Original Article
Up-regulation of ICAM-1/VCAM-1 promotes the progression of periodontitis by activating the NF-κB pathway

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Abstract: Periodontitis remains to be one of the most common oral diseases, and brings huge damage to people’s life. Inflammations play significant roles in the biology of periodontitis. This study was aimed to investigate the highly expressed proteins of ICAM-1 and VCAM-1 in the development of periodontitis at the inflammatory level and to reveal its possible mechanism. Expression of the targets in the inflamed gingiva-isolated periodontal lymphocytes and normal lymphocytes was measured. The effects of ICAM-1 and VCAM-1 expression on periodontal lymphocytes apoptosis were analyzed. Furthermore, the cell apoptosis- and signal pathway-related protein expression were also analyzed. Compared to the normal lymphocytes, ICAM-1 and VCAM-1 were highly expressed in periodontal lymphocytes. When cells were treated with the ICAM-1/VCAM-1 antibody, the cell apoptosis was enhanced compared to the controls and this effect was more apparent when cells were treated with the two antibodies. Besides, Bcl-2 and Bcl-xs expression was both significantly increased by the suppressed ICAM-1/VCAM-1, whereas the p-IKBα and p-IKKα/β levels were significantly decreased. Similarly, the decreased effects of the suppressed ICAM-1/VCAM-1 on the former proteins expression were more apparent when cells were treated with the two antibodies. Taken together, our study revealed that the overexpressed ICAM-1/VCAM-1 may contribute to the development and progression of periodontitis through inhibiting apoptosis and activating the NF-κB signal pathway.

Keywords: Periodontitis, ICAM-1, VCAM-1, cell apoptosis, NF-κB pathway

Introduction
Periodontitis remains to be one of the most common infectious diseases [1], which is characterized by the gum swelling and bleeding, alveolar bone resorption, periodontal pocket formation, and tooth mobility [2]. Previous evidence refers that periodontitis can bring huge damages on periodontium, as a result of the interaction between the toxic ingredients of periodontal pathogens and the defense mechanism of the specify host [3]. Traditional methods such as clean, drug, surgery, fixed loose tooth, and systemic treatment have obtained certain achievement in the treatment of periodontitis [4, 5], but the results still remain unsatisfactory due to the complicate pathogen mechanism. Hence, to explore the deep pathogen mechanism at the molecular level will be benefit for the treatment of periodontitis.

Studies have revealed that various kinds of factors including diabetes, genetic factors, and microorganisms introduction are the major pathogens in contributing to the development and progression of periodontitis [6-8]. Recent studies have referred that biological processes including cell apoptosis are involved in the biology development of periodontitis [9]. Apoptosis is closely associated with the biology mechanism of diseases such as tumor, autoimmune diseases, and microbial infection diseases [10, 11]. In addition, the metabolites produced by pathogenic bacteria restrained the apoptosis of leukocytes, and then resulted in the inflammatory reactions and the development of periodontitis [12].

Intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) are two immune cells that play pivotal roles in leu-
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**Table 1. Primers used for targets amplification in this study**

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>Sense</td>
<td>GGGTGAGCCAAACGGGTC</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>GAATGTCGTTTACGTCGA</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>Sense</td>
<td>TTTGGCCCTTTGAGTTGGTG</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>GGTGCCGTTGCAGTCA</td>
</tr>
<tr>
<td>Bcl-xs</td>
<td>Sense</td>
<td>AGTCAGTTGATGTC</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>GAATGTTAGATCAGGA</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Sense</td>
<td>TGAGGTCATGTC</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>TTCCGCTGGCGTATAGAG</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Sense</td>
<td>AAGATGGTCGTGATCTTGG</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>GGTGCTGCAAGCTAAGAG</td>
</tr>
</tbody>
</table>

kocytes cell-cell recognition, as well as mediate the signal transduction [13, 14]. Accumulating studies have reported that ICAM-1 is associated with the development of periodontitis [15, 16], as well as VCAM-1 in the development of periodontitis [17]. Additionally, Okada et al reported that the up-regulated ICAM-1/VCAM-1 is correlated to the pathogenesis of allergic keratopathy [18]. Although many studies have revealed the pivotal roles of ICAM-1 and VCAM-1 in the development of periodontitis, yet, the roles of ICAM-1/VCAM-1 in the inflammatory pathogenesis of periodontitis have not been fully reported.

In this study, we investigated the possible effects of the expression of ICAM-1 and VCAM-1 on the development of periodontitis using the inflamed gingiva-isolated periodontal lymphocytes. A variety of experiment methods were used to analyze the expression of the two proteins in the periodontitis and normal tissue, as well as the influences of the two proteins on periodontal lymphocytes apoptosis. This study was aimed to investigate the potential roles of ICAM-1 and VCAM-1 in the development and progression of periodontitis and to reveal its possible molecular mechanism of its action.

**Materials and methods**

**Isolation of periodontal lymphocytes**

Periodontal lymphocytes were isolated from the inflamed gingiva and normal gingiva tissues as previously described [19]. In brief, the tissue specimen in AIM V medium (Gibco BRL, Grand Island, NY) containing 125 ng/mL of amphotericin B, proteinase inhibitors (1 μg/mL of α2-macroglobulin, 0.1 μg/mL of α1-antitrypsin, 200 μg/mL of EDTA-2Na, 0.5 μg/mL of aprotinin, and 0.5 μg/mL of E-64), and 10 U/mL of heparin sodium was cut into 1-3 mm pieces with surgical scalpels and extruded through a 73.7-μm-pore-size mesh filter with glass rods. Cells were then isolated by Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) gradient centrifugation. The majority of the isolated cells (more than 99%) showed forward and side scatter identical to those of peripheral blood lymphocytes when judged by flow cytometry. These cells were considered as lymphocyte-rich fractions (periodontal lymphocytes).

**Real time (RT)-PCR**

Total RNA was isolated from cells using TRizol Reagent (Invitrogen, CA, USA) as previously described [20]. The concentration and purity of isolated RNA were measured with SMA 400 UV-VIS (Merinton, Shanghai, China). Purified RNA at density of 0.5 μg/μL with nuclease-free water was used for the complementary DNA (cDNA) synthesis using reverse transcriptase (iScript™ cDNA Synthesis Kit; Bio-Rad Laboratories). The expression levels of mRNAs were measured by SYBR green-based quantitative RT-PCR (SYBR Green Master mix; Thermo Scientific, Waltham, MA, USA). Melting curve analysis of amplification products was performed at the end of each PCR to confirm that only one product was amplified and detected. Phosphoglyceraldehyde dehydrogenase (GAPDH) was chosen as the internal control. Primers used for targets amplification were shown in Table 1.

**Flow cytometry**

Flow cytometry was used to examine the protein expression of ICAM-1 and VCAM-1 on the cell surface of cultured periodontal lymphocytes as previously described [18]. Briefly, cells were stained with the antibodies against mouse IgG1 (Invitrogen), ICAM-1 (84H10; Immunotech, Marseille, France) or VCAM-1 (1G11; Immunotech), respectively. Then cells were analyzed by FACScan (Becton Dickinson, Franklin Lane, NJ) and then analyzed using the CellQuest software (Becton Dickinson, Mountain View, CA).

**Cell apoptosis assay**

Cell apoptosis was analyzed with Annexin V-FITC cell apoptosis kit (Invitrogen, USA)
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 according to manufacturer’s protocol. Briefly, after antibody treatment, cells in each group were harvested and then washed using PBS buffer (PH 7.4) for 3 times, and then resuspended in the staining buffer. Then 5 μL of annexin-V-FITC and 5 μL of propidium iodide (PI) were mixed with the cells. After being cultivated at room temperature for 10 min, mixtures were analyzed using the FACScan flow cytometry. Annexin V-positive and propidium iodide-negative cells were considered to be apoptotic cells.

**Western blot analysis**

Cells that cultured for 48 h in each group were lapped with RIPA assay (radioimmunoprecipitation, Sangon Biotech, China) lysate containing PMSF (phenylmethanesulfonyl fluoride, Sigma, USA), and then were centrifuged at 12,000 rpm for 10 min at 4°C. Supernatant was collected for the measurement of protein concentrations using BCA protein assay kit (Pierce, Rochford, IL). For western blot analysis [21], 50 μg protein per cell lysate was subjected onto a 12% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by transferred onto the Polyvinylidenefluoride (PVDF) membranes (Millipore). Then the membranes were blocked in the Tris-Buffered Saline Tween (TBST) containing 5% non-fat milk for 1 h at room temperature. The membranes were incubated with rabbit anti-human antibodies (Bcl-2, Bcl-xs, p-IKβα, p-IKKα/β, IKβα, IKKα and IKKβ, 1:100 dilution, Invitrogen) and overnight at 4°C. Then membranes were incubated with horseradish-peroxidase labeled goat anti-rat secondary antibody (1:1000 dilution) at room temperature for 1 h. Finally, the membranes were washed 3 times with 1× TBST buffer for 10 min each. The signals were detected after incubation with a chromogenic substrate using the enhanced chemiluminescence (ECL) method. Additionally, GAPDH served as the internal control.

**Statistical analysis**

All experiments were repeated 3 times independently. Data are expressed as the mean ± SD. Statistical analysis was performed using Graph Prism 6.0 (GraphPad Prism, San Diego, CA). Significant differences for data among groups were calculated using a one-way analysis of variance (ANOVA) with post-hoc test. The P<0.05 was considered as the statistically significant.

**Results**

**mRNA expression of ICAM-1 and VCAM-1 in the periodontal lymphocytes**

The relative mRNA expression for ICAM-1 and VCAM-1 in periodontal lymphocytes was analyzed using RT-PCR (Figure 1). Compared to the normal lymphocytes, the relative mRNA expression for targets including ICAM-1 and VCAM-1 was significantly increased (P<0.01), indicating that the protein expression may involve in the pathogen of periodontal.
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Accordingly, we analyzed the median fluorescence intensity (MFI) ratio for the two proteins (Figure 2). The MFI ratio for ICAM-1 or VCAM-1 in periodontal lymphocytes was significantly increased compared to that in normal lymphocytes (P<0.01), indicating that ICAM-1 or VCAM-1 was highly expressed in the periodontal lymphocytes.

**Effects of antibody of target protein on periodontal lymphocytes apoptosis**

The results showed that the percentage of apoptotic cells in periodontal lymphocytes treated with ICAM-1 antibody (about 15.0%) or VCAM-1 antibody (about 14.1%) was significantly increased compared to the control cells (P<0.05, Figure 3). Additionally, when cells were treated with the two kinds of antibodies, the percentage of apoptotic periodontal lymphocytes was further significantly increased (about 32.6%) than the controls (P<0.01). These results suggested that the high expression of ICAM-1 or VCAM-1 may be associated with the suppressed cell apoptosis during periodontitis.

**ICAM-1 or VCAM suppression inhibited cell signal pathway-related protein expression**

We further analyzed the cell apoptosis-related and NF-κB pathway-related protein expression in periodontal lymphocytes to investigate the potential molecular mechanism of ICAM-1 or VCAM expression on periodontal progression (Figure 4). The relative mRNA and protein levels for Bcl-2 and Bcl-xs were highly expressed by the treatment of ICAM-1 or VCAM antibody in periodontal lymphocytes, and this effect was more apparent when cells were treated with the
two kinds of antibodies (Figure 4A). Besides, when cells were treated with different kinds of antibodies, the phosphorylated (p)-IKBα and p-IKKα/β were both significantly decreased compared to that in control group (P<0.05), in addition, their levels were drastically decreased when periodontal lymphocytes were treated with the two kinds of antibodies (P<0.01).

Discussion

Periodontitis remains to be one of the most common infectious diseases, and brings huge causes to people’s life [1, 22]. Previous evidence revealed the significant roles of the cell adhesion-related protein of ICAM-1 and VCAM-1 in the pathogenesis of periodontitis. However, few have mentioned their roles in the inflammatory biology of periodontitis. In this study, we investigated the expression of ICAM-1 and VCAM-1 in the inflamed gingiva-isolated periodontal lymphocytes and analyzed the effects of the two proteins in the development of periodontitis. In agreement with previous studies [16, 23], the relative mRNA expression of ICAM-1 and VCAM-1 was highly expressed in the periodontal lymphocytes (Figure 1). On the other side, flow cytometry assay showed that both ICAM-1 and VCAM-1 protein expression were up-regulated in periodontal lymphocytes (Figure 2), which was coincidence with their mRNA level. These results suggested that the abnormal expression of ICAM-1 and VCAM-1 may correlate to the pathogin of periodontitis.

Subsequently, we further analyzed the effects of ICAM-1 and VCAM-1 expression on the lymphocytes apoptosis by treating the cells with the according antibodies. Our results showed that the lymphocytes apoptosis was enhanced by the suppressed ICAM-1/VCAM-1 (Figure 3). The influence of ICAM-1/VCAM-1 expression on the apoptosis of periodontal lymphocytes has not been fully discussed. However, the roles of ICAM-1/VCAM-1 on cell apoptosis had been demonstrated in many kinds of diseases. Afford et al proved VCAM-1 promoted the inflammation by inhibiting the T cell apoptosis [24], and Yamagata et al demonstrated VCAM-1 was correlated to human endothelial cell apoptosis...
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[25]. In addition, Skowron et al proved that ICAM-1 was correlated to the apoptosis of human aortic smooth muscle cell [26]. Based on our data, we speculated that ICAM-1/VCAM-1 suppression may induce lymphocytes apoptosis during periodontitis development.

Meanwhile, we further investigated the potential molecular mechanism for the ICAM-1/VCAM-1 suppression on periodontitis progression. On one hand, the cell apoptosis promoter of Bcl-2 [27, 28] and Bcl-xs expressions [29] were both significantly enhanced by the suppressed ICAM-1/VCAM-1, and when the two kinds of antibodies were added into cells, the apoptosis-related protein was drastically enhanced (Figure 4), implying that apoptosis of periodontal lymphocytes may induced by the suppressed ICAM-1/VCAM-1 via increasing the Bcl-2 and Bcl-xs. From another point of view, cell signal transduction plays pivotal roles in the immune defense mechanism of host [30]. IκBα and IκKα/β are the enzyme complex that forms part of the NF-κB signaling pathway [31]. NF-κB signal pathway is involved in various biological processes including infection, inflammation, immune, cell apoptosis, and tumor pathogen, as well as cell cycle and differentiation [32, 33]. The activation of NF-κB including IκBα and IκKα/β could induce various factors transcription, such as cytokines, ICAM-1, and VCAM-1 [34]. In this study, when ICAM-1 and VCAM-1 were suppressed in periodontal lymphocytes, the levels of p-IκBα and p-IκKα/β were declined, and their levels were apparently decreased by the co-regulation of the two kinds of proteins, suggesting that the suppressed ICAM-1/VCAM-1 may play certain inhibit role in the inflammation by preventing the activation of NF-κB signal pathway.

In conclusion, the data presented in this study suggests that the up-regulated ICAM-1 and VCAM-1 is closely correlated to the pathogen of periodontitis through complicate mechanism. The overexpressed ICAM-1/VCAM-1 may contribute to the development and progression of periododontitis through inhibiting apoptosis and activating the NF-κB signal pathway. Our study may provide theoretical basis for the possible mechanism of ICAM-1/VCAM-1 in the pathogenesis of periodontitis and may reveal the potential application of ICAM-1/VCAM-1 in the genetic treatment of periodontitis. Further experimental studies are still needed to explore the deep mechanism at transcriptional level.

Acknowledgements

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Disclosure of conflict of interest

None.

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