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Loss of claudin-1 in lipopolysaccharide-treated periodontal epithelium

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Background and Objective: The epithelial barrier is a critical component of innate immunity and provides protection against microbial invasion. Claudin-1, a tight junction protein, is known to contribute to the epithelial cell barrier. An experimentally induced rat periodontal disease model was used to study the effects of lipopolysaccharide (LPS) on the expression of tight junction-associated molecule genes in the junctional epithelium.

Material and Methods: LPS was applied for 8 wk in the gingival sulcus, and junctional epithelium was collected by laser-capture microdissection and subjected to microarray analysis.

Results: Microarray analysis identified that expression of the claudin-1 gene was decreased in the epithelium by chronic LPS challenge. Immunohistochemical analysis confirmed the expression of claudin-1 protein in junctional epithelium and that 8 wk of chronic LPS topical application significantly reduced claudin-1 expression. The effect of LPS on claudin-1 protein expression was validated using a porcine junctional epithelial cell culture Transwell model. The epithelial barrier, as measured using transmembrane resistance, was significantly reduced after 3 wk of LPS challenge and this was associated with a decreased level of expression of claudin-1 protein.

Conclusion: These results confirm that the initiation of experimental periodontal disease is associated with reduction in the expression of claudin-1 gene and protein. This decreased level of a critical tight junction protein may result in the disruption of barrier function and may play an important role in the initiation of periodontal disease.

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The epithelial barrier is a key element of innate immunity, and the plasma membrane and intercellular tight junctions regulate the function of this barrier (1). Within the periodontium the gingival junctional epithelium functions as a barrier and serves as a first line of defense against dental biofilm-associated microorganisms and their virulence factors (2,3).

Cell–cell adhesion is maintained by adherens junctions, whereas tight junctions are located apical to adherens junctions and function to seal the paracellular pathway (4). The best understood tight-junction proteins are the claudins, a large family of at least 24 members. Claudins are tetraspan proteins with relatively short and highly variable cytoplasmic amino and carboxy termini flanking a first extracellular loop of 53 amino acids and a second, shorter, loop of 24 amino acids (5). The carboxy terminus also binds to zona occludens (ZO) -1 and to related ZO-2 and ZO-3 proteins (4). Claudin-1-deficient mice die within 1 d of birth and have severe defects in epidermal permeability (6). Conversely, claudin-1-overexpressing cells show increased transepithelial electrical resistance (7). Therefore, claudins do play an important role in regulating the epithelial barrier at tight junctions and septate junctions (8). We have recently reported that claudin-1 is present in the

junctional epithelium of rats and may contribute to the epithelial barrier function of the junctional epithelium (9).

With onset of disease, the adherent microbial biofilm matures to a gram-negative-rich microbial biofilm (10). A major virulence factor of gram-negative pathogens is lipopolysaccharide (LPS), a component of the outer bacterial membrane that disrupts normal mucosal architecture and increases mucosal permeability (11). The role of LPS in regulating the function of the junctional epithelial barrier *in vivo* can be studied most effectively using a timed disease model. Specifically, in this model, daily application of *Escherichia coli* LPS to the gingival sulci of rats resulted in the development of periodontal disease, as indicated by histological progression of the chronic inflammation, elevated oxidative stress, epithelial proliferation, alveolar bone loss and elevated monoamine oxidase activity (12,13).

In this study, this rat periodontal disease model was utilized to specifically explore the cell-junctional proteins that were negatively impacted by chronic LPS challenge. Junctional epithelium and pocket epithelium were collected from healthy and diseased animals by laser-capture microdissection and subjected to microarray analysis. This was accomplished by examining gene-array data using a number of established analysis approaches. Subsequently, immunohistochemical analyses and cell culture modeling were used to validate LPS-mediated effects on the expression of identified junctional proteins, in association with transepithelial barrier resistance.

Material and methods

Animal experiments

Animal experiments complied with the guidelines of, and were approved by, the Animal Research Committee of The University of British Columbia. A rat periodontitis model was utilized as previously described: 6-wk-old male Wistar rats (seven in the time-0 control group and seven in the 8-wk treatment group) were anesthetized daily using isoflurane (Baxter, Toronto, Canada), then 25 μg/μL of serotype O55:B5 *E. coli* LPS (Sigma-Aldrich Corp., St Louis, MO, USA) and 2.25 U/μL of *Streptomyces griseus* type XIV proteases (Sigma), in pyrogen-free water (ICN Biomedical Inc., Aurora, OH, USA), were introduced by a micropipette into the left and right palatal gingival sulcus of all three maxillary molars, as previously described (12).

Laser microdissection and RNA extraction

Before death, rats were deeply anesthetized using isoflurane and the right palatal gingival soft tissue was collected by sharp dissection, immediately embedded in optimal cutting temperature compound (Sakura Finetek USA, Inc., Torrance, CA, USA), frozen in liquid nitrogen and stored at −86°C until required for cryosectioning. Rats were then killed by intracardiac perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Serial, 8-μm-thick cryosections were cut on the day of use using a cryostat (Cambridge Instruments, Heidelberg, Germany) onto membrane slides (Zeiss P.A.L.M., Bernried, Germany), briefly thawed, stained with Mayer's hematoxylin (Sigma), rinsed with RNase-free H_2O and drained. Slides were dehydrated for 30 s each in a series of increasing concentrations of ethanol transferred into xylene for 5 min and air dried for 5 min. The slides were used immediately for laser-capture dissection (Zeiss P.A.L.M.). A sufficient number of sections were collected to yield a minimum of 2×10^6 μm^2 of epithelium from each animal. Sections from each animal were pooled and RNA was extracted using the RNeasy Micro kit (Quiagen, Mississauga, ON, Canada).

Microarray analysis

Purified RNA (10 ng) was amplified using the GeneChip® Expression 3′-Amplification Two-Cycle cDNA Synthesis kit (Affymetrix, Santa Clara, CA, USA), and the quality and quantity of complementary RNA (cRNA) was assayed using a Lab-on-a-chip/Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA). Equal amounts (15 μg) of fragmented and labeled cRNA from each sample were hybridized to a GeneChip® Rat Genome 230 2.0 Array and Fluidics Station 450 instrument (Affymetrix), with processing, data normalization and quality-control parameters as suggested by the manufacturer (12). Gene-expression data were analyzed using (i) GenMAPP/MAPPFinder 2.1 suite (http://www.genmapp.org) set at permuted $p < 0.05$ and changes $\leq / \geq 2.0$-fold, $Z \geq 4.0$, and a minimum of three changes per classification, then pruned to exclude nonsignificant/redundant nested daughters/parents; (ii) dChip (http://www.dchip.org); and (iii) Bioconductor (http://www.bioconductor.org) on the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database and differential expression via distance summary (DEDS).

Claudin-1 immunohistochemical staining

The left maxillary molar regions were resected en bloc from each rat and decalcified with 10% tetrasodium-EDTA aqueous solution (pH 7.4) for 2 wk at 4°C. Paraffin-embedded buccolingual sections of 5 μm were made and used for morphometric and immunohistochemical analyses. For general histomorphometric analysis, sections were stained with hematoxylin and eosin. Sections to be used for immunohistochemical staining, endogenous horseradish peroxidase was inactivated with 3% hydrogen peroxide in phosphate-buffered saline (PBS), then the sections were incubated for 30 min at room temperature with 0.2% casein in Tris-buffered saline (TBS; 20 mM Tris–HCl, 0.15 M NaCl, pH 7.6), then with anti-claudin-1 IgG in PBS (Invitrogen, Camarillo, CA, USA) for 24 h at 4°C. The claudin-1 antibody used (37-4900; Invitrogen) was generated from a synthetic peptide derived from the C-terminal region of the human and mouse proteins. Hence, it was specifically designed to recognize claudin-1 in multiple species. It has also been

shown to cross-react with claudin-1 from dog and rat. It was therefore considered reasonable to use this claudin-1 antibody in the present study also for staining porcine periodontal ligament epithelial cells. The slides were rinsed in PBS, incubated for 30 min with secondary antibody – biotinylated anti-rabbit IgG – in PBS, rinsed with PBS, incubated with peroxidase-conjugated streptavidin for 30 min and then rinsed with PBS. The color was developed with 0.025% 3,3′-diaminobenzidine tetrahydrochloride in TBS plus hydrogen peroxide. The specimens were counterstained with methyl green, dehydrated, mounted, observed by light microscopy (ECLIPSE E600; Nikon, Tokyo, Japan) and photographed (DXM1200; Nikon). Negative control samples were prepared by replacing the anti-rabbit IgG with normal rabbit IgG. The intensity of staining of claudin 1 was examined in six control and five diseased samples, and the relative staining intensity was determined using Image-J (http://rsb.info.nih.gov/ij). Statistical significance was determined using the Student's *t*-test ($p < 0.05$).

Cell culture

Porcine periodontal ligament epithelial cells were isolated as previously described (14) and cultures were maintained in α-MEM medium (Gibco Life Technologies, Carlsbad, CA, USA) supplemented with 15% fetal bovine serum (PAA Laboratories Inc., Etobicoke, ON, Canada). Experiments were performed by plating 20,000 cells/cm^2 into 24-mm Transwell® culture wells (Corning Inc., Corning, NY, USA). Experimental groups were treated every 3 d with 250 ng/mL of LPS/1.5% fetal bovine serum starting 3 d after plating for up to 21 d. Transepithelial permeability was measured every 3 d using a Millicell ERS2 (Millipore, Billerica, MA, USA) followed by a change of medium. Cell viability was assayed using CellTiter 96® (Promega, Madison, WI, USA).

Immunoassay for claudin-1 in culture

Cells from control and LPS-treated Transwell® cultures were recovered by trypsinization or left *in situ* for immunostaining, then fixed in 2% formaldehyde/5% sucrose for 1 h at room temperature. Cell samples that were blocked and permeabilized (in 3% bovine serum albumin, 0.2 M glycine, 0.1% saponin) were incubated with anti-claudin-1 primary IgG, washed, then incubated with Alex 488-conjugated secondary antibody (Molecular Probes, Eugene, OR, USA). Five groups of 10,000 cells from triplicate experiments were analysed by flow cytometry using a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA) to generate geometric mean fluorescence. Parallel cultures were similarly stained *in situ* then digitally photographed under epifluorescence. Samples stained with secondary antibody alone were included to standardize the results relative to autofluorescence.

Results

Analysis of gene expression

An 8-wk LPS-induced rat periodontitis model was used to induce periodontal disease, and disease onset was confirmed histologically relative to healthy controls (data not shown), as previously described (12). Cryosections from gingival soft tissue of healthy and diseased rats were processed by laser-capture microdissection to isolate junctional epithelium. RNA purification and cRNA preparation and quality control were performed as previously reported (12). Total RNA yields, amplified cRNA and cRNA per unit area were highly consistent between healthy and diseased tissue and showed consistent size profiles, indicating that LPS treatment was not associated with increased RNA degradation (data not shown). Gene-expression changes were assayed by applying cRNA from healthy and diseased tissue to expression microarray analysis.

Microarray data analysis was focused on genes and pathways associated with the epithelial barrier. First, the dChip normalized microarray data set was analyzed with GenMAPP/MAPPFinder 2.1, which uses a *Z*-statistic to determine if groups of genes within gene ontology (GO) annotations are over-represented. This transcriptome analysis produces a statistically ranked list of GO structure and function categories (15). Analysis parameters were set as $Z \geq 4.0$ (< 0.005 of total distribution), a minimum of three genes per GO pathway changed, with the changes being ≥ twofold greater or less than control values. Within the cellular component category, enrichment at the plasma-membrane level was identified and cell adhesion within the biological processes category was also identified (Table 1). These data identified significant changes in adhesion molecules associated with the plasma membrane.

Microarray analysis was further focused using Bioconductor on the KEGG pathway database. This showed that the tight junction pathway group ranked at 24 of 164 KEGG groups that were affected by LPS treatment with a *p*-value set at 0.05 and a false discovery rate (FDR) of 0.23 (Table 2). With the same *p*-value setting, the Bioconductor package for

Table 1. Gene ontology analysis of LPS-induced changes in epithelial cell gene expression

Analysis	GO ID	GO name	>2-fold change	Total changed	Number local	*Z* score
Component	0005886	Plasma membrane	7	220	342	4.25
Process	0007155	Cell adhesion	9	13	239	4.15

Oral junctional epithelia from control (0 wk) and LPS-treated (8 wk) rats were separately isolated by laser capture microscopy, pooled and RNA extracted. Amplified complementary RNA (cRNA) was analyzed using the GeneChip rat genome 230 2.0 array. Normalized data analysed by GenMAPP/MAPPfinder 2.1 software ($Z > 4.00$; $p < 0.05$) analysis of gene ontology (GO) structural components and biological processes categories.

determining DEDS showed that the only barrier-related gene that was significantly changed in the disease state corresponded to claudin-1 (Table 3; probe set ID 1387470). Post hoc inspection of both MAPPFinder GO classifications and the identified KEGG group revealed claudin-1 as a constituent. Cross-referencing to the dChip analysis data (Table 4) confirmed that all three claudin-1 probe sets showed a similar negative fold-change (−2.96 to −3.07) in diseased tissues. No other tight junction constituents (i.e. claudins, occludins or catenins) were found to exhibit significant LPS-induced change after 8 wk of treatment.

Analysis of protein expression

Using immunohistochemistry and cell culture model testing we confirmed changes in the expression of claudin-1 protein. Control samples showed characteristic knife-edge junctional epithelium, which differed from the downgrowth characteristic of disease-associated pocket epithelium (Fig. 1A and 1B). All samples from five control rats stained strongly for claudin-1 (Fig. 1C and 1E). The intense staining in the time-zero control junctional epithelium was localized to cell–cell contact sites; the intensity of staining was significantly weaker in the sulcular and oral epithelium (Fig. 1C and 1E). In contrast, chronic LPS topical application for an 8-wk period resulted in weaker and discontinuous staining of claudin-1 protein (Fig. 1D and 1F). Overall, the reduction in expression of claudin-1 protein was reduced by 54% in diseased tissues ($p < 0.01$) (Fig. 1G).

The effect, on expression of claudin-1, of chronic LPS exposure of epithelial cells was studied further using an *in vitro* cell-culture barrier model. The primary porcine periodontal epithelial line used is an established model for junctional epithelium by virtue of its identical cytokeratin profile but can be cultured for much longer than primary human cultures (16). Porcine periodontal ligament cells were plated in Transwell culture chambers to allow the initial formation of a barrier (3 d), then treated with low-dose LPS and the barrier measured by determining the transmembrane resistance every 3 d for 21 d. Chronic LPS treatment reduced barrier formation (Fig. 2A). There was no statistically significant reduction in the number of cells at the end of the experimental period so this would not account for the decrease in transmembrane epithelial resistance (data not shown). Cells from replicate Transwell cultures were recovered by trypsinization after 0, 1, 2 or 3 wk of treatment with LPS, immunostained and subjected to flow cytometry analysis. Claudin-1 protein in the LPS-treated group was reduced by 46% relative to untreated, 3-wk, controls (Fig. 2B). Treatment with LPS caused a steady decrease in the expression of claudin-1 protein over the 3-wk experimental period (Fig. 2C). Immunostaining of Transwell cultures also showed that relative to controls (Fig. 2D), LPS treatment was associated with a markedly decreased intensity of claudin-1 protein staining (Fig. 2E).

Discussion

In this study, timed application of LPS to the gingival sulcus consistently induced histological changes habitually with periodontal disease. Specific laser-

Table 2. KEGG analysis of LPS-induced changes in epithelial cell gene expression

Rank	KEGG group	Statistic Q	Expected Q	SD of Q	*p*-value	FDR.adjusted
24/164	Tight junction	25.04	14.19	5.85	0.05	0.23

Microarray gene expression assay of oral junctional epithelia from control (0 wk) and LPS-treated (8 wk) rats. Normalized data was analysed using Bioconductor software on the Kyoto encyclopedia of genes and genomes pathway database (KEGG).
FDR, false discovery rate.

Table 3. DEDS analysis of LPS-induced changes in epithelial gene expression

Value	*t*	abs *t*	*fc*	abs *fc*	Sample	Probe set ID
0.29	−2.6	2.6	−1.58	1.58	−1.93	1387470

Microarray expression assay of oral junctional epithelia from control (0 wk) and LPS-treated (8 wk) rats. Normalized data was analysed by bioconductor analysis of differential expression via distance synthesis (DEDS) package.
SAM, significance analysis of microarrays; *fc*, fold-change; abs, absolute; *t*, *t*-statistic.

Table 4. Microarray analysis of LPS-induced changes in epithelial claudin-1 gene expression

Probe set ID	Gene	Accession	Locuslink	Description	Fold-change	*p*-value
1387470	Claudin 1	NM_031699	65129	gb:NM_031699.1	−2.96	0.057
1383946	Claudin 1	AI137640	65129	gb:AI137640	−3.07	0.049
1396150	Claudin 1	AW917275	65129	gb:AW917275	−2.97	0.063

Oral junctional epithelia from control (0 wk) and LPS-treated (8 wk) rats were separately subject to microarray expression assay using GeneChip rat genome 230 2.0 array. Normalized dChip data of array probe sets of claudin 1.

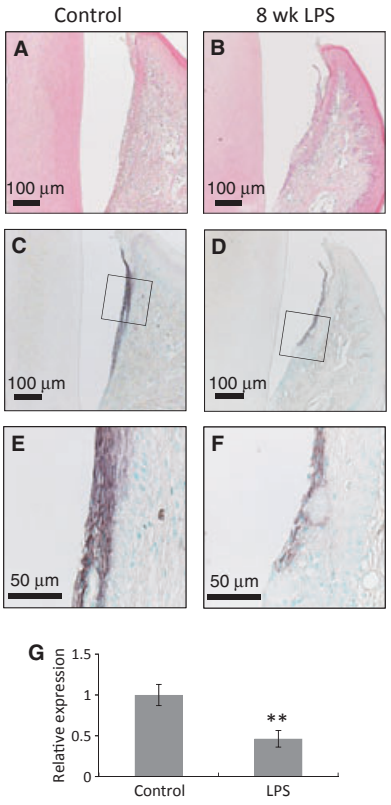

Fig. 1. Reduction in the expression of claudin-1 protein in disease. (A, B) Hematoxylin and eosin staining of healthy (A) and diseased (B) tissues. Immunohistochemical staining of claudin-1 in gingival tissues of rats. (C) Immunolocalization of claudin-1 protein in control animals. (E) Higher magnification of the boxed area in (C). (D) Reduced claudin-1 protein expression in lipopolysaccharide (LPS)-treated rats. (F) Higher magnification of the boxed area in (D). (G) Digital quantification of claudin-1 staining intensity in control ($n = 6$) and lipopolysaccharide (LPS)-treated ($n = 5$) groups. The results are given as mean ± standard deviation (SD). Claudin-1 protein expression was significantly reduced by treatment with LPS. (**t-test, $p < 0.01$).

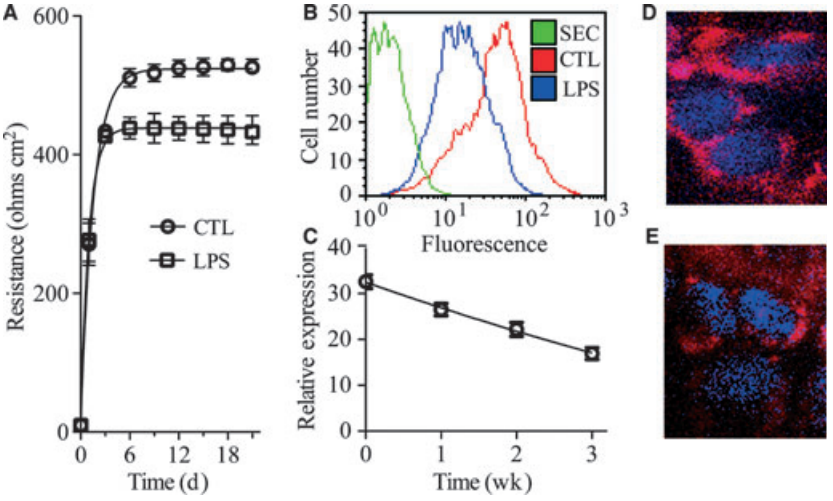

Fig. 2. Chronic lipopolysaccharide (LPS) treatment of epithelial cell cultures reduces transepithelial resistance and the expression of claudin-1 protein. Porcine periodontal ligament epithelial cells were cultured in Transwell® inserts for up to 3 wk, with or without LPS. (A) Transepithelial barrier resistance in control (CTL) and LPS-treated (LPS) cultures. (B) Representative sample of flow-cytometric analysis of total cell claudin-1 expression at 3 wk. SEC, secondary antibody alone. (C) Flow cytometric analysis of total relative claudin-1 in LPS-treated culture samples over 3 wk ($n = 3$; mean ± standard deviation). (D) Representative samples of control cell cultures and (E) of cell cultures after 3 wk of LPS treatment, immunostained for claudin-1 ($n = 3$).

capture microscopic dissection of healthy and diseased epithelium ensured that the confounding effects of connective tissue-associated gene expression were eliminated. Previously, we demonstrated that principal component analysis of the gene array data separately grouped healthy from diseased tissue samples (12).

Multiple bioinformatic applications were utilized to examine changes in the expression of genes from health to disease states. All of these analyses were consistent in identifying LPS-induced changes in tight junction-associated gene categories. Within these categories, consistent, significant fold-reduction was observed in LPS-induced claudin-1 gene expression. Interestingly, although the claudin family consists of at least 24 members (4), no significant changes in the expression of other claudin genes were identified in diseased tissues. Although tight junctions in gingival junctional epithelium are much less developed (17), we have recently reported that claudin-1 exists in rat gingiva junctional epithelium (9). In this study a reduction in expression of the claudin-1 gene was validated with expression of the claudin-1 protein, which was also significantly lower in the same experimental animals. Consistent with this, chronic LPS treatment of epithelial cell cultures was associated with decreased claudin-1 protein in association with a reduced functional barrier.

Claudin-1 is known to be a major structural protein of tight junctions. In invertebrates, septate junctions circumscribe epithelial cells and have been regarded as the functional counterparts of tight junctions (8). *Drosophila* exhibits six claudin sequences, one of which, Megatrachea, is located at septate junctions (8). Mutations of Megatrachea disrupt the barrier and result in defects in the size and shape of the tracheal epithelium (18). Conversely, claudin-1 over-expressing cells exhibit higher transepithelial electrical resistance and reduced paracellular flux compared with wild-type MDCK cells (7). Our *in vivo* data suggest that claudin-1 may play a significant role in the maintenance of a junctional epithelium barrier, and the presence of biofilm-associated LPS can reduce barrier effectiveness. Support for this is provided by our cell culture model. Specifically, chronic application of LPS over a 3-wk period consistently reduced transmembrane resistance, a marker of cell barrier efficiency and in association with total claudin-1 protein levels and localization to the cell–cell contacts. This reduction in barrier was not associated with a reduction in cell number following LPS-mediated apoptosis (data not shown). These findings are consistent with a previous report that *Porphyromonas gingivalis*

had a destructive effect on both barrier function and claudin-1 expression in human oral epithelial cells (19).

In conclusion, this study provides the first *in vivo* evidence that chronic LPS challenge is associated with a significant reduction in the expression of claudin-1 gene and protein in junctional epithelium. The periodontopathogenic bacteria-mediated decrease in tight junction proteins and associated increased permeability would allow penetration of bacteria and associated virulence factors into the subjacent connective tissue via the paracellular pathway between epithelial cells (20–22). This increased penetration may play a significant role in the initiation and progression of periodontal disease.

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