Structural and recovery mechanisms of 3D dental pulp cell microtissues challenged with *Streptococcus mutans* in extracellular matrix environment

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Cariopathogen *Streptococcus mutans* exists in infected dental pulp of deciduous teeth and is frequently linked with heart diseases. Organotypic (3D) dental pulp stem cell (DPSC) cultures/microtissues, developed to mimic the physiological conditions in vivo, were utilized to assess the bacterial impact on their (i) 3D structural configuration and (ii) recovery mechanisms. The cultures, developed in extracellular matrix (ECM) bio-scaffold (Matrigel®), interacted with WT and GFP-tagged bacterial biofilms by permitting their infiltration through the ECM. Challenged cell constructs were visualized by F-actin/nuclei staining. Their pluripotency (Sox2) and differentiation (osteocalcin) markers were assessed by immunocytochemistry. Secreted mineral was detected by alizarin red, and 3D structural arrangements were analysed by epi-fluorescence and confocal scanning microscopy. Bacterial biofilm/ECM-embedded DPSC interactions appeared in distinct areas of the microtissues. Bacterial attachment to the cell surface occurred without evidence of invasion. Surface architecture of the challenged versus unchallenged microtissues was apparently unaltered. However, significant increases in thickness (138.42 vs 106.51 µm) and bacterial penetration were detected in challenged structures causing canal-like microstructures with various diameters (12.94 – 42.88 µm) and average diameter of 20.66 to 33.42 µm per microtissue. Challenged constructs expressed pluripotency and differentiation markers and secreted the mineral. Presented model shows strong potential for assessing pulp–pathogen interactions in vivo. *S. mutans* infiltrated and penetrated the microtissues but did not invade the cells or compromise major cell repair mechanisms. These findings would suggest reexamining the role of *S. mutans* as an endodontic pathogen and investigating DPSC resistance to its pathogenicity.

INTRODUCTION

*Streptococcus mutans* is recognized as a primary aetiological agent of dental caries, affecting almost 100% of adults and 60% to 90% of school children worldwide. This virulent bacterium adheres and colonizes on the tooth surface, creates biofilms highly resistant to environmental changes, produces acid that degrades the tooth enamel and dentin and invades deep dentinal tubules to interact with the pulp tissue (Love & Jenkinson, 2002; Petersen, 2003). Dental caries frequently leads to chronic pulp inflammation by triggering type I immune response and, ultimately, pulpal pathology (Hahn et al., 2000).

*S. mutans* is found in 30% of primary teeth root canals, and it is the dominant species in infected pulp of deciduous teeth pulpitis as well as in tissues from root and coronal caries (da Silva et al., 2006). Furthermore, the microbiota of vital infected pulp in primary teeth with severe early childhood caries is similar to that of advanced caries lesion biofilms (Chalmers et al., 2015). Dissemination of *S. mutans* into the bloodstream has been strongly associated with pulp infections and tooth extraction, endodontic treatments and periodontal surgeries (Abranches et al., 2009), infective endocarditis (Li et al., 2000; Babu & Gomes, 2011) and coronary atherosclerosis (Mattila et al., 1993). Significantly, both planktonic and biofilm forms of *S. mutans* cultured with human gingival fibroblasts or coronary artery endothelial cells show a similar percentage of survival within the host cells (Berlutti et al., 2010) and a persistence for more than 24 h (Abranches et al., 2009), respectively.
Recently, a connection between the binding capabilities of the pathogen to the extracellular matrix (ECM) and its potential to invade the host cells has been proposed (Westerlund & Korhonen, 1993). It is understood that ECM macromolecules become exposed when tissue integrity is damaged by lesions or trauma. During infection, ECM’s components such as collagen, fibronectin and laminin serve as receptors for bacteria (Westerlund & Korhonen, 1993). *S. mutans* surface adhesion molecules, adhesins, exhibit profound affinity to bind ECM proteins (Ajdic et al., 2002) such as collagen and fibronectin. In contrast to the SpaP mutants (Love et al., 1997), surface polypeptide SpaP adeptly invades dentinal tubules and adheres to collagen. The wall-associated protein A (WapA), expressed in the cariogenic strain, also significantly binds to collagen. It has been demonstrated that WapA includes a putative collagen-binding domain which is considered responsible for the binding activity of *S. mutans* (Han et al., 2006). Another surface protein, SmFnB, binds fibronectin in a concentration-dependent manner (Miller-Torbert et al., 2008). The putative protein SMU.1449 sequence possesses 66% identity and 77% similarity to the fibronectin-binding proteins from *Streptococcus pneumoniae* and *Streptococcus pyogenes*, respectively (Ajdic et al., 2002), thus indicating that SMU.1449 might play a role in the fibronectin binding of the strain.

The pathogenesis of *S. mutans* is associated with their ability to form biofilms on both solid surfaces and within 3D tissue structures (Krzysciak et al., 2014). The 3D structural arrangements of the bacterial biofilms generally provide a resistance to antimicrobial agents and changes in the environmental conditions by facilitating interbacterial interactions and exopolysaccharide cover (Krzysciak et al., 2014; Davey & O’toole 2000; Zijin et al., 2010). Recently, it was demonstrated that ECM components such as platelets and fibronectin have the potential to modulate *S. mutans* biofilm formation (Jung et al., 2012; Bedran et al., 2013). Another study revealed that the biofilm-inducible protein BrpA is essential for the persistence and the virulence of the bacteria in the blood (Nakano et al., 2005).

Conclusively, *S. mutans* is considered to be not only a cariogenic bacterium but also a potential endodontic pathogen capable of invading soft tissues and cells and interacting with the surrounding ECM.

Our group has recently developed 3D cell culture models (microtissues) (Kaufman et al., 2016), composed of immortalized mouse oral and dental-tissue-derived stem cells that include the dental pulp stem cell (DPCPS) line IMDP-3 in ECM surrounding platform called Matrigel (Kleinman & Martin, 2005). In this study, we extend the utility of our unique model to investigate the virulence capacities of *S. mutans* under conditions resembling the DPCPS multilayers and the bacterial infection process in low-oxygen and low-nutrient conditions *in vivo*. Specifically, we examined the changes in 3D structural architecture of DPCPS microtissues penetrated by *S. mutans* in ECM scaffolds, expression of the pluripotency and differentiation markers [SRY (sex determining region Y)-box 2, Sox2] and osteocalcin, respectively] and mineral secretion. Our 3D study of DPCPS microtissues challenged with *S. mutans* in ECM environment could easily be extended to determine interactions with other oral pathogens and multispecies or to measure the effectiveness of oral antimicrobials.

**METHODS**

**Cell and bacterial cultures.** WT (ATCC 700610, American Type Culture Collection) and green fluorescent protein (GFP)-tagged (Liu et al., 2011) *S. mutans* strain UA159 were grown overnight by incubation in brain–heart infusion broth (Becton, Dickinson and Company). The next day, cultures were diluted 1:10 into 3 ml fresh broth and grown to a mid-log phase of OD600 0.3 to 0.4. DPCPS, represented by the immortalized mouse dental papilla mesenchymal cells (iMDP-3) (Wang et al., 2013), were cultured in standard media containing α-MEM (Invitrogen) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U ml⁻¹ of penicillin and 100 µg ml⁻¹ of streptomycin. Since DPCPS contain the G418 resistance gene, the cells were incubated with 600 µg ml⁻¹ of G418 (Sigma-Aldrich). When the cells reached about 80% confluence, cultures were split at 1:50 ratios. All incubations were performed in a 5% CO₂ humidified incubator at 37°C.

**Organotypic cultures and bacterial challenge.** 3D cell culture microtissues were prepared as described previously by Kaufman et al. (2016) and Lee et al. (2007). Briefly, 0.17×10⁶ iMDP-3 cells were cultured in an eight-well chambered cover glass (Lab-Tek; Nunc) coated with 100 µl of growth-factor-reduced Matrigel® (BD Biosciences). The adherent cells were covered with 10% Matrigel® diluted in the standard medium for 14 days (Fig. 1a). Matured 3D DPCPS cultures were cultivated with 5×10⁴ bacterial cells taken from the mid-log culture and suspended in 100 µl of standard media (Fig. 1b). After 24h incubation, the media were replaced with fresh media without bacteria and incubated for an additional 7 days (Fig. 1a).

**Cell staining.** 3D DPCPS microtissues were washed twice with Dulbecco’s PBS (Invitrogen) and fixed with 4% paraformaldehyde (Electron Microscopy Sciences). Cell nuclei and F-actin were stained with 0.1 µM phalloidin and 1 µg ml⁻¹ Hoechst for 30 min (Molecular Probes), respectively, after permeabilization with 0.1% Triton X-100 (Alfa Aesar) for 5 min.

For immunostaining, cells were washed twice with 0.1% BSA (Invitrogen), blocked for 45 min with 10% normal donkey serum (GeneTex), 0.3% Triton X-100 and 1% BSA, then incubated with 10 µg ml⁻¹ of mouse anti-mouse/human Sox2 (MAB2018; R&D Systems) or rat anti-rat/human osteocalcin (MAB1419; R&D Systems) mAbs overnight at 4°C. Following the incubation, cells were washed twice and incubated with 1:200 dilution of donkey anti-mouse IgG (NL009; R&D Systems) or goat anti-rat IgG (NL013; R&D Systems) Northern Lights® NL557-conjugated polyclonal antibodies, respectively, for an hour at room temperature. After washing and incubation with 14.3 mM 4’6-diamidino-2-phenylindole (Molecular Probes) for 5 min at room temperature, the cells were washed again and kept in PBS at 4°C before being analysed.

**Mineralization.** For detection of the mineral dentin secretion, DPCPS constructs were cultured in a calcifying medium containing the standard medium supplemented with 50 µg ml⁻¹ ascorbic acid and 10 mM sodium β-glycerophosphate (Sigma-Aldrich) at 37°C for 14 days. The cells were fixed in 4% formaldehyde neutral solution and then stained with 1,2-dihydroxyanthraquinone (alizarin red S; Ward’s Science) according to the manufacturer’s instructions.
Microscopy. Phase-contrast and fluorescence images of the organotypic DPSC cultures were taken by stitching sliced images along the z-axis (motorized inverted Eclipse Ti-E epifluorescence microscope; Nikon Instruments) using the NIS-Elements software version 3.0 (Nikon Instruments). Cross-sectional analysis was performed by Z-stacking images in each channel into a single image representing both fluorescence channels. The images were collected using laser scanning confocal microscopy [3.5 mm intervals, two fluorescence channels, laser confocal scanning microscope (LCSM); Leica Microsystems]. Thickness of the constructs was measured along both x–z and y–z planes using Leica Application Suite Advanced Fluorescence software (Leica Microsystems). 3D structures were created by stacking images obtained by LCSM and analysed using Image J software (V.1.48; NIH).

Statistical analysis. The thickness of 3D structure and the canal diameters were expressed as means±1 SD of at least three to five separate experiments, performed in triplicate. Statistical comparisons were performed using one-way ANOVA followed by two-tailed Student’s t-test for the unpaired samples. Results were considered statistically significant when P<0.05.

RESULTS

S. mutans biofilm-like structures preferentially colonize at distinctive areas of ECM-embedded 3D DPSC microtissues

Infiltration of biofilm forms of WT S. mutans UA159 through the ECM layers (Fig. 1) and bacteria/DPSC surface interactions are shown in Fig. 2(a, b). S. mutans attached and colonized distinctive areas of the constructs (noted by the thick arrows) without engulfing the entire surface. The interactions were observed primarily along the horizontal and vertical planes. They were demonstrated by the series of optical sectioned images along z-axis (denoted in the black box; Fig. 2c–e). The bacteria colonized and proliferated to create elongated biofilm structures (noted by the thin arrows). These structures extended from one construct to the next within the Matrigel™ milieu.

S. mutans penetrates and modifies the internal architecture of 3D DPSC microtissues

After 7 days of cultivation, 3D cell constructs that interacted with GFP-tagged S. mutans maintained their spherical aggregated structures (Fig. 3a–d). Similar patterns were seen in the unchallenged 3D DPSC constructs (Fig. 3e–h). However, after the infection of 3 days culture (white arrows; Fig. 3i–l), bacteria attached to the cell surface without any evidence of cell invasion. 3D DPSC structures responded to the bacterial challenge by enhancing the proliferation rate and significantly increasing (P<0.01) the constructs thickness compared to the unchallenged ones (138.42±15.2 vs 106.51±10.9 µm, respectively; Fig. 3m). Cross-sectional analysis of stacked LCSM images revealed bacterial penetration into the 3D cell constructs and a network of canal-like microstructures, created by the bacteria. Some of the canals were occupied by the bacteria (white arrows; Fig. 3n), while others remained unoccupied (yellow arrows; Fig. 3n). The canals were detected in all five challenged microtissues with a varying diameter from 12.94 to 41.88 µm. The average diameter of the canals per microtissue was between 20.66
Canals were not detected in the unchallenged microtissues.

**3D DPSC microtissues challenged with *S. mutans* continue to express the pluripotency marker Sox2**

After 7 days of cultivation, GFP-tagged *S. mutans*-challenged cell cultures expressed the pluripotency and the self-renewal marker Sox2 (Fig. 4a–h) to the same extent as the unchallenged constructs (Fig. 4i–p). The expression of the marker appears equal in areas where cells interacted with the bacteria (white box, black arrows; Fig. 4a, c) and in areas where cells did not.

**Challenged 3D DPSC microtissues have the potential to differentiate and secrete the mineral**

3D DPSC cultures challenged with the bacteria and cultured in mineralization media to stimulate the differentiation process expressed the osteogenic marker osteocalcin (Fig. 5a–l). Furthermore, mineral secretion in challenged cell constructs occurred at the same extent as in the unchallenged controlled systems. Accumulation of the mineral in the matrix and its detection by alizarin red reagent after 7 days of incubation with the bacteria are shown in Fig. 5(m–o).

**DISCUSSION**

As a major pathogen of dental caries, *S. mutans* attaches to dentin, colonizes the tissue even after caries removal and sealing (Damé-Teixeira et al., 2014) and penetrates deep into dentinal tubules via SpaP (Love & Jenkinson, 2002; Love et al., 1997). Bacterial biofilms colonizing the deep layers of the dentin are identified as primary cause of endodontic infections and irreversible pulpitis (Rôças et al., 2015). These pieces of evidence accentuate the role of *S. mutans* as a potential endodontic bacterium capable of colonizing inflamed dental pulp (Nomura et al., 2016).

Even though *S. mutans* was reported as one of the aetiopathogenic agents isolated from infected pulp tissues, its abilities to interact, invade and interfere with major functions of the 3D pulp tissue have not been documented yet. Some of these properties are associated with the regeneration and differentiation capacities of the dental pulp tissue to recover after an injury (Zhang & Yelick, 2010). In this study, we evaluate structural and recovery mechanisms of 3D dental pulp cell cultures challenged with *S. mutans* in ECM environment. We utilize our unique biomimetic 3D infection model, which assimilates the interactions of the bacteria with organotypic DPSC cultures in ECM-Matrigel®. Matrigel® is considered a biomimetic basement membrane platform, which is used to provide a suitable stem cell niche for the development of 3D structures and recapitulate the

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**Fig. 2.** *S. mutans* UA159 (WT) interacts and colonizes particular areas of DPSC microtissues along the 3D coordinates after 7 days of incubation. 2D phase-contrast imaging of (1) spatial views of bacteria interacting with and colonizing 3D cell cultures (a, b), (2) three subsequent optical sectioned images along the z-axis of the interaction area (indicated by the black box) between the bacterial biofilm and the 3D cell cultures (c–e). The black thick arrows indicate the bacterial biofilm development on the cell constructs, while black thin arrows point toward the elongated biofilm structures.
Fig. 3. The challenged DPSC microtissues are penetrated by the GFP-tagged *S. mutans* to create intercellular canal-like microstructures after 7 days of incubation. Fluorescence images (a–h) show GFP-tagged *S. mutans* (white arrows indicate bacteria location) colonization of 3D cell constructs [white boxes; images (e) and (g)]. Bacteria are attached to the surface of the fibroblasts after 3 days of growth [images (i–l); attached bacteria are indicated by white arrows in (j) and (k)]. Thickness of the challenged versus the unchallenged 3D cell constructs is represented by a bar graph (m). LCSM images representing cross-sectional analysis of cell aggregates penetrated by the bacteria [white arrows indicate the penetrating bacteria; yellow arrows indicate empty intercellular gaps, (n)]. The diameter distribution of the canal-like microstructures per each one of the five challenged microstructures is presented by a boxplot graph (o). Orange, F-actin; green, GFP-tagged *S. mutans*. 
natural environment of the interactions between *S. mutans* and DPSCs, and was utilized before in host–pathogen interaction assays (Ma & Baumgartner, 2013; Schlaermann et al., 2016). Our approach is encouraged by studies suggesting that *S. mutans* virulence adhesins (SpaP, WapA and SmFnB) play a significant role in ECM binding and bacterial pathogenicity (Westerlund & Korhonen, 1993). Bacteria that infiltrate via the ECM are exposed to decreasing levels of oxygen and nutrients, which lead to oxidative stress and the activation of *bprA* gene playing a major role in biofilm formation (Wen et al., 2006; Bitoun et al., 2012; Gambino & Cappitelli, 2016). The DPSC cultures were incubated at 5% CO₂ conditions required to maintain physiological pH.

Results of our study suggest that *S. mutans* biofilms attach to and colonize distinct areas of the 3D DPSC microtissues. The adherence mechanism of the bacteria to local areas presumably involves the host cell receptor recognition by bacterial surface adhesins. These proteins may also trigger host cell signalling cascades and modulate their behaviour (Nobbs et al., 2009). *S. mutans* cell wall polysaccharides rhamnose–glucose polymers and the adhesin SpaP (antigen I/II polypeptide) effects on epithelial and endothelial cells reportedly include (i) release of cytokines by binding to their integrin α5β1 receptor and stimulation of the mitogen-activated protein kinase signalling pathways (Al-Okla et al., 1999) or (ii) binding through lectin interactions (Vernier et al., 1996). Although *S. mutans* was found to adhere to the cell membranes, in contrast to gingival fibroblasts (Berlutti et al., 2010), there was no evidence of invading them. This phenomenon could be explained either by the pathogen strain lacking the Cnm protein that typically enables the invasion into the host cells (Abranches et al., 2011) or by well-established cellular immune response that dental pulp cells develop against bacterial invasion. The latter would suggest the involvement of pattern recognition receptors such as the Toll-like receptor (TLR) family. TLRs trigger the effector phase of the innate immune response, including antimicrobial agents and pro-inflammatory cytokines and chemokines that recruit and activate tissue resident and blood borne immune/inflammatory cells (Farges et al., 2015a). The intrinsic capability of DPSCs to resist...
bacterial invasion and survival and as a result preventing alterations in regulatory systems and basic functions such as reproduction and differentiation may be explained by the antibacterial agents known to be produced by odontoblasts, such as β-defensins (BDs) that function as broad-spectrum, cationic antimicrobial peptides. BD-2 was shown to possess antibacterial activity against *S. mutans* (Song et al., 2009), except for its pro-inflammatory role, and it was found to be regulated by the activation of TLR4. These pieces of evidence suggest that BDs are differentially produced by odontoblasts to combat Gram-positive bacteria such as *S. mutans*. Another important antimicrobial agent produced by challenged odontoblasts is nitric oxide (NO). This antibacterial free radical agent is produced through oxidation by the inducible NO synthase 2. Evidence showed that NO synthase 2 synthesis and NO production are amplified upon TLR2 activation and that NO can inhibit the growth of *S. mutans*, consequently suggesting the role of this odontoblast-derived molecule in the limitation of intradental progression of caries-related micro-organisms (Farges et al., 2015b). Further studies may be required to analyse the role and control of these agents in DPSC 3D cultures surrounded by ECM environment.

The healing sequence of dental pulp starts with an initial inflammatory process, continues with tissue regeneration (pulp repair) and ends with cell differentiation. The repair occurs at a later stage, when the infection is under control and the inflammation is modulated (Goldberg et al., 2015). DPSCs are considered adult self-renewable progenitor cells that express the embryonic stem cell markers, including the major transcription factor Sox2, and increase their proliferation rate upon infection (Ronay et al., 2014). They also possess a multi-lineage differentiation potential with the ability to migrate to the injured site, proliferate and

**Fig. 5.** Challenged 3D DPSC microtissues retain potential to differentiate and secrete mineral after 7 days of incubation. Expression of osteocalcin in cells unchallenged [images (a–d)] versus challenged with GFP-tagged *S. mutans* [images (e–l); white boxes in images (e) and (g), white arrow indicates bacteria location] (a–l) (colour legend: red, osteocalcin; green, bacteria). Mineral secretion in challenged versus the unchallenged 3D cell cultures stained with alizarin red (m–o). Colour legend: orange indicates mineral formation. Challenged standard media is shown as a comparative control.
differentiate to the specific cell type that facilitates the healing of the tissue. DPSCs can differentiate into odontoblasts, which makes them the most promising candidates for dentin–pulp complex regeneration by expressing osteogenic markers such as alkaline phosphatase and osteocalcin and producing and secreting the dentin mineral (Zhang & Yelick, 2010). Consistent with our observations, odontoblasts have been shown to survive, proliferate and continue producing the dentin barrier beneath the decayed or injured dentin. Our bacterial challenges could be representative of S. mutans interactions with DPSC constructs based on similar results that were obtained by other studies, demonstrating infected periodontal granulation tissues which harbour cells expressing embryonic stem cell markers and osteogenic capacities (Ronay et al., 2014). Although injured dental pulp tissue has limited potential for self-recovery, mild stimulation or slow progress of dental caries may facilitate self-recovery according to the irritation level.

The penetration of S. mutans into the DPSC constructs by creating a network of canal-like microstructures may be explained by the collagenolytic activity associated with this strain (Jackson et al., 1997). Since collagen is a major ECM protein which binds cells together via the cellular integrin receptors α2β1, degradation of this protein by collagenases may deteriorate the bonding and create intercellular gaps (observed in our structures). Two of these putative collagenases of the peptidase U32 family have already been isolated and cloned (Han et al., 2006).

Our 3D bacterial challenged ECM/DPSC model shows strong potential for assessing cell–pathogen interactions in the ECM environment, which simulates the in vivo conditions of the pulp tissue.

The main findings of this study would suggest reexamining the role of S. mutans as an endodontic pathogen. In order to develop deeper understanding of DPSC resistance to S. mutans pathogenicity, further studies based on our biomimetic model may be prudent.

In our DPSC microtissue study, S. mutans mildly resemble the typical endodontic pathogenic bacteria. The modifications to the internal architecture of the 3D cell layers caused by the bacteria did not interfere with their recovery mechanisms. However, we speculate that these inner structural alterations may increase the susceptibility of the constructs to other pathogen infections. The 3D ECM/DPSC microtissue model was found to be a useful biomimetic platform that mimics the microenvironment of the pulp bacteria challenges and may be used as a tool to determine interactions with other oral pathogens or even multispecies or to measure the efficiency of antimicrobial drug release in the ECM environment.

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