TNF-α Expression in Oral Candida albicans-Infected Human Gingival Epithelial Cells

Yoko Tanaka,1 Lin Zhang,2 Tonami Ikuta,2 Joutaro Omori,2 Hirotaka Omine,3 Junichi Mega,1 Noboru Kuboyama,4 and Yoshimitsu Abiko2

Departments of 1Dentistry for the Disabled, 2Biochemistry and Molecular Biology, 3Maxillo-Facial Orthodontics, and 4Pharmacology, Nihon University School of Dentistry at Matsudo, Matsudo, Chiba 271-8587, Japan

Correspondence to:
Yoshimitsu Abiko
E-mail: abiko.yoshimitsu@.nihon-u.ac.jp

Abstract
The presence of yeasts in periodontal pockets is well documented and Candida albicans is the species most commonly isolated from the oral cavity. The immune response and antifarindal activity of oral epithelial cells play a key role in host defense against C. albicans infection. Human gingival epithelial cells were primarily cultured from healthy human gingival tissues and challenged with C. albicans ATCC90029. After 8 hours, total RNA was extracted and mRNA levels were analyzed using Affymetrix GeneChip (Human Genome U133 plus 2.0 Array, ca. 47,000 genes). Numerous genes showed altered gene expression, and among these, a pro-inflammatory cytokine, tumor necrotizing factor alpha (TNF-α), was up-regulated by more than two-fold over normal levels after C. albicans infection. Altered mRNA levels on GeneChip results were confirmed by reverse transcription-polymerase chain reaction (RT-PCR) and real-time RT-PCR. Rat gingiva was infected with C. albicans cells and immunohistochemical examination for TNF-α production was then carried out. Stronger immunoreactivity against TNF-α was observed in rat gingival epithelium infected with C. albicans.

Keywords:
Candida albicans, gingival epithelial cells, TNF-α

Introduction
The presence of yeasts in periodontal pockets has been documented 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 antifarindal activity of oral epithelium play a key role in 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) reported that C. albicans infection enhanced IL-6, IL-8 and TNF-α gene expression in oral mucosa epithelial cells. However, little is known about the expression of pro-inflammatory cytokines, such as TNF-α, in human gingival epithelial cells. Moreover, TNF-α expression in gingival tissues has not been assessed in in vivo experimental models of C. albicans infection. To clarify the involvement of TNF-α in human gingival epithelial (HGE) cells in local defenses against C. albicans infection, we examined gene expression profiles of the TNF gene family in C. albicans-infected HGE cells in primary cell culture system. We also examined TNF-α protein expression in C. albicans-infected rat gingival epithelium using immunohistochemistry.
Materials and Methods

Cell culture
Healthy human gingival tissues were obtained when teeth were extracted by orthodontic therapy. Patients gave informed consent for the surgery and for the use of their tissue in research. The study was approved by the Ethics Committee of Nihon University School of Dentistry at Matsudo (No. 04–014). Explants were treated with 6 mg/ml dispase (Sigma Chemical, St. Louis, MO, USA) in phosphate-buffered saline at 4 °C for 16 hours in order to separate the epithelial layer from the underlying fibrous connective tissue. The epithelial layer was then cut and incubated at 37 °C in trypsin–EDTA (0.05% trypsin, 0.53 mM EDTA) for 10 minutes, and was repeatedly pipetted in order to prepare a single–cell suspension. Cell pellets were collected and resuspended in EpLife medium with supplemental S7 and antibiotics (penicillin, streptomycin, and amphotericin B) (Cascade Biologics, Portland, OR, USA). Cells were plated on Biocoat Collagen I cellware (BD Biosciences, Bedford, MA, USA). Cultures were kept at 37 °C in a humidified incubator in the presence of 95% air and 5% CO₂. Subculturing was performed when cells reached 70–80% confluence, and cells were used within five passages. HGE cells were seeded at $1 \times 10^8$ cells/dish and medium was replaced with the new medium containing $1.5 \times 10^7$ cells/ml *C. albicans* ATCC90029, followed by incubation at 37 °C. Eight hours later, total cellular RNA was isolated using an RNeasy kit. Total RNA samples were subjected to GeneChip analysis.

Animal experiment
The gingival tissue of Sprague–Dawley rats was infected with *C. albicans* (ATCC90020). The same volume of saline was given to the control group. Rats were maintained and handled in accordance with the Guidelines on the Care and Use of Laboratory Animals of Nihon University, School of Dentistry at Matsudo (#08–0004). *C. albicans* infection was carried out as reported previously (9). Twenty–four hours after infection, rats were injected with sodium pentobarbital and their gingival tissues were removed and subjected to immunohistochemistry.

Gene Chip analysis
Total RNA samples (100 ng) were subjected to two–cycle target labeling according to the Affymetrix instructions. Antisense complimentary RNA (cRNA) derived from double–stranded complimentary DNA (cDNA) was labeled in the presence of biotinylated deoxyribonucleotide triphosphate (dNTP) derivatives to produce cRNA probes. Probes were then fragmented and hybridized onto the Gene Chip Mouse Genome 430 2.0 Array. Washing and staining were performed for each sample using a Gene Chip Fluidics station 450 (Affymetrix, Santa Clara, CA, USA). Chip performance, background levels, and the presence or absence of signals were assessed using Microarray Suite software (Affymetrix). Probe intensities were normalized against each chip in a given set. 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 (Silicone Genetics, Redwood, CA, USA).

Reverse transcription–polymerase chain reaction and real–time polymerase chain reaction analysis
Reverse transcription–PCR (RT–PCR) and real–time PCR were carried out using a DNA thermal analyzer (Rotor–Gene™ 6000 ; Corbett Life Science, Sydney, Australia). For RT–PCR, amplification products were electrophoresed on agarose gels, and were subsequently stained with ethidium bromide. Real–time PCR was performed using SYBR Premix Ex Taq™ (Perfect Real–Time PCR, Takara, Ohtsu, Japan) and a green PCR kit (Qiagen GmbH, Düsseldorf, Germany). Amplification by PCR was started with an initial incubation at 95 °C for 15 s to activate the Taq DNA polymerase, followed by 95 °C for 5 s and 56 °C for 15 s for 40 cycles. To calculate the fold changes in gene expression, the initial template concentration was derived from the cycle number at which the fluorescent signal crossed the threshold in the exponential phase of the real–time PCR. The
mRNA copy unit was given by the cycle threshold value from the fluorescent signal of all samples, including the standard curve and target genes, in accordance with the method provided by Corbett Life Science using RCx43-Gene™ 6000 software. Details were as described in the operation manual (version 1.7.40, 2006). The respective primer sequences were; 5'-GAAACCTGGGATTCAAGA TG-3' (forward primer for TNF-α gene); 5'-GTCT CAAGGAGTGCTGGAAAC-3' (reverse primer for TNF-α gene), (predicted size=246 bp); 5'-AAAAG CCTGGAAGTGACGAAG-3' (forward primer for TNFRSF1A); and 5'-GGGACTGAAGCTGGGT TG-3' (reverse primer for TNFRSF1A gene), (predicted size=267 bp); 5'-ATCACATCTCCAG GAG-3' (forward primer for GAPDH); and 5'-ATC GACTGTGGTCAGG-3' (reverse primer for GAPDH gene), (expected product size=318 bp). Values were calculated as means±standard deviation (SD). Comparisons were made between two groups by Student's t-test.

**Immunohistochemistry**

Formalin-fixed, paraffin-embedded sections were deparaffinized in xylene and rehydrated in a graded alcohol series and water. To examine the invasion of *C. albicans* into rat gingival epithelium, the gingival tissues were excised using aseptic techniques and immediately fixed in 10% (vol/vol) neutral buffered formalin, followed by staining with hematoxylin–eosin (HE) and periodic acid–Schiff (PAS) for observation by light microscopy.

For immunostaining, sections were incubated with 0.3% H2O2 in methanol for 30 min in order to inhibit endogenous peroxidase activity, and sections were washed with PBS three times for 5 min. Sections were then incubated with the monoclonal primary antibody (Mouse anti-Rat TNF-α; Yanaihara Institute Inc. (Fujinomiya, Shizuoka, Japan) 1: 1000, diluted in 1% BSA–PBS) for 60 min at 37°C, followed by washing three times with PBS. Control staining was performed by omitting primary antibody. Sections were incubated with biotinylated anti-mouse IgG secondary antibody solution (1: 200 in 0.1% PBS–BSA containing 2% normal rat serum) for 1 h at room temperature. After three washes with PBS for 30 min, sections were incubated with streptavidin–Horse Radish Peroxidase; 1: 200, diluted with 1% BSA–PBS for 1 h at room temperature. Colorization was carried out using diaminobenzidine tetrahydrochloride/PBS with H2O2. Finally, slides were washed with tap water, and were then counterstained with hematoxylin. 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 imaging application software (Win Roof Ver. 5.5.0, Mitani Corp, Tokyo, Japan).

**Results**

We examined the mRNA levels of the TNF gene family by Affymetrix GeneChip analysis using “Present” and “Absent” flags. Among the genes analyzed, only TNF-α (*TNFSF2*) showed 2.5-fold higher

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene Bank ID</th>
<th>Common Name</th>
<th>Product Description</th>
<th>Control</th>
<th>Candida</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFSF2</td>
<td>NM_000594</td>
<td>TNFα</td>
<td>TNFα</td>
<td>49.2(P)</td>
<td>122.8(P)</td>
<td>2.5</td>
</tr>
<tr>
<td>TNFRSF1A</td>
<td>NM_001065</td>
<td>TNFR1</td>
<td>TNF receptor 1</td>
<td>429.7(P)</td>
<td>423.0(P)</td>
<td>1</td>
</tr>
<tr>
<td>TNFRSF3</td>
<td>NM_002342</td>
<td>TNFR-RP</td>
<td>TNF receptor 3</td>
<td>265.3(P)</td>
<td>221.1(P)</td>
<td>0.8</td>
</tr>
<tr>
<td>TNFRSF5</td>
<td>X60592</td>
<td>TNFRSF5</td>
<td>TNF receptor 5</td>
<td>54.2(P)</td>
<td>52.8(P)</td>
<td>1</td>
</tr>
<tr>
<td>TNFRSF6</td>
<td>X83493</td>
<td>TNFRSF6</td>
<td>TNF receptor 6</td>
<td>224.9(P)</td>
<td>217.5(P)</td>
<td>1</td>
</tr>
<tr>
<td>TNFRSF10A</td>
<td>W65310</td>
<td>TNFRSF10A</td>
<td>TNF receptor 10a</td>
<td>352.7(P)</td>
<td>238.1(P)</td>
<td>0.7</td>
</tr>
<tr>
<td>TNFRSF10B</td>
<td>AF153687</td>
<td>DR5 ; CD282</td>
<td>TNF receptor 10b</td>
<td>1000.1(P)</td>
<td>1130.0(P)</td>
<td>1.1</td>
</tr>
<tr>
<td>TNFRSF10D</td>
<td>AI783556</td>
<td>TNFRSF10D</td>
<td>TNF receptor 10d</td>
<td>156.5(P)</td>
<td>127.2(P)</td>
<td>0.8</td>
</tr>
<tr>
<td>TNFRSF11A</td>
<td>AW026379</td>
<td>TNFRSF11A</td>
<td>TNA receptor 11a</td>
<td>21.6(P)</td>
<td>14.1(P)</td>
<td>0.7</td>
</tr>
<tr>
<td>TNFRSF11B</td>
<td>BF433902</td>
<td>TNFRSF11B</td>
<td>TNF receptor 11b</td>
<td>6.7(M)</td>
<td>5.2(A)</td>
<td>0.8</td>
</tr>
</tbody>
</table>
levels in *C. albicans*-infected HEE shown in Table 1. A scatter plot of the DNA microarray analysis results after normalization against the median of the corresponding control is shown in Fig. 1. *C. albicans* infection altered the expression of numerous genes in HGE cells when compared with controls. Among the altered genes, TNF-α increased by 2.5-fold, whereas other genes of the TNF family, including TNFSF1A (TNF receptor 1), showed no changes.

RT-PCR experiments were then carried out in order to confirm the DNA microarray results regarding the increases in TNF-α mRNA levels. As shown in Fig. 2, a significantly higher level of TNF-α DNA was detected in HGE infected by *C. albicans*, as compared to controls. In contrast, mRNA levels of GAPDH, the housekeeping control, showed no changes between *C. albicans* infection and controls in HGE.

In other experiments, in order to confirm the elevated mRNA levels of TNF-α, real-time PCR analysis was carried out. The increase in TNF-α gene expression levels was confirmed to be significantly different between the *C. albicans*-infected cells and controls, as shown in Fig. 3.

Finally, in order to examine the phenotypic expression of TNF-α, we performed immunostaining for TNF-α in *C. albicans*-infected rat gingival tissue. As shown in Fig. 4, we demonstrated infection and invasion by *C. albicans* into the epithelium of rat gingival tissue. Furthermore, the *C. albicans*-infected epithelium in rats showed elevated TNF-α protein expression in the regions of *C. albicans* invasion.
Discussion

In the present study, we demonstrated significant induction of TNF-α mRNA in the HGE cells in the presence of C. albicans, and we demonstrated elevated TNF-α production in the rat epithelium after infection by C. albicans.

It is 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 infection (10, 11). Epithelial cells are now considered to play a role in host defense, including antigen presentation (12–14), and the production of cytokines/chemokines in response to infection (15, 16).

Periodontitis is a disease resulting in the destruction of tissues supporting the teeth, subsequent pocket formation, and ultimately, tooth loss. It is an inflammatory response in the gingival and connective tissues to a mixed bacterial infection originating in the subgingival plaque. Järvensivu et al. (17) reported that C. albicans plays a role in the infrastructure of periodontal microbial plaque and its adherence to periodontal tissues. C. albicans may thus have a role in the immune evasion of 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, such as prostaglandins, and this leads to production of lytic enzymes and stimulates the production of chemokines (18). Recent studies have demonstrated that cytokines, such as TNF-α, can be harmful in the context of tissue destruction, and that they play important roles in the control of periodontal infection (19).

Based on these findings, the enhancement of TNF-α expression by C. albicans infection appears to be involved in the initiation and/or amplification of inflammatory responses to periodontitis.

Acknowledgments

This study was supported in part by the “Academic Frontier” Project for Private Universities: a matching fund subsidy from the Ministry of Educa-
tution, 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 would like to thank Drs. Ying Li and Jian Zhao for their technical assistance.

References