Celecoxib suppresses lipopolysaccharide-stimulated oral squamous cell carcinoma proliferation *in vitro* and *in vivo*

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Abstract. Periodontitis is one of the most common chronic oral inflammatory conditions worldwide and is associated with a risk of developing oral squamous cell carcinoma (OSCC). *Porphyromonas gingivalis* is a major pathogen in periodontitis, and its lipopolysaccharide (LPS) promotes the expression of cyclooxygenase-2 (COX-2) in OSCC both *in vivo* and *in vitro*. Celecoxib is a selective COX-2 inhibitor; however, its antitumor effects on *P. gingivalis* LPS-stimulated OSCC and the underlying molecular mechanism remain unclear. To elucidate the association between periodontitis and OSCC, the effect of *P. gingivalis*-derived LPS on OSCC cell proliferation was examined both *in vitro* and *in vivo* in the present study. The expression levels of COX-2 and p53 in OSCC cells with/without celecoxib treatment were determined via western blotting. The therapeutic potential of celecoxib in LPS-stimulated OSCC was evaluated by staining for Ki-67 and p21, as well as with terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling staining. LPS treatment significantly increased OSCC cell proliferation *in vitro*, and celecoxib significantly inhibited cell proliferation *with/without* LPS treatment. Celecoxib treatment of OSCC cells downregulated the protein expression levels of COX-2 compared with untreated cells, but there was little change in p53 expression. In the mouse xenograft model, oral administration of celecoxib significantly suppressed tumor growth, reduced the expression of Ki-67, increased the apoptosis index and induced p21 expression *with/without* LPS treatment. The results from the present study demonstrate that *P. gingivalis’* LPS can stimulate tumor growth by interacting with OSCC cells. In conclusion, these results suggest that celecoxib could be used for the effective prevention and treatment of LPS-stimulated OSCC.

Introduction

Oral squamous cell carcinoma (OSCC) is one of the most common malignant epithelial tumors that arises in the oral cavity (estimated 263,900 new cases in 2008 worldwide) (1). Although combination treatments employing surgical resection and adjuvant treatments such as radiotherapy and/or chemotherapy have improved, the disease-free and overall survival rates in advanced OSCC have not improved (2,3).

Periodontitis is a common chronic oral inflammatory disease that causes destruction of periodontal tissues (4). Recent studies have demonstrated that patients with periodontal disease have a 2-5-fold greater risk of developing OSCC (4). One of the major pathogens in periodontal disease is the gram-negative, anaerobic bacterium *Porphyromonas gingivalis*. It can adhere to both epithelial cells and gingival fibroblasts, and induce the expression of pro-inflammatory cytokines involved in the progression of periodontitis. Its primary virulence factor is lipopolysaccharide (LPS) (5), which has recently been revealed to play an important role in the migration, invasion, lymphangiogenesis and metastasis of various types of malignant tumor, including OSCC (6,7).

The induction of cyclooxygenase-2 (COX-2), an enzyme that promotes cell proliferation and invasion, and suppresses apoptosis (8), is considered to be one of the key mechanisms by which LPS affects OSCC cells (9,10). Upregulated COX-2 is frequently observed in head and neck squamous cell carcinoma (HNSCC) (11), where it is associated with a lower survival rate in patients with this disease (12). Inhibition of COX-2 thus represents a potential approach for OSCC therapy.

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Celecoxib is a selective COX-2 inhibitor that has recently been demonstrated to possess antitumor effects in HNSCC (13,14); however, its method of administration in OSCC has not yet been established.

In the present study, to elucidate the association between chronic periodontitis and OSCC progression, the effect of P. gingivalis-derived LPS on OSCC cell proliferation was examined both in vitro and in vivo. The antitumor effects of celecoxib in an LPS-stimulated OSCC xenograft were evaluated by assessing cell proliferation, apoptosis and the cell cycle. Finally, the present study discusses the molecular mechanisms underlying chronic periodontitis and OSCC, and the therapeutic potential of celecoxib.

Materials and methods

Cell culture. The human OSCC cell line HSC-3 (Japanese Cancer Research Resources Bank) was maintained in α-minimum essential medium (α-MEM; Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Biowest), 100 IU/ml penicillin and 100 µg/ml streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C in a 5% CO2 atmosphere.

Cell viability assay. The effect of celecoxib (Combi-Blocks, Inc.) or P. gingivalis’ LPS (InvivoGen, Inc.) on cell viability was assessed using an MTS assay. Celecoxib was dissolved in dimethyl sulfoxide (DMSO; Wako Pure Chemical Industries, Ltd.). The levels of DMSO were minimized to avoid any potential DMSO-associated toxic effects in the MTS assay. In all cases, the final DMSO concentration was <0.1% in the cell cultures. HSC-3 cells were plated at a density of 5,000 cells/well in 96-well plates and incubated for 48 h at 37°C. Cell cultures were exposed to celecoxib (0, 100 and 200 µM), LPS (10 µg/ml), or a combination of the two, for 24 or 48 h. Following this, 20 µl of CellTiter 96 AQueous One Solution Reagent (Promega Corporation) was added to each well and incubated for 2 h at 37°C. The absorbance at 490 nm in each well was then determined (SpectraMax M5; Molecular Devices, LLC).

Western blot analyses of COX-2 and p53. HSC-3 cells were lysed in RIPA lysis buffer (Santa Cruz Biotechnology, Inc.) containing 2 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovandate and 2% protease inhibitor cocktail. The protein concentration was quantified by DC Protein Assay (Bio-Rad Laboratories, Inc.). Protein samples (10 µg/lanne) were separated using 10-20% gradient gels and electro-transferred to Immun-Blot PVDF Membranes (Bio-Rad Laboratories, Inc.). Membranes were blocked using EzBlock Chemi (1:5; ATTO Corporation) for 1 h at room temperature and then probed using the appropriate primary antibodies, including anti-COX-2 (1:1,000; catalog no. ab15191; Abcam), anti-p53 (1:1,000; catalog no. ab1101; Abcam) and anti β-actin (1:1,000; catalog no. 8H10D10; Cell Signaling Technology, Inc.) overnight at 4°C. After washing with Tris-buffered saline with Tween 20 (TBS-T) three times, the membranes were incubated with a horseradish peroxidase (HRP)-conjugated AffiniPure goat anti-mouse IgG antibody (1:10,000; catalog no. 115-035-072; Jackson ImmunoResearch Laboratories, Inc.) for anti-p53 and anti β-actin or an HRP-conjugated AffiniPure goat anti-rabbit IgG antibody (1:10,000; catalog no. 111-035-144; Jackson ImmunoResearch Laboratories, Inc.) for anti-COX-2 for 1 h at room temperature. Chemiluminescent signals were developed with Western Lightning ECL Pro (PerkinElmer, Inc.) and detected using a cooled charge-coupled device camera (LAS 4000 Mini; GE Healthcare Life Sciences).

Nude mouse tumor model. A total of 32 5-week-old female nude BALB/c nu/nu mice (21.4±1.2 g; CLEA Japan, Inc.) were maintained at 21-25°C and 40-70% humidity in a 12-h dark/light cycle, with continuous free access to food and water. For stimulation, HSC-3 cells were exposed to 10 µg/ml LPS for 48 h before implantation. HSC-3 and LPS-stimulated HSC-3 cells (5x105 cells in 50 µl α-MEM) were then mixed with an equal volume of Matrigel (BD Biosciences) and injected into the flanks of mice during a short period of anesthesia with 2% isoflurane (Abbott Pharmaceutical Co., Ltd.). Tumor-bearing mice were then randomly divided into four groups (n=8 in each group): i) A control group; ii) an LPS-treated group; iii) a celecoxib-treated group; and iv) an LPS + celecoxib-treated group. In all cases, tumors were allowed to grow to ~60 mm3 prior to the treatment. The celecoxib-treated group and LPS + celecoxib-treated group were fed powder feed containing 1,500 ppm celecoxib, as previously described (15); the control and LPS-treated groups were fed common powder feed alone. Mice were assessed twice a week for 28 days, and tumor growth was determined by estimating tumor volume, using the formula 0.5 x length x width2, as previously described (16). Each cage contained four mice; the dietary intake of powdered feed per day in the cage was measured to calculate an estimate of the dietary intake for each mouse. The limitation associated with this method of drug administration was that dietary intake was not an actual dose, but estimated dose. One mouse was euthanized when bleeding from the ulceration of the tumor was observed. On day 28 post-administration, the mice were euthanized using carbon dioxide gas (20%/min gradual displacement) and monitored for 5 min to confirm cardiac arrest, and the tumors were removed along with the surrounding tissue and overlying skin (2 mm from the tumor) for subsequent histological examination as previously described (16). The specimens were fixed immediately with 4% paraformaldehyde for 24 h at room temperature and embedded in paraffin. All animal experiments were approved by the Animal Ethics Committee of the University of Fukui (no. 29105) and performed in accordance with the Guide for the Care and Use of Laboratory Animals (published by the National Institutes of Health) (17).

Immunohistochemistry. The paraffin-embedded tissues were sliced into 4 µm-thick sections. Ki-67 and p21 were stained using indirect immunoperoxidase staining (ImmPRESS Reagent kit; Vector Laboratories, Ltd.). Tissue sections were deparaffinized in Clear Plus (Falma, Co., Ltd.), dehydrated in 100% ethanol for 15 min at room temperature and autoclaved in 1 mM EDTA 2Na and 10 mM Tris buffer (pH 9.0) at 95°C for 30 min for antigen retrieval. Endogenous peroxide activity was eliminated by treatment with 0.3% H2O2 in methanol for 30 min at room temperature. The ImmPRESS reagent with 2.5% normal horse serum (undiluted; Vector Laboratories, Ltd.) was used to block non-specific immunoreactions.
for 10 min at room temperature. After incubation with a primary monoclonal rabbit anti-human antibody against either Ki-67 (1:100; catalog no. ab16667) or p21 (1:100; catalog no. ab109520) (both from Abcam) for 2 h at room temperature, the sections were incubated with ImmPRESS polymer anti-rabbit IgG reagent (undiluted; cat. no. MP-7800; Vector Laboratories, Ltd.) for 30 min at room temperature. Immunoreactivity was visualized using 3,3′-diaminobenzidine (Dojindo Molecular Technologies, Inc.) for 5 min at room temperature. The sections were rinsed in PBS between all steps. Ki-67-stained or p21-stained tissue sections were observed under an Olympus AX80 light microscope (Olympus Corporation) at x400 magnification, and the number of Ki-67- or p21-positive cells were counted in each individual microscopic field. At least five microscopic fields per section were used for the subsequent analysis.

Cell death assay. The terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling (TUNEL) method was used to evaluate apoptosis using the in situ Apoptosis Detection kit (Takara Bio, Inc.). Briefly, the sections were deparaffinized for 15 min, dehydrated in 100% ethanol for 15 min and permeabilized using 10 µg/ml proteinase K (Invitrogen; Thermo Fisher Scientific, Inc.) for 10 min at room temperature. Endogenous peroxide activity was blocked with 3% H₂O₂, for 5 min at room temperature. The sections were incubated with 50 µl labeling reaction mixture (consisting of TdT Enzyme 5 µl + Labeling Safe Buffer 45 µl) for 90 min at 37°C and reacted with 70 µl anti-FITC horseradish peroxidase (undiluted; cat. no. MK503; Takara Bio, Inc.) for 30 min at 37°C. Immunoreactivity was visualized using 3,3′-diaminobenzidine (Dojindo Molecular Technologies, Inc.). The sections were counterstained with hematoxylin for 5 min at room temperature. The apoptosis index was determined by calculating the ratio of the number of TUNEL-positive cells to the total number of tumor cells (avoiding necrotic tumor areas) from a minimum of five randomly selected microscopic fields in each individual section using an Olympus AX80 light microscope (Olympus Corporation) at x400 magnification as previously described (16).

Statistical analysis. All experiments were independently repeated at least three times. Numerical values are expressed as the mean ± standard deviation. Differences between experimental groups were analyzed using one-way analysis of variance followed by Dunnett's test (cell viability at 24 and 48 h after celecoxib treatment), Turkey-Kramer multiple comparison test (cell viability, tumor volumes, mouse body weights, the expression levels of Ki-67 and p21 and apoptosis index in response to celecoxib with/without LPS treatment) and unpaired Student's t-test (cell viability 24 and 48 h after LPS treatment and the COX-2/β-actin ratio). P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of celecoxib on OSCC cells in vitro. LPS treatment for 24 and 48 h increased the viability of OSCC-3 cells (P<0.01; Fig. 1A); whereas, celecoxib decreased cell viability in a dose- and time-dependent manner (P<0.01; Fig. 1B), indicating that the cells were sensitive to celecoxib. The proliferation of LPS-treated OSCC-3 cells was significantly inhibited by treatment with celecoxib (100 µM for 48 h) (P<0.01; Fig. 1C). The protein expression levels of COX-2 and p53 with/without celecoxib treatment were also examined in HSC-3 cells via western blotting. Compared with untreated cells, treatment of HSC-3 cells with 100 µM celecoxib downregulated the protein expression levels of COX-2 after 12 h, but there was little change in p53 expression levels (Fig. 1D). The COX-2/β-actin ratios in the HSC-3 cells were significantly decreased by the celecoxib treatment (Fig. 1E).

Effect of celecoxib on OSCC tumor growth in vivo. The antitumor effects of celecoxib were examined in control and LPS-treated OSCC tumors in nude mice. After 28 days of treatment, tumor volumes were significantly decreased in both celecoxib-treated and LPS + celecoxib-treated mice compared with control and LPS-treated mice (P<0.01; Fig. 2A and B). There were no significant differences body weight in the four treatment groups (Fig. 2C). Dietary intake throughout the experimental period (3.7-4.6 g/day) is presented in Table 1.

<table>
<thead>
<tr>
<th>Group</th>
<th>Estimated quantity of dietary intake per day for each mouse, g/day</th>
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<tbody>
<tr>
<td>0-7 days</td>
<td>3.8</td>
</tr>
<tr>
<td>8-14 days</td>
<td>4.1</td>
</tr>
<tr>
<td>15-21 days</td>
<td>4.5</td>
</tr>
<tr>
<td>22-28 days</td>
<td>4.1</td>
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LPS, lipopolysaccharide.

Effect of celecoxib on OSCC cells in vivo. Ki-67 expression levels were significantly decreased in celecoxib-treated and LPS + celecoxib-treated groups when compared with the control and LPS-treated groups (P<0.01) and increased in the LPS-treated group compared with the control group (P<0.01), indicating that cell proliferation was decreased (Fig. 3A and B). Furthermore, TUNEL staining revealed that the apoptotic indices in the celecoxib-treated and LPS + celecoxib-treated groups were significantly higher than in the control and LPS-treated groups (P<0.01), suggesting that celecoxib induced apoptosis in the control and LPS-treated OSCC xenografts (Fig. 4A and B). The apoptotic indices in the LPS + celecoxib-treated group were significantly lower compared with those in the celecoxib-treated group (P<0.01; Fig. 4A and B). Apoptosis was not induced in non-tumorous skin and mucosal tissues surrounding the tumors following celecoxib treatment (Fig. 4C). Furthermore, there were no significant changes in p21 expression levels between the control and LPS-treated groups (Fig. 5A and B), whereas the celecoxib-treated and LPS + celecoxib-treated groups had significantly increased levels

Table 1. Estimated amount of dietary intake of powdered feed with/without celecoxib.
of p21 compared with the control and LPS-treated groups (P<0.01), indicating that celecoxib upregulates p21 expression in OSCC xenografts (Fig. 5A and B). The levels of p21 in the LPS + celecoxib-treated groups were significantly decreased compared in the celecoxib-treated groups (Fig. 5A and B).

**Discussion**

In the present study, *P. gingivalis*-derived LPS was used to investigate the molecular mechanisms underlying periodontitis in the Toll-like receptor (TLR) 4-expressing OSCC cell line...
HSC-3 (7). LPS binds directly to the TLR4/myeloid differentiation factor 2 receptor complex (18) activating the myeloid differentiation factor 88 signaling pathway, which in turn activates mitogen-activated protein kinase (MAPK) and the transcription factor nuclear factor-κB, which play an important role in cell proliferation (19). Previous reports have demonstrated that LPS stimulation of TLR4 promotes breast cancer growth in nude mice (6), and, consistently, LPS caused a significant increase in OSCC cell proliferation in vitro in the present study.

To assess whether COX-2 plays a role in LPS-induced proliferation of OSCC cells, the antitumor effect of the COX-2 inhibitor celecoxib was investigated. Celecoxib significantly decreased the proliferation of OSCC cells and inhibited tumor growth in a xenograft model. The expression of Ki-67 was also decreased, and apoptosis was significantly increased in the celecoxib-treated group. This coincides with previous reports that celecoxib significantly decreases the viability of OSCC cells by inhibiting phosphorylated protein kinase B, cyclin D1 or the epithelial-to-mesenchymal transition, as well as by inhibiting tumor growth in OSCC xenografts (14,20).

In the present study, celecoxib treatment downregulated the protein expression levels of COX-2 in OSCC cells. This...
finding is supported by previous reports that celecoxib attenuates COX-2 expression and inhibits the growth of OSCC cells (21,22). On the other hand, celecoxib has been indicated to inhibit the cell survival of both COX-2-expressing and non-expressing colon carcinoma cells (23), indicating that the effects of celecoxib are independent of COX-2, and there could be other targets/effects that have yet to be defined.

The concentrations of celecoxib used in the present study were much higher than those used in oral administration for humans (24), so the results obtained from the present study may not be directly extrapolatable to humans. However, the antitumor effects of celecoxib have previously been established in epidemiological studies and in clinical trials (24), indicating that celecoxib may be effective even at lower concentrations in humans. Furthermore, no significant weight loss was observed in celecoxib-treated mice in the present study, suggesting that celecoxib did not exhibit toxicity or cause adverse effects. Nonetheless, other studies have suggested that adverse effects may be decreased and controlled using the oral administration of antitumor drugs (25,26), and that celecoxib may be eligible for such application as well. Furthermore, a recent study has revealed that, in HNSCC, a combined metronomic oral celecoxib treatment is advantageous when compared with intravenous single-agent therapy (24); thus, this approach may also improve future results using celecoxib therapy.

In the LPS-stimulated OSCC xenografts, celecoxib upregulated the expression of p21 which induces cell cycle arrest and inhibits tumor development (27). Celecoxib has been demonstrated to inhibit the G₀/G₁-to-S-phase transition by increasing the expression of p21, thereby decreasing tumor growth in colon cancer (23). In the present study, the expression levels of p53 exhibited little change in the OSCC cells exposed to celecoxib. Since it is known that p21 expression is upregulated by both p53-dependent and independent pathways (27), celecoxib may exhibit its antitumor effect in LPS-stimulated OSCC xenografts by upregulating p21 expression and halting the cell cycle through a p53-independent pathway.

In the present study, HSC-3 cells were stimulated with LPS for 48 h before implantation. This protocol has been supported by reports that describe the pre-treatment of carcinoma cells with LPS prior to implantation in nude mouse xenograft models (6,28). Stimulation of breast carcinoma cells with LPS for 48 h before implantation increased tumor volumes and weights in a xenograft model of breast carcinoma (6). Similarly, stimulation of cluster of differentiation 133 hepatoma cells with LPS for 1 week before implantation caused an increase in the growth of tumors in nude mice compared with those without LPS (28). Although the duration of LPS stimulations prior to implantation varied between these two reports, both clearly indicate that pre-treatment with LPS has growth-promoting effects in vivo. In the present study, LPS-induced tumor growth was not observed in vivo, although LPS treatment significantly increased the proliferation of OSCC cells in vitro. The limitation of the method of LPS administration used in the present study is that this was a one-time LPS treatment in vivo. In a murine breast cancer model, LPS was intraperitoneally injected for 3 consecutive days and it was observed that this promoted lung metastasis (29). Therefore, intraperitoneal injection of LPS following implantation may improve the experimental model used in the present study.

Understanding the mortality and clinical abnormalities caused by celecoxib is important for chemotherapy. One study reported the adverse effects of celecoxib in patients with locoregionally advanced nasopharyngeal carcinoma (30). According to this report, administration of celecoxib concomitantly with nasopharyngeal radiotherapy caused toxicities such as mucositis, weight loss, dermatitis and otitis, although no episodes of toxic mortality occurred with the treatment (30). Furthermore, a regimen of celecoxib combined with radiation was well tolerated in patients with brain metastases, and celecoxib-associated toxicity was limited to a mild skin reaction (31). These reports suggest that celecoxib can be safely administrated to patients with carcinomas such as lung or breast carcinoma or melanoma, including those with metastases.
A limitation of the present study is that it utilizes a single cell line, HSC-3, for both the in vitro and in vivo experiments. In a previous study, an HSC-3-bearing nude mouse model was successfully established and utilized (16). For the in vitro experiments, previous studies support the data from the present study that celecoxib inhibits the growth of OSCC cells (14,20).

The clinical implications of the present study are important. First, *P. gingivalis*-derived LPS can stimulate tumor growth by interacting with OSCC cells. Thus, it poses a risk for developing OSCC (4). Secondly, celecoxib could be used for the effective prevention and treatment of LPS-stimulated OSCC. Further experiments are required for the future clinical development of celecoxib as an OSCC treatment.

In conclusion, the data from the present study revealed that *P. gingivalis*-derived LPS can stimulate the growth of OSCC tumors. Celecoxib suppressed the proliferation of LPS-stimulated OSCC cells and inhibited tumor growth by increasing p21 expression levels and inducing apoptosis in OSCC xenografts. These results suggest that celecoxib administration could provide an effective prevention and treatment strategy for LPS-stimulated OSCC.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions
HisY, HitY and KS designed the experiments. HisY, HitY, SM, SY, KO, HI and KS performed the experiments. HisY, HitY, MO, TR, TK and MK analyzed the data. HisY, HitY and KS wrote the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate
All animal experiments were approved by the Animal Ethics Committee of the University of Fukui (approval no. 29105).

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

References


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