**Stimulation of CCL2 Expression in Human Gingival Epithelium by Candida albicans**

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**Abstract**

The presence of yeasts in periodontal pockets is well known and *Candida albicans* (*C. albicans*) is the species most commonly isolated from the oral cavity. The immune response and anti-candidal activity of oral epithelium cells play a key role in the host defense against *C. albicans* infection. Rat gingival tissue was infected with *C. albicans* ATCC90029. Total RNA was extracted from the epithelium and mRNA levels were monitored using DNA microarray. By ingenuity pathway analysis demonstrated that TNF and IL-1 stimulated gene expression of CC type chemokine CCL2, also known as monocyte chemoattractant protein-1 (MCP-1), through NF-κB pathway. Altered mRNA level of CCL2 by rat gingival tissue was infected with *C. albicans* was confirmed by reverse transcription polymerase chain reaction. The immunohistochemical examination for CCL2 production was carried out. As a result, stronger immunoreactivity against CCL2 was observed in the rat gingival epithelium infected with *C. albicans*.

**Introduction**

The presence of yeasts in periodontal pockets has been described and *Candida albicans* is the species most commonly isolated from the oral cavity (1). Oral candidiasis is associated with significant morbidity (2) and may predispose severely immunocompromised patients to invasive disease (3). The immune response and the anti-candidal activity of oral epithelium play a key role in the host defense against localized *C. albicans* infection (4). Oral epithelial cells provide the first line of host defense against mucosal *C. albicans* infections. These cells actively respond to candidal invasion and cell injury by producing proinflammatory cytokines and chemokines (5).

Recently, increased induction of IL-8 (6) and IL-1α (7) in human oral epithelial cells by *C. albicans* infection has been investigated. Mostefaoui et al (8) also reported that *C. albicans* infection enhanced IL-6, IL-8 and TNF-α gene expressions in oral mucosa epithelial cells. We demonstrated *C. albicans*-infection enhanced expression of TNF-α (9), c-met (10), ICAM, COX2 (11) genes in human gingival epithelial cells. Although chemokine IL-8 expression was enhanced its expression by *C. albicans* infection (6), however, little is known about the expression of chemokines in gingival epithelium by *C. albicans* in vivo.

In this study, *C. albicans* was infected into rat gingival tissues and monitored gene expression of chemokine using DNA microarray.

**Materials and Methods**

**Animal experiment**

*C. albicans* (ATCC90020) was grown and infected to the gingival tissue of Sprague-Dawley rats according to the previous study (9). The same volume of saline was given to the control group. Rats were maintained and used in accordance with the guide the Care and Use of Laboratory Animals of Nihon University, School of Dentistry at Matsudo (#08-0004). Rats were injected with sodium pentobarbital and their gingival papilla were removed and used for DNA microarray analysis and immunohistochemistry.

**DNA microarray analysis**

Total cellular RNA was isolated from gingival tissues using Trizol (GIBCO BRL, Life Technologies, Rockville, MD,
USA) by a FastPrep machine (FP120; BIO 101), and cDNA was synthesized using a Superscript II RNaseH(-) reverse transcriptase system with oligo d(T)12-18 primer at 42℃ for 1 hour. Total RNA was extracted with the Ambion RNAqueous Kit (Ambion, Austin, TX) according to the manufacturer’s protocol. Fluoro-dye labeled cRNA was synthesized using the Agilent Low RNA Input Fluor Linear Amp Kit (Agilent, Santa Clara, CA). For hybridization, 3.5μg of Cy3-labeled cRNA from treated and vehicle-treated cells was combined and hybridized to an Agilent 44K Whole Rat Genome Oligo Microarray (41,000 rat genes and transcripts; Agilent) according to the manufacturer’s protocol. Fluoro-dye labeled cRNA was synthesized using the Agilent Low RNA Input Fluor Linear Amp Kit (Agilent, Santa Clara, CA). For hybridization, 3.5μg of Cy3-labeled cRNA from treated and vehicle-treated cells was combined and hybridized to an Agilent 44K Whole Rat Genome Oligo Microarray (41,000 rat genes and transcripts; Agilent) according to the manufacturer’s protocol. Finally, the arrays were scanned using a GeneArray Scanner (Agilent), and the scanned images were analyzed using GeneSpring 4.0 software (Silicon Genetics, Redwood City, CA). The presence or absence of signals was re-evaluated and intensity normalization was performed across all eight of the arrays. Data analysis was performed using the GeneChips Expression Analysis software (Affymetrix) and GeneSpring software (Silicon Genetics, Redwood, CA, USA).

Reverse transcription-polymerase chain reaction

Reverse transcription-PCR (RT-PCR) was carried out using a DNA thermal analyzer (Rotor-GeneTM 6000; Corbett Life Science, Sydney, Australia). RT-PCR amplification products were electrophoresed on agarose gels and subsequently stained with ethidium bromide. Real-time PCR reactions were performed using SYBR Premix Ex Taq™ (Perfect Real-Time PCR, Takara, Ohtsu, Japan) and a green PCR kit (Qiagen GmbH, Dusseldorf, Germany). Amplification by PCR was started with an initial incubation at 95℃ for 15 s to activate the Taq DNA polymerase, and then performed at 95℃ for 5 s and 56℃ for 15 s for 40 cycles. DNA primer sequences were: 5’-gtgctgaccccaataaggaa-3’ (forward primer for CCL2 gene) and 5’-tgaggtggttgtggaaaaga-3’ (reverse primer for CCL2 gene) (predicted size=185 bp); and 5’-atcaccatcttccaggag-3’ (forward primer for GAPDH) and 5’-atcgactgtggtcatgag-3’ (reverse primer for GAPDH gene) (predicted size=318 bp). Values are provided as means ± standard deviation (SD). Comparisons were made between two groups using Student’s t-test.

Ingenuity pathway analysis (IPA)

IPA software version 4.0 (Ingenuity Systems, Mountain View, CA, USA) was used to search for possible biological processes, pathways, and networks. A detailed description of IPA can be found on www.Ingenuity.com. As previously described, lists of genes with significant changes in gene expression based on the GeneChip experiments were moved from GeneSpring GX software into IPA software. The functional analysis of a network identified the biological functions and/or diseases that were most significant to the genes in the network.

Immunohistochemistry

Formalin-fixed, paraffin-embedded sections were deparaffinized in xylene and rehydrated in graded alcohol series and in water. To examine the invasion of C. albicans into rat gingival epithelium, the gingival tissues were excised using aseptic technique and immediately fixed in 10% (vol/vol) neutral buffered formalin and stained with periodic acid-Schiff (PAS) for observation under the light microscopy. Formalin-fixed, paraffin-embedded specimens were subjected to antigen retrieval and endogenous peroxidase blocking (30 min), and then rinsed with phosphate-buffered saline (PBS). Immunostaining was performed using Elite ABC kits (Vector, Burlingame, CA, USA) and dianobenzidine (Kirkegaard & Perry, Gaithersburg, MD, USA) as chromogen using rabbit polyclonal anti-CCL2 antibody (ABCM, CA, USA). Peroxidase-conjugated goat anti-rabbit immunoglobulin (Funakoshi, MP Biomedicals, Inc, Tokyo, Japan) in PBS supplemented with 2-vol% heat-inactivated normal human serum was used. Peroxidase activity was visualized with 0.06% diaminobenzidine (Walter, Kiel, Germany) and 0.01 vol% H2O2.

Finally, the slides were washed with tap water and then counterstained with haematoxylin. The specimens were examined with an Olympus light microscope (AX80T RF-2, Olympus Corporation, Tokyo, Japan) and a digital color camera (Fujix HC-2500; Fujifilm Co. Ltd, Osaka, Japan) together with an imaging application software (Win Roof Ver. 5.5.0, Mitani Corp, Tokyo, Japan).

Results

In first, we analyzed IPA gene network search by the top functions of connective tissue disorders and genetic disorders, and found the network with up-regulation of CCL2 gene expression, shown in Fig.1. Canonical pathway analysis was also carried out using IPA database and found that TNF and IL-1 signaling...
stimulated CCL2 gene expression through NF-κB pathway (Fig. 2).

Then, we searched the raw intensity signals of mRNA levels of the CCL2 gene from DNA microarray analysis results with “Present” and “Absent” flags. Among the genes analyzed, CCL2 gene was presented with 1.7-fold higher level in *C. albicans*-infected gingival tissues shown in Table 1. The scatter plot analysis result of DNA microarray analysis was shown in Fig. 3. *C. albicans* infection altered many gene expressions in gingival tissues when compared with a control including CCL2 gene.

Next, RT-PCR experiments were carried out to confirm DNA microarray result of the increase of CCL2 mRNA level. Fig. 4 shows that higher level of CCL2 amplified DNA band was detected in gingival tissues infected by *C. albicans* compared to control. In contrast, the mRNA levels of
Table 1 Raw intensity signal and Flag of genes in DNA mic

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<tr>
<th>Gene</th>
<th>Hour</th>
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<th>Flag</th>
<th>C. albicans Raw signal</th>
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<td></td>
<td>(Flag*)</td>
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<td>(Flag*)</td>
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<td>31.2 (P)</td>
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Flag (P) or (A) indicate the reliability of the data according to present (P) or absent (A) of gene expression in GeneChip. * Fold, fold change by normalization against the median of the corresponding to control.

Fig. 2 IPA Canonical pathway analysis

Fig. 3 Scatter plot of mRNA levels of CCL2 gene.

Fig. 4 RT-PCR analysis of CCL2 gene expression. An ethidium bromide staining pattern of the amplified polymerase chain reaction products using agarose gel electrophoresis is shown. 1, control (24-hr); 2, C. albicans (24-hr); 3, control (36-hr); 4, C. albicans (36-hr).
GAPDH, the housekeeping control, shows not big change between *C. albicans* infection and control.

Finally, to examine the phenotypic expression of CCL2 protein, we performed the immunostaining in *C. albicans*-infected rat gingival tissue. As shown in Figure 5A, the invasion of *C. albicans* cells into the epithelium of rat gingival tissue. Furthermore, the *C. albicans*-infected area of epithelium in rat showed more CCL2 protein expressions in the region of *C. albicans* invasion (Fig. 5B).

**Discussion**

In the present study, we demonstrated an induction of CCL2 gene expression and the elevated CCL2 protein production in the rat gingival tissues by *C. albicans* infection.

It is well known that oral epithelial cells can take part in inflammatory processes, both protective and destructive, through signals that are essential for initiating and amplifying inflammatory responses to the infection (12, 13). Epithelium is considered to play a role in host defense, including antigen presentation (14–16), and the production of cytokines/chemokines in response (17, 18).

Periodontitis is a disease resulting in the destruction of tissues supporting the teeth, subsequent pocket formation, and ultimate tooth loss. It is an inflammatory response in gingival and connective tissues in response to a mixed bacterial infection originating in the subgingival plaque. Jøensviv et al. (19) investigated that *C. albicans* could have a role in the infrastructure of periodontal microbial plaque and its adherence to the periodontal tissues. *C. albicans* may thus have a role in the immune evasion of the plaque in periodontal infections and in the provocation of destructive inflammation in the underlying tissues. IL-1 and TNF induce expression of other mediators that amplify the inflammatory response and lead to production of chemokines (20). IPA canonical pathway analysis result shown in Fig. 4 agreed to this report.

CCL2 is a chemokine belonging to the CC subfamily, also known as monocyte chemoattractant protein-1 (MCP-1), and is produced by macrophages, endothelia, synovial fibroblasts, and chondrocytes. CCL2 stimulates the chemotaxis of monocytes and also several cellular events associated with chemotaxis thus causes recruitment of inflammatory cells (21). In fact, the major periodontal pathogen *Porphyromonas gingivalis* stimulated CCL2 expression in human coronary artery endothelial cells and may cause the development of subsequent atherosclerosis (22).

Furthermore, the CCL2 concentration increased proportionally with the severity of periodontal disease showing positive correlation of CCL2 with clinical parameters (23). Gingival crevicular fluid CCL2 levels increased progressively with the progression of disease and decreased after treatment of periodontitis, thus CCL2 can be considered as an inflammatory biomarker in periodontal disease and also deserves further consideration as a therapeutic target (24).

More recently, Goto et al. (reported that hyperocclusion induced CCL2 expression in periodontal ligament tissues and may promote chemotaxis and osteoclastogenesis (25).

Taking together with our findings, the enhancement of CCL2 expression by *C. albicans* infection might involve in
the initiation and/or amplification of inflammatory responses to the periodontitis.

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