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J Dent Res 93(2):169-174, 2014

ABSTRACT

In type 1 diabetes (T1D), a Toll-like receptor (TLR)-hyper-inflammatory monocytic phenotype has been implicated as a mechanism of exacerbated tissue destruction. Other cells of the periodontium, including oral epithelial cells (OECs), express innate immune receptors, including TLRs. To delineate the TLR responses of OECs derived from T1D participants and to determine effects of the anti-inflammatory agent triclosan on the TLR-hyper-inflammatory phenotype, primary human OECs from individuals with T1D and diabetes-free individuals were stimulated with TLR ligands in the presence and/or absence of triclosan. The expression of pro-inflammatory cytokines and micro-RNAs (miRNAs) was evaluated. While the repertoire of TLRs expressed by OECs is similar to that expressed by macrophages (Mφ), the relative amounts and ratios are significantly different. OECs demonstrate a TLR-response profile similar to that of Mφ, yet attenuated. OECs have a unique response to *P. gingivalis* LPS, where miR146a and miR155 play a regulatory role in responsiveness. OECs from T1D participants are TLR-hyper-responsive, due to dysregulated induction of miR146a and miR155, which is abrogated by pretreatment with triclosan. The aberrant TLR-activation of OECs in T1D has the potential to contribute to excessive soft- and hard-tissue destruction. Importantly, triclosan's anti-inflammatory property is effective in abrogating TLR-induced OEC hyperactivity.

KEY WORDS: triclosan, inflammation, cytokines, chemokines, periodontal disease.

DOI: 10.1177/0022034513516345

Received September 16, 2013; Last revision November 12, 2013; Accepted November 13, 2013

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Type 1 Diabetes-associated TLR Responsiveness of Oral Epithelial Cells

INTRODUCTION

An association between diabetes mellitus and destructive periodontal diseases has been reported in the literature since 1960 (Williams and Mahan, 1960). A well-described bi-directional relationship between diabetes mellitus and periodontal diseases has been established, whereby diabetes is a major risk factor for periodontitis and the severity of periodontitis influences glycemic control (Preshaw *et al.*, 2012). In addition, it has been suggested that controlling periodontal inflammation has the potential to reduce the systemic inflammation responsible for secondary complications of diabetes (Preshaw *et al.*, 2012).

Periodontal diseases are microbially induced inflammatory disorders (Pihlstrom *et al.*, 2005). Multiple reports have concluded that the nature of the bacterial challenge in patients with diabetes mellitus and periodontitis does not seem to differ from that of individuals without diabetes mellitus (Lalla *et al.*, 2006; Lalla and Papapanou, 2011). Thus, it seems that the host response to the bacterial challenge is what drives the enhanced severity of periodontal disease in patients with diabetes. In type 1 diabetes (T1D), a hyper-inflammatory monocytic phenotype characterized by enhanced concentrations of pro-inflammatory mediators following challenge with lipopolysaccharide (LPS) has been implicated as a mechanism of exacerbated tissue destruction (Lalla and Papapanou, 2011).

Toll-like receptors (TLRs) respond to various pathogen-associated molecular patterns (PAMPs). TLRs are predominantly expressed in cells of the innate immune system (Hans and Hans, 2011). Research has established that TLRs are also expressed in periodontal tissues on non-hematopoietic immune cells such as epithelial cells (Kusumoto *et al.*, 2004). TLR responses of oral epithelial cells (OEC) are the first line of defense against potential infectious pathogens within the oral cavity. However, under steady-state conditions, activation of TLRs by commensal bacteria is critical for the maintenance of oral health (Hatakeyama *et al.*, 2003). Thus, TLR-induced signaling in OECs results in divergent cellular outcomes, which can be a 'two-edged-sword'. Over-production of pro-inflammatory cytokines by OECs, due either to chronic stimulation or inappropriate TLR-responsiveness, can lead to exacerbated soft- and hard-tissue destruction. TLR-responsiveness can be controlled by microRNAs (miRNAs) through their regulation of important intermediate molecules of the signaling cascade. miRNAs are short non-coding RNAs which have emerged as key regulators of gene expression acting at the post-transcriptional level (Filipowicz *et al.*, 2008). The objective of this study was to evaluate the TLR-responsiveness of OECs derived from participants with T1D and to define mechanisms associated with aberrant responsiveness.

A treatment regimen which is directed at microbial colonization as well as the restoration of proper innate immunity would be beneficial for the treatment of periodontal disease in patients with T1D. 2,4,4-trichloro-2-hydroxydiphenyl ether (triclosan) is a bisphenolic and non-cationic agent used in oral care products because of broad-spectrum anti-microbial and anti-plaque activity (Blinkhorn *et al.*, 2009). We have previously published that pre-treatment of OECs with triclosan can inhibit TLR-induced inflammatory responses (Wallet *et al.*, 2013). Here, the effects and mechanism(s) of the action of triclosan on TLR-hyper-responsiveness in OECs derived from patients with T1D were explored.

MATERIALS & METHODS

Participant Cohorts

The institutional review board (IRB) for the protection of human subjects at the University of Florida approved this protocol. Twenty participants [diabetes-free or diagnosed with type 1 diabetes (T1D)] were recruited from the University of Florida, College of Dentistry, Department of Periodontology and/or Department of Orthodontics. Inclusion criteria were that they were 13 to 75 yrs old, required at least one premolar tooth extraction, or needed surgical treatment. Exclusion criteria were that they had clinically significant neurological, hepatic, renal, gastrointestinal, hematologic, dermatologic, metabolic, autoimmune, or immune deficiency diseases (other than T1D); were receiving any immunosuppressive medication, or antibiotic or glucocorticoid therapy; smoked or used oral tobacco; were pregnant or lactating; were taking medications known to affect the gingival; or had obvious gingival inflammation. There was no significant difference in the average age of diabetes-free (17.11 ± 1.47 yrs) and T1D (18.33 ± 6.31) participants. All T1D participants were under good glycemic control ($HbA1c = 7.07 \pm 0.40$), with an average length of disease of 6.00 yrs (± 5.79). All data are reported as average $\pm$ standard deviation (SD).

Cell Culture

Tissue harvest for OEC culture was performed during third molar extractions, where soft tissues (1 mm waste) associated with the extraction were collected, and epithelial cells were isolated and expanded as previously described (Amir *et al.*, 2011). The buffy coat was separated by centrifugation (300 $\times$ g for 15 min) from 10 mL of heparinized blood. CD14⁺ cells were sorted by magnetic bead separation (Miltenyi Biotech, Auburn, CA, USA). Purified cells were cultured with 1 ng/mL of M-CSF (Sigma-Aldrich, St. Louis, MO, USA) for 7 days to differentiate monocyte-derived macrophages (M ϕ) as previously described (Lamont *et al.*, 1995; Wallet *et al.*, 2010). OECs were cultured in a serum-free HEPES and bicarbonate buffered keratinocyte medium (KM) (ScienCell Research Labs, Carlsbad, CA, USA). M ϕ were cultured in DMEM complete media [10% human serum, 0.005% β -2 mercaptoethanol, and 1% penicillin/streptomycin (Fisher Scientific, Pittsburgh, PA, USA)].

A 1×10^6 quantity of OECs, the OECs derived from T1D participants (dOECs), or M ϕ were left untreated or stimulated with 100 ng/mL of agonist [TLR2 (*P. gingivalis* LPS), TLR4 (*E. coli* LPS), TLR2/1 (Pam3CKS4), and TLR2/6 (FSL-1)] (Invivogen,

San Diego, CA, USA). In some experiments, OECs and dOECs were left untreated or were stimulated following 24-hour pre-treatment of 0.75 μ g/mL of triclosan (Sigma-Aldrich, St. Louis, MO, USA). Zero, 6, and 24 hrs post-stimulation, culture supernatants and mRNAs were collected for analysis. All assays were performed with 10 donors using primary cells passaged fewer than 5 times.

Immunohistochemistry

OECs were grown on glass coverslips, fixed with 4% PFA, permeabilized with 0.1% triton-X, probed with human anti-pancytokeratin conjugated to FITC (1:200), and mounted with a DAPI-containing mounting medium. Images were acquired by spinning-disk confocal microscopy.

Flow Cytometry

Cells were allowed to dissociate from the bottoms of UpCell (ThermoFisherScientific, Pittsburgh, PA, USA) coated plates. Suspended cells were washed with FACS Buffer (1x PBS + 5% FBS + 0.372 g EDTA) and probed with human anti-pancytokeratin conjugated to FITC (1:200). Cells were acquired on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) and analyzed with FCS Express (De Novo Software, Los Angeles, CA, USA).

Soluble Factors

Human Cytokine ELISA kits (Millipore, Billerica, MA, USA) were used to quantify interleukin-8 (IL-8) and transforming growth factor beta 1 (TGF- β 1) in culture supernatants, according to manufacturer's instructions. Concentrations were determined by spectrophotometry with standard curves.

Real-time PCR (qPCR)

Total RNA was harvested with an RNAeasy extraction kit (Qiagen, Valencia, CA, USA). RNA was reverse-transcribed to generate cDNA. Primers specific for GAPDH (Integrated DNA Technologies, Coralville, IA, USA) or hTLR-1, hTLR-2, hTLR-4, and hTLR-6 RT² qPCR primers (SABiosciences, Frederick, MD, USA) and Cyber Green 2x Master Mix (BioRad, Hercules, CA, USA) were used for qPCR reactions. Standard curves were used to determine mRNA transcript copy number.

Similarly, total RNA, TaqMan MicroRNA Reverse Transcription Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), and miR146a or miR155 primers (Applied Biosystems) were used for miRNA qRT-PCR analysis. miRNA expression values were expressed as fold change over miR146a expression in unstimulated cells at 0 hrs.

Statistics

Statistical analyses were performed with GraphPad Software (GraphPad Software Inc., La Jolla, CA, USA), where one-way analysis of variance (ANOVA) with Bonferroni's multiple-comparisons test was used to determine significance ($p < .05$ was considered significant). All error bars represent standard deviation (SD).

RESULTS

Unique TLR-responsiveness by Human OECs

Primary OECs were isolated, expanded, and characterized (Fig. 1). Cells exhibited epithelial cell morphology where 100% of the cells were pancytokeratin-positive (Figs. 1a, 1b). Others have detected the expression of multiple TLRs in both M ϕ and OECs, although the relative levels of expression have never been described (Mahanonda and Pichyangkul, 2007). Thus, the mRNA transcript numbers of TLRs 1, 2, 4, and 6 were determined by qPCR in M ϕ and OECs from the same donors (Fig. 1c). While both OECs and M ϕ express mRNA for TLRs 1, 2, 4, and 6, OECs express significantly fewer copies of *tlr1*, *tlr2*, and *tlr4* (Fig. 1c). While M ϕ express more copies of *tlr4* than *tlr2*, OECs express more copies of *tlr2* than *tlr4* (Fig. 1c).

The unique functions of M ϕ and OECs suggest that cellular outcomes following TLR ligation may be divergent. Thus, M ϕ and OECs were stimulated with TLR ligands, and the expression of IL-8 and TGF- β 1 was evaluated. All ligands induced IL-8 but not TGF- β 1 expression in M ϕ (Figs. 1d, 1e). Similarly, *E. coli* LPS, Pam3CKS4, and FSL-1 stimulation induced IL-8 expression by OECs, albeit at significantly lower concentrations than observed in M ϕ (Fig. 1d). Interestingly, *P. gingivalis* LPS induced not IL-8 but rather TGF- β 1 expression in OECs, a phenotype which was not observed in M ϕ (Figs. 1d, 1e).

Type 1 Diabetes-derived OECs are TLR-hyper-responsive

Although monocytic TLR-hyper-responsiveness has been well-described in T1D, the TLR-responsiveness of OECs has not. OECs derived from participants with T1D (dOECs) and diabetes-free participants were stimulated with the indicated TLR ligands, and the induction of IL-8 and TGF- β 1 was evaluated. Significantly higher concentrations of IL-8 were induced in dOECs when compared with OECs regardless of the ligand utilized (Fig. 2a). In addition, *P. gingivalis* LPS induced significantly higher concentrations of IL-8 along with significantly lower concentrations of TGF- β 1 expression in dOECs, when compared with those isolated from diabetes-free participants (Figs. 2a, 2b).

MicroRNA Regulation of OEC TLR-responsiveness

miR146a and miR155 have been implicated in the negative and positive regulation of TLR signaling, respectively (Taganov *et al.*, 2006). Therefore, to investigate the mechanism(s) associated with the unique responsiveness of OECs to *P. gingivalis*

Figure 1. TLR repertoire of human oral epithelial cells. (a) Representative image of cytochrome-positive (green) primary human oral epithelial cells (OECs). (b) Flow cytometry histogram of pan-cytokeratin-positive OECs. Solid line = isotype control; dashed line = anti-human pancytokeratin FITC. (c) Total RNA was harvested from 1×10^6 primary human M ϕ and OECs from diabetes-free participants. Data are expressed as mRNA transcript copy number normalized to GAPDH $\pm$ standard deviation. Normalized mRNA copy number = [raw transcript copy number] $\times$ [GAPDH corrective ratio]. GAPDH corrective ratio = [lowest GAPDH copy number within sample set] / [GAPDH copy number for cell of interest]. * $p < .05$ M ϕ vs. OEC. ^ $p < .05$ *tlr2* vs. *tlr4* and *tlr6* vs. *tlr4*. One-way ANOVA with Bonferroni's multiple-comparisons correction. All assays were performed on 10 separate donors and thus represent 10 biological replicates. (d,e) 1×10^6 primary human M ϕ (white) and OECs (black) from diabetes-free individuals were left unstimulated or were stimulated for 24 hrs with 100 ng/mL of indicated ligands, after which the supernatants were used to quantify (d) IL-8 and (e) TGF- β 1 expression. Data are expressed as pg/mL $\pm$ standard deviation. * $p < .05$. One-way ANOVA with Bonferroni's multiple-comparisons correction. All assays were performed on 10 separate donors.

LPS, the expression of miR146a and miR155 was evaluated following stimulation with *E. coli* or *P. gingivalis* LPS. *E. coli* LPS stimulation of OECs induced peak expression of miR146a at 24 hrs, while significant induction of miR155 could be observed as early as 6 hrs (Figs. 2c, 2d, black bars). Following stimulation with *P. gingivalis* LPS, peak induction of miR146a was again observed at 24 hrs, although expression of miR146a could be observed as early as 6 hrs, while induction of miR155 was almost non-existent at both 6 and 24 hrs post-stimulation (Figs. 2c, 2d, black bars).

Dysregulation of miRNA Expression in T1D-derived OECs

To investigate the mechanism(s) associated with dOEC TLR-hyper-responsiveness, the expression of miR146a and miR155 was again evaluated by qPCR 6 and 24 hrs following stimulation with *E. coli* or *P. gingivalis* LPS. Following stimulation of dOECs with *E. coli* LPS, little miR146a was induced at 6 or 24 hrs post-stimulation, whereby levels were significantly lower than those observed in OECs derived from diabetes-free participants (Fig. 2c, gray bars). Conversely, significant amounts of miR155 were induced both 6 and 24 hrs post-stimulation, whereby levels at 6 hrs were significantly higher than in OECs (Fig. 2d, gray bars). Following stimulation with *P. gingivalis* LPS, again, little

Figure 2. Oral epithelial cell (OEC) TLR-hyper-responsiveness in type 1 diabetes. A 1×10^6 quantity of primary human OECs from diabetes-free individuals (black) and individuals with type 1 diabetes (dOECs) (gray) was left unstimulated or was stimulated for 6 or 24 hrs with 100 ng/mL of the indicated ligand, after which the (a–b) supernatants (24 hr) and (c–d) mRNAs (6 and 24 hrs) were used to quantify (a) IL-8, (b) TGF- β 1, (c) *miR146a*, and (d) *miR155* expression, respectively. Protein data are expressed as pg/mL $\pm$ standard deviation. miRNA data are presented as normalized fold change over unstimulated cells $\pm$ standard deviation. Normalized miRNA fold change = [(corrected C_t value of sample of interest) / (corrected C_t value of unstimulated cells at 0 hrs) – 1]. Corrected C_t value = (raw C_t value) $\times$ (RNU6 corrective ratio). RNU6 corrective ratio = (lowest RNU6 C_t within sample set) / (RNU6 copy number for cell of interest). * $p < .05$ OECs vs dOECs; ^ $p < 0.05$ *E.c.* LPS vs. *P.g.* LPS. One-way ANOVA with Bonferroni's multiple comparisons correction. All assays were performed on 10 separate donors.

miR146a was induced at either 6 or 24 hrs post-stimulation, which was accompanied by significant amounts of *miR155*, which, again, were higher than those observed in OECs (Figs. 2c, 2d, gray bars).

Triclosan Restores TLR-responsiveness of T1D-derived OECs through the Regulation of miRNA

We have previously published that triclosan can also act as an anti-inflammatory agent on epithelial cells through modulation of *miR146* expression (Wallet *et al.*, 2013). Therefore, to determine if triclosan can ameliorate TLR-hyper-responsiveness of dOECs, cells were pre-treated with triclosan 24 hrs prior to TLR stimulation. As previously demonstrated, triclosan significantly reduced the expression of IL-8 regardless of the ligand utilized, but had no effect on the expression of TGF- β 1 following stimulation with *P. gingivalis* LPS (Figs. 3a, 3b) (Wallet *et al.*, 2013). Importantly, pre-treatment of dOECs with triclosan resulted in significant reductions in IL-8 expression and was able to restore TGF- β 1 expression following stimulation with *P. gingivalis* LPS (Figs. 3c, 3d).

Pre-treatment with triclosan also resulted in an increase of *miR146a* induction in dOECs, restoring peak induction at 24 hrs

following stimulation with *E. coli* LPS and *P. gingivalis* LPS (Fig. 3e). In addition, triclosan concomitantly inhibited induction of *miR155* in dOECs following stimulation with either *E. coli* LPS or *P. gingivalis* LPS (Fig. 3f).

DISCUSSION

Oral epithelial cells function as non-hematopoietic innate immune cells that contribute directly to innate immunity and play an important role in mucosal homeostasis. Here we demonstrate that while the repertoire of TLRs expressed by OECs is similar to that expressed by M ϕ , the relative amounts and ratios are significantly different. The expression levels and locations of TLR expression on the epithelium have been suggested as mechanisms to regulate inflammatory responses at the mucosal surface (Wells *et al.*, 2010). Here we demonstrate that, in OECs, along with a differential expression of TLRs was a similar yet attenuated TLR response profile when compared with M ϕ for most ligands tested, whereby OECs also had a unique response to *P. gingivalis* LPS when compared with M ϕ . Whether this is an effect of the relative ratio of expression or differential localization of the TLRs was not evaluated.

Over-activation of innate immunity or disruption of this homeostasis can result in initiation and/or exacerbation of localized inflammation as seen in periodontal disease associated with T1D. Here we demonstrate that dOECs are hyper-responsive to TLR ligation, as has been observed in T1D-derived monocytes (Poligone *et al.*, 2002; Sen *et al.*, 2003). The exacerbated TLR-induced inflammatory response from dOECs is concomitant with an absence of TLR-induced regulatory or homeostatic responsiveness. Specifically, significantly higher concentrations of IL-8 are induced in response to *P. gingivalis* and *E. coli* LPS in T1D. IL-8 has significant pleiotropic activities, where IL-8 causes an increase in: (1) the release of enzymes from granules, (2) the metabolism of reactive oxygen species (ROS), and (3) the chemotaxis and retention of immune cells, all of which can amplify the inflammatory cascade (Rosenkilde and Schwartz, 2004). Importantly, IL-8 can also induce osteoclastogenesis and osteoclast activation (Bendre *et al.*, 2003). Thus, overexpression of IL-8 as seen in T1D-derived OECs can contribute to the exacerbation of both soft- and hard-tissue destruction observed in T1D-associated periodontal disease.

Conversely, *P. gingivalis* LPS-induced TGF- β 1 expression was abrogated in T1D. TGF β suppresses cell activation and deactivates many innate immune cells, thus preventing the induction of adaptive immunity. Specifically, TGF β suppresses the expression

of MHC II and the production of IL1, IL6, and TNF α , along with inhibition of the proliferation of most adaptive immune cells (Letterio and Roberts, 1998). TGF β can also induce the synthesis of bone matrix proteins in osteoblasts as well as most major extracellular matrix proteins (Letterio and Roberts, 1998). Thus, the absence of TGF β induction in T1D prevents the control of inflammation and progression of wound healing necessary for the control of periodontal disease.

miRNAs, short non-coding RNA, have emerged recently as key regulators of gene expression acting at the post-transcriptional level (Filipowicz *et al.*, 2008). miR-146a and miR-155 are important for the regulation of the activation of TLR pathways in myeloid cells (Hou *et al.*, 2009; Ruggiero *et al.*, 2009). As previously mentioned, the unique purposes of M ϕ and OECs suggest that regulation of their TLR signaling may be divergent. Here we demonstrate that miR146a and miR155 play a regulator role in the unique responsiveness of OECs to *P. gingivalis* LPS, whereby this level of regulation is disrupted in T1D, directly contributing to exacerbated pro-inflammatory cytokine expression.

We have previously established that triclosan can modulate TLR-signaling through effects on miR146a expression (Wallet *et al.*, 2013). Here we confirm that triclosan can modulate miR146a expression and expand its effects to the modulation of miR155. The triclosan/copolymer, which is used in toothpastes and mouthwashes, has demonstrated a therapeutic effect beyond conventional periodontal therapy (Blinkhorn *et al.*, 2009), whereby twice-daily use of triclosan significantly improves clinical plaque control and slows periodontal disease progression (Cullinan *et al.*, 2003). Here we demonstrate a potential mechanism whereby triclosan prevents TLR-induced hyper-responsiveness of T1D-derived OECs by inducing miRNA regulation of the TLR signaling pathway.

We have demonstrated a TLR hyper-responsiveness in T1D-derived OECs along with mechanisms that could explain the exacerbated clinical presentation of periodontal disease in diabetes. In addition, we have demonstrated that an anti-inflammatory agent, triclosan, can (1) inhibit this TLR-induced hyper-responsiveness and (2) affect miRNA expression, which is appreciated to be involved in the regulation of TLR signaling (Nahid *et al.*, 2009). Whether these phenomena are linked is still under investigation.

Figure 3. Triclosan suppresses TLR-hyper-responsiveness of T1D-derived oral epithelial cells (OEC). OECs from diabetes-free individuals (OECs) (white) and individuals with type 1 diabetes (dOECs) (gray) were pre-treated with 0.75 μ g/mL of triclosan (hatched) for 24 hrs, after which they were stimulated with 100 ng of the indicated ligand. The resulting (a-d) supernatants (24 hrs) and (e-f) mRNA (6 and 24 hrs) were used to quantify (a,c) IL-8, (b,d) TGF- β 1, (e) miR146a, and (f) miR155 expression, respectively. Protein data are expressed as pg/mL $\pm$ standard deviation. miRNA data are presented as normalized fold change over unstimulated cells $\pm$ standard deviation. Normalized miRNA fold change = [(corrected C_t value of sample of interest) / (corrected C_t value of unstimulated cells at 0 hrs) - 1]. Corrected C_t value = (raw C_t value) $\times$ (RNU6 corrective ratio). RNU6 corrective ratio = (lowest RNU6 C_t within sample set) / (RNU6 copy number for cell of interest). * p < .05. One-way ANOVA with Bonferroni's multiple-comparisons correction. All assays were performed on 10 separate donors.

Analysis of these data will now direct the investigation into novel accessible targets for personalized intervention in periodontal patients with T1D.

ACKNOWLEDGMENTS

This study was supported by both the American Diabetes Association (ADA) 1-08-JF and the Colgate-Palmolive Corporation, A-2008-634-OC. KGN was supported by NIH/NIDCR T32DE 007200. F. Panagakos declares the following potential conflict of interest: FP is an employee of Colgate-Palmolive. All other authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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