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

Noboru Kuboyama¹, Eriko Hayakawa², Manabu Yaguchi³, Takato Nomoto³, and Yoshimitsu Abiko²

Departments of ¹Oral Molecular Pharmacology, ²Biochemistry and Molecular Biology, and ³Dentistry for the Disabled, Nihon University School of Dentistry at Matsudo, Matsudo, Chiba 271-8587 Japan

Article History

Received 5 June 2012

Accepted 7 June 2012

Keywords :

Candida albicans, rat, gingival epithelium, CCL2

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- κ B 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-1 α (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,

Correspondence to :

Yoshimitsu Abiko

E-mail : abiko.yoshimitsu@nihon-u.ac.jp

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°C for 1 hour. Total RNA was extracted with the Ambion RNA queous 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, 3.5 µg 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 GeneChip 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-Gene™ 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°C for 15 s to activate the Taq DNA polymerase, and then performed at 95°C for 5 s and 56°C for 15 s for 40 cycles. DNA primer sequences were: 5'-gtgctgaccccaataaggaa-3' (forward primer for CCL2 gene) and 5'-tgagtggtgttggaaga-3' (reverse primer for CCL2 gene) (predicted size=185 bp); and 5'-atcaccatcttcaggag-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 diaminobenzidine (Kirkegaard & Perry, Gaithersburg, MD, USA) as a chromogen using rabbit polyclonal anti-CCL2 antibody (ABCAM, 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

Fig. 1 IPA gene network analysis.

stimulated CCL2 gene expression through NF- κ B 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

Fig. 2 IPA Canonical pathway analysis

Table 1 Raw intensity signal and Flag of grnes in DNA mic

Gene	Hour	Control		<i>C. albicans</i>		Fold
		Raw signal	(Flag*)	Raw signal	(Flag*)	
<i>CCL2</i>	12	34.1	(P)	31.2	(P)	0.9
	24	31.3	(P)	52.3	(P)	1.7
	36	33.2	(P)	40.6	(P)	1.2
<i>IL-1 α</i>	24	45.6	(P)	57.4	(P)	1.3
<i>IL-1 β</i>	24	47.3	(P)	283.5	(P)	8.1
<i>IL-1R1</i>	24	126.7	(P)	186.1	(P)	1.3
<i>IL-1R2</i>	24	106.8	(P)	149.8	(P)	1.4
<i>TNF- α</i>	24	37.2	(P)	52.4	(P)	1.4
<i>TNFR1A</i>	24	343.2	(P)	381.8	(P)	1.1
<i>TNFR1B</i>	24	121.5	(P)	254.3	(P)	2.1
<i>NF-κ B1</i>	24	279.8	(P)	371.15	(P)	1.3

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. 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).

Fig. 5 Immunostaining of CCL2 protein expression. (A) PAS staining of rat gingival tissue. (B) Immunostaining of CCL2 in rat gingival tissue.

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. J?rvensivu 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 know 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.

Acknowledgments

This study was supported in part by the "Academic Frontier" Project for Private Universities: a matching fund subsidy from Ministry of Education, Culture, Sports, Science and Technology, 2007-2011, and by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (B21390497). The authors thank Dr. Lin Zhang for technical assistance.

References

1. Samaranayake LP, MacFarlane TW: *Oral Candidosis*, 1990, Wright Publications Ltd. Holmfirth, UK..
2. Lynch DP: Oral candidiasis. History, classification, and clinical presentation. *Oral Surg Oral Med Oral Pathol*, 78: 189-193, 1994.
3. Edwards JE Jr, Lehrer RI, Stiehm ER, Fischer TJ, Young LS: Severe candidal infections: clinical perspective, immune defense mechanisms, and current concepts of therapy. *Ann Intern Med*, 89: 91-106, 1978.
4. Challacombe SJ: Immunologic aspects of oral candidiasis. *Oral Surg Oral Med Oral Pathol*, 78: 202-210, 1994.
5. Dongari-Bagtzoglou A, Kashleva H: *Candida albicans* triggers interleukin-8 secretion by oral epithelial cells. *Microb Pathog*, 34: 169-177, 2003.
6. Egusa H, Nikawa H, Makihira S, Jewett A, Yatani H, Hamada T: Intercellular adhesion molecule 1-dependent activation of interleukin 8 expression in *Candida albicans*-infected human gingival epithelial cells. *Infect Immun*, 73: 622-626, 2005.
7. Dongari-Bagtzoglou A, Kashleva H, Villar CC: Bioactive interleukin-1 α is cytolytically released from *Candida albicans*-infected oral epithelial cells. *Med Mycol*, 42: 531-541, 2004.
8. Mostefaoui Y, Bart C, Frenette M, Rouabhia M: *Candida albicans* and *Streptococcus salivarius* modulate IL-6, IL-8, and TNF- α expression and secretion by engineered human oral mucosa cells. *Cell Microbiol*, 6: 1085-1096, 2004.
9. Tanaka Y, Zhang L, Ikuta T, Omori J, Omine H, Mega J, Kuboyama N, Abiko Y: TNF- α Expression in Oral *Candida albicans*-Infected Human Gingival Epithelial Cells. *Int J Oral-Med Sci*, 10: 77-82, 2011.
10. Omori J, Ikuta T, Tsushima K, Aoki A, Kuboyama N and Abiko Y: Stimulation of mesenchymal-epithelial transition factor (*c-Met*) expression in *Candida albicans*-infected human gingival epithelial cells and rat gingival epithelium. *Int J Oral-Med Sci*, 10: 2012.
11. Ikuta T, Bhawal UK, Tsushima K, Aoki A, Kuboyama N, Abiko Y: Identification by DNA microarray of genes involved in *Candida albicans*-treated gingival epithelial cells *J Oral Pathol Med*, 2012, in press.
12. Villar CC, Kashleva H, Mitchell AP, Dongari-Bagtzoglou A: Invasive phenotype of *Candida albicans* affects the host proinflammatory response to infection. *Infect Immun*, 73: 4588-4595, 2005.
13. Page RC: The role of inflammatory mediators in the pathogenesis of periodontal disease. *J Periodontol Res*, 26: 230-242, 1991.
14. Gonella PA, Neutra MR: Membrane-bound and fluidphase macromolecules enter separate prelysosomal compartments in absorptive cells of suckling rat ileum. *J Cell Biol*, 99: 909-917, 1984.
15. Gonella PA, Wilmore DW: Co-localization of class II antigen and endogenous antigen in the rat enterocyte. *J Cell Sci*, 106: 937-940, 1993.
16. Wira CR, Rossoll RM. Antigen-presenting cells in the female reproductive tract: Influence of the estrous cycle on antigen presentation by uterine epithelial and stromal cells. *Endocrinology*, 136: 4526-4534, 1995.
17. Hedges SR, Agace WW, Svanborg C: Epithelial cytokine responses and mucosal cytokine networks. *Trend Microbiol*, 3: 266-270, 1995.
18. Hedges SR, Agace WW, Svensson M, Sjogren A, Ceska M, Svanborg C: Uroepithelial cells are part of a mucosal cytokine network. *Infect Immun*, 62: 2315-2321, 1994.
19. Järvensivu A, Hietanen J, Rautemaa R, Sorsa T, Richardson M: Candida yeasts in chronic periodontitis tissues and subgingival microbial biofilms *in vivo*. *Oral Dis*, 10: 106-112, 2004.
20. Deo V, Bhongade ML: Pathogenesis of periodontitis: role of cytokines in host response. *Dent Today*, 29: 60-62, 2010.
21. Deshmane SL, Kremlev S, Amini S, Sawaya BE :Monocyte chemoattractant protein-1 (MCP-1): an overview. *J Interferon Cytokine Res*, 29: 313-326, 2009.
22. Maekawa T, Takahashi N, Honda T, Yonezawa D, Miyashita H, Okui T, Tabeta K, Yamazaki K: *Porphyromonas gingivalis* antigens and interleukin-6 stimulate the production of monocyte chemoattractant protein-1 via the upregulation of early growth response-1 transcription in human coronary artery endothelial cells. *J Vasc Res*, 474: 346-345, 2010.
23. Pradeep AR, Daisy H, Hadge P: Serum levels of monocyte chemoattractant protein-1 in periodontal health and disease. *Cytokine*, 472: 77-81, 2009.
24. Pradeep AR, Daisy H, Hadge P: Gingival crevicular fluid levels of monocyte chemoattractant protein-1 in periodontal health and disease. *Arch Oral Biol*, 54: 503-509, 2009.
25. Goto KT, Kajiya H, Nemoto T, Tsutsumi T, Tsuzuki T, Sato H, Okabe K: Hyperocclusion stimulates osteoclastogenesis via CCL2 expression. *J Dent Res*, 906: 793-798, 2011.