL-dependent pathway [13]. Bekerik et al. [11] report lower LIF level in GCF with chronic periodontitis. In addition, it shows significant positive correlation with GCF IL-11 level observed in the same study. LIF which clearly has effect on the differentiation and growth of cells might have possible correlation with periodontitis since various types of cells are involved in the progression and severity of periodontitis.

The most important feature of the periodontitis is the destruction of alveolar bone following the attachment loss and osteoclast plays important role during the process of alveolar bone destruction. In order to play bone resorption role at the inflammatory periodontal tissue, osteoclast should go through several processes including fusion of osteoclast precursor such as formation, differentiation and activation. During the process, complex regulation of stimulation and inhibition becomes accomplished by various factors involved in different pathways [14]. As mentioned above, PMCA-1 plays dual role (suppressing the differentiation through RANKL-dependent pathway in immature cell while maintaining osteoclastic activity by preventing apoptosis in mature cell) and both IL-11 and LIF have function of differentiation stimulus each through RANKL-independent pathway and RANKL-dependent pathway respectively.

Since these three factors have possibility to play certain role in osteoclastic activity, it is also possible that expression of these factors might show different features depending on the osteoclastic activity which is varied according to
the severity of periodontitis.

**MATERIALS AND METHODS**

**Study population and tissue sampling**

Gingival tissue specimens were collected from patients with periodontal disease and from healthy individuals, at the Department of Periodontics, Kyungpook National University Dental Hospital. Exclusion criteria included pregnancy, smoking, and any kind of systemic disease that might influence response to treatment such as diabetes mellitus, hypertension, cardiovascular disease, arthritis, liver disease, renal disease, malignant tumor, autoimmune disease, and metabolic bone disease, etc. Specimen collection occurred during periodontal surgery including surgical crown lengthening or tooth extraction by internal bevel incision. The gingival tissue samples are comprised of junctional and crevicular epithelium and connective tissue. Before any surgical procedures, informed consent was achieved from all the subjects. The diagnosis and classification depending on the severity of chronic periodontitis were established based on the clinical and radiographic criteria. The sulcus bleeding index (SBI) value [15] and probing pocket depth (PPD) [16] were measured to define the clinical periodontal parameters. The study protocol was approved by the Ethical Committee of Clinical Experiments, Kyungpook National University Hospital (IRB no. KNUH 2013-05-036-002).

| Variable          | Group 1 (n=16) | Group 2 (n=16) | Group 3 (n=16) | p-value |
|-------------------|----------------|----------------|----------------|---------|
| Sex (male/female) | 7/9            | 9/7            | 9/7            | 0.816   |
| Age (y)           | 51.31±10.96    | 48.13±7.13     | 50.44±6.22     | 0.542   |
| (from 24 to 68)   |                |                |                |         |
| PD                | ≤3 mm          | 3 mm<PD≤6 mm   | >6 mm          |         |

Values are presented as number only or mean±standard deviation (range).

| Variable          | Group 1 (n=16) | Group 2 (n=16) | Group 3 (n=16) | p-value |
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| (from 24 to 68)   |                |                |                |         |
| PD                | ≤3 mm          | 3 mm<PD≤6 mm   | >6 mm          |         |

Values are presented as number only or mean±standard deviation (range).

Demographic characteristics of the study subjects are shown in Table 1. There were no significant differences in sex and age among each group according to the results of the chi-square test and one-way ANOVA analysis each performed for the gender and age respectively. After periodontal surgery, tissue specimens were immediately placed on liquid nitrogen and subsequently frozen (-70°C).

**Protein isolation and immunoblotting (Western blot analysis)**

For Western blotting, as previously described [17,18], frozen tissues were mechanically broken down using a homogenizer in RIPA lysis buffer (10 mM EDTA, 0.15M NaCl) with 1:30 diluted protease inhibitor cocktail (Roche, Mannheim, Germany) [19]. The lysates were centrifuged at 12,000 g for 15 min at 4°C. Quantitative analysis on protein concentrations of supernatant liquid were performed by a Bradford protein assay (Quick Start; BIO-RAD, Hercules, CA, USA) using bovine serum albumin (BSA) as standard. Lysates were boiled for 3 min in sodium dodecyl sulfate (SDS) samples buffer (1 M Tris-Cl [pH 6.8], 40% glycerol, 8% SDS,
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Prepared samples were separated by 15% SDS-polyacrylamide gels using gel electrophoresis, which maintains polypeptides in a denatured state, and transferred to a polyvinylidene difluoride membrane to make the proteins accessible to antibody detection. Blocking of the membranes was subsequently achieved in Tris-buffered saline (TBS) with a min percentage of detergent such as Tween 20 (Sigma Aldrich, St. Louis, MO, USA) containing 5% powdered milk for 1 hr. Then, the membranes were incubated with polyclonal anti-PMCA-1, anti-IL-11 and anti-LIF antibody (diluted 1:1,000 in TBS: Santa Cruz Biotechnology Inc., Dallas, TX, USA) for overnight at 4°C. The membranes were washed in TBS with Tween 20 three times for 10 min and incubated with a horseradish peroxidase-linked donkey anti-goat secondary antibody for anti-PMCA-1, anti-IL-11, and anti-LIF antibody (diluted 1:2,000 in TBS) for 1 hr at room temperature. After that, membranes were rinsed three times for 10 min with Tween 20. The membranes were developed with an ECL Plus development kit (Amsterdam, Buckinghamshire, UK). The quantitative analysis of PMCA-1, IL-11, and LIF along with their β-actin (Abcam, Cambridge, UK) was performed using a densitometer (Image Gauge V 3.46, Koshin Graphic Systems; Fuji Photo Film Co., Tokyo, Japan). After normalization of each sample, comparative levels of PMCA-1, IL-11, and LIF were expressed as a ratio of PMCA-1, IL-11, and LIF/β-actin then the differences among groups were evaluated.

**Statistical analysis of the Western blot results**

All data were presented as means±standard deviation and results were statistically analyzed via SPSS ver. 23.0 (IBM Corp., Armonk, NY, USA). The PMCA-1, IL-11, and LIF levels among the different groups were compared using one way ANOVA followed by Dunnett post-hoc test. \( p<0.05 \) was considered to be statistically significant. The correlations between gingival PMCA-1, IL-11, and LIF levels were evaluated and compared using Spearman correlation coefficients. \( p<0.05 \) was considered significant.

**RESULTS**

The gingival tissue from the patients of normal group (group 1), moderate periodontitis group (group 2), and severe periodontitis group (group 3) showed the expression of PMCA-1 corresponding to molecular weight about 130 kDa in all samples. One representative band of PMCA-1 is shown Fig. 1. The expression levels of PMCA-1 were increased in order of group 1, group 2, and group 3. The quantification of PMCA-1 expression is shown in Table 2 and summarized as a graph in Fig. 2. The mean normalized value of PMCA-1 expression relative to its β-actin was

![PMCA-1 and β-actin expression](image)

**Table 2. Normalized PMCA-1 expressions by PMCA-1/β-actin**

| Sample | Group 1 | Group 2 | Group 3 |
|--------|---------|---------|---------|
| 1      | 0.060   | 0.258   | 0.296   |
| 2      | 0.044   | 0.164   | 0.264   |
| 3      | 0.065   | 0.170   | 0.170   |
| 4      | 0.114   | 0.213   | 0.213   |
| 5      | 0.067   | 0.095   | 0.095   |
| 6      | 0.086   | 0.090   | 0.090   |
| 7      | 0.064   | 0.093   | 0.093   |
| 8      | 0.060   | 0.198   | 0.198   |
| 9      | 0.087   | 0.156   | 0.156   |
| 10     | 0.138   | 0.242   | 0.242   |
| 11     | 0.190   | 0.191   | 0.191   |
| 12     | 0.206   | 0.180   | 0.180   |
| 13     | 0.118   | 0.109   | 0.109   |
| 14     | 0.082   | 0.064   | 0.064   |
| 15     | 0.092   | 0.087   | 0.087   |
| 16     | 0.088   | 0.154   | 0.154   |

Mean±standard deviation

|          | 0.098±0.046* | 0.154±0.059† | 0.242±0.107‡ |

PMCA, plasma membrane calcium ATPase; group 1, normal group; group 2, moderate periodontitis group; group 3, severe periodontitis group.

*Significant difference between group 1 and group 2 (\( p<0.05 \)). †Significant difference between group 2 and group 3 (\( p<0.05 \)). ‡Significant difference between group 1 and group 3 (\( p<0.05 \)).
0.098±0.046 in group 1, 0.154±0.059 in group 2, 0.242±0.107 in group 3. Significant difference could be found between group 1 and group 2 (p=0.016), group 2 and group 3 (p=0.023), and group 1 and group 3 (p<0.001) (p<0.05).

The gingival tissue from the patients of normal group (group 1), moderate periodontitis group (group 2), and severe periodontitis group (group 3) showed the expression of IL-11 corresponding to molecular weight about 20 kDa in all samples. One representative band of IL-11 is shown Fig. 3. The expression levels of IL-11 were increased in order of group 1, group 2, and group 3.

The quantification of IL-11 expression is shown in Table 3 and summarized as a graph in Fig. 4. The mean normalized value of IL-11 expression relative to its β-actin was 0.096±0.047 in group 1, 0.208±0.092 in group 2, 0.344±0.122 in group 3. Significant difference could be found between group 1 and group 2 (p=0.001), group 2 and group 3 (p=0.004), and group 1 and group 3 (p<0.001) (p<0.05).

**Table 3. Normalized IL-11 expressions by IL-11/β-actin**

| Sample | Group 1 | Group 2 | Group 3 |
|--------|---------|---------|---------|
| 1      | 0.034   | 0.255   | 0.334   |
| 2      | 0.094   | 0.277   | 0.399   |
| 3      | 0.160   | 0.350   | 0.417   |
| 4      | 0.190   | 0.374   | 0.478   |
| 5      | 0.088   | 0.079   | 0.185   |
| 6      | 0.087   | 0.187   | 0.222   |
| 7      | 0.066   | 0.157   | 0.247   |
| 8      | 0.089   | 0.142   | 0.467   |
| 9      | 0.098   | 0.184   | 0.391   |
| 10     | 0.078   | 0.147   | 0.259   |
| 11     | 0.053   | 0.236   | 0.445   |
| 12     | 0.188   | 0.283   | 0.579   |
| 13     | 0.099   | 0.055   | 0.197   |
| 14     | 0.053   | 0.127   | 0.159   |
| 15     | 0.047   | 0.183   | 0.364   |
| 16     | 0.109   | 0.294   | 0.367   |

Mean±standard deviation: 0.096±0.047* 0.208±0.092† 0.344±0.122‡

IL, interleukin; group 1, normal group; group 2, moderate periodontitis group; group 3, severe periodontitis group.

*Significant difference between group 1 and group 2 (p<0.05). †Significant difference between group 2 and group 3 (p<0.05). ‡Significant difference between group 1 and group 3 (p<0.05).
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The gingival tissue from the patients of normal group (group 1), moderate periodontitis group (group 2), and severe periodontitis group (group 3) showed the expression of LIF corresponding to molecular weight about 20 kDa in all samples. One representative band of LIF is shown Fig. 5. The expression levels of LIF were decreased in order of group 1, group 2, and group 3.

The quantification of LIF expression is shown in Table 4 and summarized as a graph in Fig. 6. The mean normalized value of LIF expression relative to its β-actin was 0.428±0.127 in group 1, 0.186±0.073 in group 2, 0.054±0.027 in group 3. Significant difference could be found between group 1 and group 2 (p<0.001), group 2 and group 3 (p<0.001), and group 1 and group 3 (p<0.001) (p<0.05).

Table 5 demonstrates the correlations between gingival PMCA-1, IL-11, and LIF levels in normal gingiva and inflamed gingiva of chronic periodontitis. The correlations were given by Spearman correlation coefficient. There was positive relationship between PMCA-1 and IL-11 and relatively high correlation could be found. However, between PMCA-1 and LIF, as well as, IL-11 and LIF, negative rela-

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**Table 4.** Normalized LIF expressions by LIF/β-actin

| Sample | Group 1 | Group 2 | Group 3 |
|--------|---------|---------|---------|
| 1      | 0.559   | 0.274   | 0.084   |
| 2      | 0.453   | 0.182   | 0.067   |
| 3      | 0.360   | 0.158   | 0.047   |
| 4      | 0.417   | 0.126   | 0.047   |
| 5      | 0.474   | 0.199   | 0.068   |
| 6      | 0.552   | 0.313   | 0.051   |
| 7      | 0.424   | 0.241   | 0.057   |
| 8      | 0.329   | 0.137   | 0.046   |
| 9      | 0.675   | 0.320   | 0.047   |
| 10     | 0.512   | 0.168   | 0.038   |
| 11     | 0.294   | 0.082   | 0.019   |
| 12     | 0.368   | 0.060   | 0.022   |
| 13     | 0.532   | 0.198   | 0.131   |
| 14     | 0.449   | 0.200   | 0.064   |
| 15     | 0.283   | 0.145   | 0.038   |
| 16     | 0.163   | 0.173   | 0.031   |

Mean±standard deviation

|     | 0.428±0.127 | 0.186±0.073 | 0.054±0.027 |

LIF, leukemia inhibitory factor; group 1, normal group; group 2, moderate periodontitis group; group 3, severe periodontitis group.

*Significant difference between group 1 and group 2 (p<0.05). †Significant difference between group 2 and group 3 (p<0.05). ‡Significant difference between group 1 and group 3 (p<0.05).

**Table 5.** The correlations between gingival PMCA-1, IL-11, and LIF levels

| Variable | PMCA-1 | IL-11 | LIF |
|----------|--------|-------|-----|
| r        | 0.655* | -0.641* |     |
| p-value  | <0.001 | <0.001 |     |
| number of samples | 48 | 48 |     |

| IL-11 | 0.655* | -0.788* |
| p-value | <0.001 | <0.001 |
| number of samples | 48 | 48 |

| LIF | -0.641* | -0.788* |
| p-value | <0.001 | <0.001 |
| number of samples | 48 | 48 |

PMCA, plasma membrane calcium ATPase; IL, interleukin; LIF, leukemia inhibitory factor; r, correlation coefficient.

*Statistically significant (p<0.05).
tionship could be observed and high correlation could be found between IL-11 and LIF.

**DISCUSSION**

Periodontitis is characterized as the disease with the host-mediated destruction of soft and hard tissue. Such destruction is occurred by induced production and activation of lytic enzymes along with stimulated osteoclastogenesis [20]. During this process, inflammatory mediators secreted from the host cells play important role and especially, bone resorption related factors, which is associated with osteoclast activity causing the resorption and destruction of alveolar bone, seems to have important role in the development and the process of periodontal disease.

There are only limited studies available focusing on the direct relation of PMCA-1 toward dental tissue though it is known to be related to the osteoclastogenesis and regulation of bone homeostasis [7]. Likewise, IL-11 and LIF which is well known inflammatory mediators yet has not been investigated to find out their correlation with periodontitis at the gingival tissue level in many studies. Therefore, in this study, we investigated expression pattern of PMCA-1, IL-11, and LIF, which are suggested to be possible bone-resorption related factors, at the gingival tissue level in the different groups classified depending on the severity of periodontitis.

Generally, as chronic periodontitis progresses, osteoclastic activity becomes predominant [21]. From the result of this study, higher expression of PMCA-1 could be found as severity of chronic periodontitis aggravates. In other words, higher PMCA-1 expression could be seen as degree of alveolar bone resorption increases. This result can be explained by correlating it with the increased activity of bone-resorbing mature osteoclast. During the bone resorption process, excessive Ca\(^{2+}\) excreted from the bone goes into osteoclast. However, since such excessive amount of Ca\(^{2+}\) intake might be toxic to osteoclast, Ca\(^{2+}\) needs be continuously secreted to the extracellular space [22,23]. At this time, PMCA which is located at the basolateral membrane of mature osteoclast protects the osteoclast from the Ca\(^{2+}\) induced apoptosis by secreting Ca\(^{2+}\) toward outside of the cell [7]. However, considering the role of PMCA-1 which suppresses the osteoclastogenesis and increased osteoclastogenesis in chronic periodontitis, it is difficult to relate it with our study result [24]. Thus, further studies on the exact action mechanism and effect of PMCA-1 seems necessary in near future.

Until now, studies investigating on the correlation between periodontitis and IL-11 only performed at the GCF level [11]. In this study based on the gingival tissue level, IL-11 shows increased expression pattern as severity of periodontitis aggravates and differences in each group were all statistically significant. On the contrary, LIF shows highest level of expression in normal gingival tissue and expression level was decreased with statistical significance as severity of periodontitis aggravates. Above results are very similar with the result came from Becerik et al. [11]. They discovered that IL-11 GCF levels get increased in gingiva with periodontitis more than in normal gingiva, and reported their positive correlations with PD, CAL, and BOP. Meanwhile, in case of GCF LIF level, it was decreased in gingiva with periodontitis comparing to normal gingiva, and its negative correlations with PD and CAL were suggested. Based on limited results of this study, there is possibility that IL-11 might have a role as an inflammatory biomarker whereas LIF might have a beneficial role in the modulation of inflammatory response. If we could successfully utilize such factors with opposite characteristics in combination, it would be possible to accomplish more precise diagnosis and set up more effective treatment plan of periodontitis. Although this study results show relatively clear expression pattern of IL-11 and LIF as severity of periodontitis aggravates, it should be noted that there are still many conflicting results and only limited study available which directly observed amount of expression in gingival tissue level. Thus, further study on the expression pattern of IL-11 and LIF depending on the severity of periodontitis in GCF or gingival tissue level seems necessary.

Based on our study result synthesized with previous ones, it is clear that these factors all commonly affect the osteoclast formation. In addition, based on the high level of correlation, there is possibility that these factors might interact with each other in chronic periodontitis. However, evidence shows that IL-11 supports the osteoclast formation through RANKL-independent mechanism [25] whereas LIF stimulates bone resorption through stimulat-
ing Ca\textsuperscript{2+} release and increasing RANKL mRNA expression [13]. Therefore, these cytokines are likely to affect the osteoclast formation with different mechanism. On the other hands, in case of PMCA-1, there is evidence that PMCA-1 suppresses the RANKL-induced Ca\textsuperscript{2+} oscillations and osteoclast differentiation [7]. Hence, there is possibility that LIF and PMCA-1 might affect each other through the pathway related to RANKL.

Considering the characteristic of periodontitis which tends to increase osteoclast formation, it would be probable result based on evidence up to present that expression of IL-11 and LIF should be increased while PMCA should be decreased in the aspect of osteoclast formation. However, since actual results of this study shows increased expression of PMCA-1 and IL-11 and decreased expression of LIF, there is gap between actual result and expected result. Such discrepancy might be due to characteristic of cytokine that functions can be varied depending on the target cell, cell environment/condition and the presence of other cytokine.

A forementioned ‘dual role’ of PMCA-1 would be easier to understand in the same context. Therefore, in order to understand the role and interaction of PMCA-1, IL-11, and LIF more properly, enhanced understanding on the role of individual factor would be necessary supported by further studies including comparative studies under controlled condition.

Periodontitis is inflammatory disease that accompanies alveolar bone resorption and destruction of periodontal connective tissue. Thus, it is important to understand the inflammatory destructive process of both soft and hard tissue and find out the related factors in this process for proper diagnosis and treatment of periodontitis. As inflammatory process of periodontal disease and role of regulatory factors toward it gets easier to understand through many studies, new strategies are being developed to prevent and treat periodontal disease. Factors subjected to this research were also studied for supporting such strategies.

In conclusion, PMCA-1, IL-11, and LIF seem to be associated with severity of chronic periodontitis and its existence, and these factors could affect each other during the progress of periodontitis. These factors are expected to be applied in the diagnosis and treatment of periodontitis through more comprehensive studies.

**CONFLICTS OF INTEREST**

The authors declare that they have no competing interests.

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