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DOI:
[10.1111/jre.12546](https://doi.org/10.1111/jre.12546)

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Document Version
Peer reviewed version

Citation for published version (Harvard):
Abdulkareem, A, Cooper, P, Milward, M, Landini, G & Shelton, R 2018, 'Potential role of periodontal pathogens in compromising epithelial barrier function by inducing epithelial-mesenchymal transition', *Journal of Periodontal Research*. <https://doi.org/10.1111/jre.12546>

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Publisher Rights Statement:

Checked for eligibility: 21/02/2018

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Potential role of periodontal pathogens in compromising epithelial barrier function by inducing epithelial-mesenchymal transition

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ABSTRACT

Background and Objective: Epithelial-mesenchymal transition (EMT) is a process by which epithelial cells acquire a mesenchymal-like phenotype and this may be induced by exposure to Gram-negative bacteria. It has been proposed that EMT is responsible for compromising epithelial barrier function in the pathogenesis of several diseases. However, the possible role of EMT in the pathogenesis of periodontitis has not previously been investigated. The aim of this study therefore was to investigate whether Gram-negative, anaerobic periodontal pathogens could trigger EMT in primary oral keratinocytes *in vitro*.

Methods: Primary oral keratinocytes were harvested from labial mandibular mucosa of Wistar Han rats. Cells were exposed to heat-killed *Fusobacterium nucleatum*, *Porphyromonas gingivalis* (100 bacteria/epithelial cell), and 20µg/ml *E. coli* lipopolysaccharide (LPS) over 8-days. Exposure to bacteria did not significantly change epithelial cell number or vitality in comparisons with unstimulated controls at the majority time-points examined. Gene expression of EMT-markers were determined by semi-quantitative reverse transcriptase-polymerase chain reaction (sqRT-PCR) at 1, 5, and 8 days following stimulation. The expression of EMT-markers was also assessed by immunofluorescence (E-cadherin and vimentin) and using immunocytochemistry to determine Snail activation. The loss of epithelial monolayer coherence, in response to bacterial challenge, was determined by measuring trans-epithelial electrical resistance. The induction of a migratory-phenotype was investigated using scratch-wound and transwell-migration assays.

Results: Exposure of primary epithelial cell cultures to periodontal pathogens was associated with a significant decrease in transcription (~3 fold) of E-cadherin and the upregulation of N-cadherin, vimentin, Snail, matrix metalloproteinase-2 (MMP2) (~3-5 fold) and Toll-like receptor 4 (TLR-4). Bacterial stimulation (for 8-days) also resulted in an increased percentage of vimentin-positive cells (20% for *P. gingivalis* and 30% *F. nucleatum* stimulation, compared with controls). Furthermore, periodontal pathogens significantly increased the activation of Snail (60%) and cultures exhibited a decrease in electrical impedance ($P < 0.001$) in comparison with unexposed controls. The migratory ability of the cells increased significantly in response to bacterial stimulation as shown by both the number of migrated cells and scratch-wound closure rates.

Conclusion: Prolonged exposure of primary rat oral keratinocyte cultures to periodontal pathogens generated EMT-like features which introduces the possibility that this process may be involved in loss of epithelial integrity during periodontitis.

INTRODUCTION

Periodontitis is a polymicrobial, inflammatory condition affecting tooth-supporting tissues, characterised by a progressive loss of epithelial attachment and resorption of alveolar bone (1). If not correctly managed, it eventually leads to tooth loss (2). Periodontitis is associated with a wide diversity of bacterial species in the oral environment; however, a small group of bacteria has been shown to be strongly associated with disease progression and tissue destruction, particularly *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, *Bacteroides forsythus* and *Aggregatibacter actinomycetemcomitans* (3). These bacteria possess potent virulence factors including lipopolysaccharide (LPS) and bacterial DNA, which can induce an intense immune response stimulating the release of range of pro-inflammatory cytokines and metalloproteinase (MMP) enzymes (4, 5, 6). Sulcular and junctional epithelium are the first defense against dental biofilms, acting both as a mechanical barrier and by secreting inflammatory cytokines and enzymes (7) which can orchestrate the local host immune response. Loss of epithelial integrity has been associated with invasion of bacteria into underlying connective tissues potentially increasing the inflammatory response and the subsequent tissue damage, which is characteristic of periodontal disease (8).

Epithelial-mesenchymal transition (EMT) has been proposed as a reversible process that shifts the cell phenotype from epithelial to mesenchymal-like (8). This is mainly characterised by the dissociation of cellular adhesion junctions, upregulation of mesenchymal markers, the downregulation of epithelial markers and an increased migratory ability of the cells (9, 10). Persistent inflammation and cytokines elicited by bacteria have been reported among potential predisposing factors for EMT-induction elsewhere in the body, for example in the lungs, liver and intestine (11-13). Furthermore, inflammatory signaling induced by microbial challenge, reportedly shares common pathways with EMT (14). Indeed gingival samples collected from patients with chronic periodontitis showed significant downregulation of E-cadherin compared with healthy controls (15). This finding was supported by another study showing that E-cadherin downregulation occurred following co-culturing of human gingival epithelium with *P. gingivalis*-LPS (16). Furthermore, prolonged application of LPS to epithelium of gingival sulcus of rats was shown to result in loss of cellular adhesion (17). These cellular changes are considered hallmarks of EMT (9, 18) and are regulated by the transcriptional factor Snail-1 (19, 20). Interestingly, overexpression of Snail-1 is known to be associated with vimentin upregulation (21, 22) and evidence from previous studies has indicated that Gram-negative bacteria are a potent Snail-stimulus through activation of LPS-TLR4 signaling (23, 24).

The potential role of EMT as a mechanism involved in compromising epithelial barrier function in periodontal diseases has not yet been fully investigated. While the influence of a plaque biofilm on the host response is well established, the cellular level mechanisms involved remain to be fully characterised. Indeed, it is possible that EMT is involved in loss of pocket epithelium integrity. The aim of this study therefore was to investigate whether EMT was induced in primary oral keratinocytes, obtained from rats, through exposure to periodontal pathogens in an *in vitro* model of periodontitis.

MATERIALS AND METHODS

Bacterial cultivation and heat-killing

Lyophilised stocks of *F. nucleatum* (ATCC 10953) and *P. gingivalis* (ATCC 33277) purchased from the American Type Culture Collection (ATCC, Rockville, MD) were reconstituted then plated onto a trypticase soy agar (with 5% sheep blood) and incubated anaerobically at 37°C in an atmosphere of 80% nitrogen, 10% carbon dioxide, 10% hydrogen for 48hr. Pure cultures, indicated by colony morphology, Gram stain and biochemical test, were inoculated into 10ml trypticase soy broth and incubated at 37°C in anaerobic conditions for 24hr. Post-inoculation, the bacterial suspension was pelleted, washed with sterile PBS and then heat-inactivated at 100°C for 10min in an autoclave (Prestige Medical, UK). Bacteria concentration of the suspension was determined by measuring the optical density (OD) using a spectrophotometer (Jenway, Dunmow, UK) at 600nm. A final suspension containing 4×10^8 bacteria/ml was produced using a standard curve (OD vs. bacterial count). Fifty μ l of the suspension were re-plated on blood agar and cultured anaerobically for 48hr to confirm the death of the bacteria.

For each experiment a ratio of 100 bacteria per epithelial cell (25) in an attempt to mimic the cell-bacteria ratio reported in periodontal pockets. Twenty μ g/mL *E. coli* LPS (Sigma, UK), a well-characterised EMT inducer, was used as a positive control (26).

Primary oral keratinocytes isolation and culturing on feeder layer

Gingival samples were excised from mandibular labial gingiva of 6-week old euthanized male Wistar Han rats (Charles River Laboratory, UK). Keratinocyte culture medium (KCM) was prepared, consisting of 20ml of Dulbecco's modified Eagle's medium (DMEM) (Sigma, UK), supplemented with 2.5mM L-glutamine, 10% foetal calf serum (FCS) (Sigma, UK), 10 μ l/ml of penicillin–streptomycin, 2 $\times 10^{-2}$ μ l/ml cholera toxin, 5 $\times 10^{-2}$ μ l/ml of epidermal growth factor (ThermoFisher, UK) and 5 $\times 10^{-2}$ μ l/ml Amphotericin-B (Sigma, UK). Following excision, samples were incubated overnight in tubes containing 0.25% trypsin-EDTA at 4°C. The following day, the sheet of epithelium was carefully peeled away from connective

tissue using forceps. Then the epithelial sheet was dissected into smaller pieces (approximately 2x2mm) and seeded into T75 flasks containing Mitomycin-C-inhibited 3T3 fibroblasts and KCM, and incubated at 37°C in 5% CO₂. Keratinocyte growth was identified by the formation of round-tightly adherent epithelioid colonies. Once cultures reached ~70-80% confluence, the cells were sub-cultured up to 5 times and this was the maximum passage number used before they were discarded.

For each experiment, epithelial cells cultures were fed every two days. Experimental groups were repeatedly stimulated with bacteria every time media were changed, taking in consideration the increasing number of epithelial cells. Further, bacteria number was adjusted to keep the ratio between epithelial cells and bacteria constant (100 bacteria per cell) throughout experiment period.

Cell count and viability assay

The number and viability of oral keratinocytes were assessed using Trypan blue (Sigma, UK) and a haemocytometer (Improved Neubauer, Hawksley, UK). Viable and non-viable cells were counted separately to determine the percent of viable cells and the total cell number in each sample using an inverted microscope (Primovert, Zeiss, Germany) with a 10x objective. The procedure was repeated in triplicate and mean readings used to calculate total cell count and percentage viability.

Semi-quantitative reverse transcriptase-polymerase chain reaction (sq-RT-PCR)

Total RNA was extracted from stimulated and unstimulated cultures, after 1, 5, and 8 days, using RT lysis buffer (Qiagen, UK). Single stranded cDNA was synthesised from 1µg of RNA using the Tetro kit (Bioline, UK). cDNA templates were added to RedTaq (Sigma, UK) mastermix then amplified in a thermal cycler (Mastercycler, Eppendorf, Germany) using selected primers (Suppl. Table 1) for between 18-40 cycles. Each cycle consisted of denaturation, 5min at 94°C, followed by an amplification cycle, 94°C for 20s, then 60–61°C for 20s and 72°C for 20s ending with a 10min extension at 72°C. Amplification products were visualised following separation in 1.5% agarose gels supplemented with ethidium bromide (10mg/ml) (Sigma, UK). Images were captured using GeneSnap software (Syngene, USA) and analysed using the GeneTools software (Syngene, USA). Relative levels of PCR products were calculated and normalised against the housekeeping gene GAPDH. All analyses were performed in duplicate.

Immunofluorescence (IF) and immunocytochemistry (ICC) technique

Keratinocytes were cultured on sterilised glass coverslips (22x22mm) and stimulated with bacteria as described previously for 8 days. Cultures were prepared for IF and ICC by fixing in 4% paraformaldehyde

for 15min. Cells were permeabilised with 0.25% Triton X-100 in PBS (10min). Non-specific binding of antibodies was blocked by incubating samples for 30min in 5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS). Incubation with primary antibodies (1:100) for Anti-E-cadherin (rabbit polyclonal IgG), Anti-vimentin (mouse monoclonal IgG) (Santa Cruz, USA), and Anti-Snail (rabbit polyclonal IgG) (Abcam, UK) was undertaken overnight at 4°C. For IF, secondary antibodies were applied for 1hr at room temperature. The antibodies were FITC-conjugated (bovine anti-rabbit) and TRITC-conjugated (goat anti-mouse) for immunofluorescence (Santa Cruz Biotechnology (USA)). Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Sigma, UK) and images captured using a confocal microscope (LSM 700, Zeiss, Germany). The number of vimentin-positive cells were counted in 5 random fields and expressed as percentage of the total number of cells (27).

For Snail activation, biotin-conjugated (mouse anti-rabbit) (Abcam, UK) was used and counterstained with Mayer's haematoxylin. Slides were viewed (10x objective) using a bright field microscope (Olympus BX50, Japan). A total of 4 random fields from each sample were used to calculate the percent of Snail-positive cells in the fields examined. Both assays were performed in triplicate.

Transepithelial electrical resistance (TEER)

This assay was used to examine integrity of epithelial monolayers *in vitro* by measuring their resistance to the passage of an electric current. Tissue culture inserts with 0.4µm pore membrane (Greiner Bio-one, Germany) mounted in 24-well plates were seeded with keratinocytes (7×10^4) in the upper chamber (28). Cultures were exposed separately to media only (unstimulated control), or the bacterial components described previously. Measurement of the transepithelial electrical resistance (TEER) was initiated at a point where stable recording was obtained (after 3-days) from the culture using an AC-based ohmmeter (EVOM2, USA). Experiments were performed in triplicate.

Scratch wound assay

Primary oral keratinocytes were grown to confluency in 6-well plates and either treated with media only or exposed to bacterial components over 8 days. The epithelial cultures were "scratched" using a sterile 10µm pipette tip and rinsed with phosphate buffered saline (PBS) to remove debris prior to media replenishment. Only samples with the same initial gap width (about 500µm) were selected for this experiment to avoid discrepancy in the closure rate. Images were captured immediately after scratching (time 0hr), then at 12, 24, and 36hr using phase contrast microscopy (Primo Vert, Zeiss, Germany). Images were superimposed by grid and the distance between the wound margins at ten points was

measured and analysed using Java-based ImageJ (29). Three images were captured at each time-point and data were averaged. Experiments were repeated in triplicate

Transwell migration assay

Cells were either treated with media only or heat-inactivated bacteria or 20µg/ml *E. coli* LPS for 8 days. Then the cells were passaged and seeded (1×10^5 cells) in the upper compartment of 8µm-pore tissue culture insert (Greiner Bio-One, Germany), containing 200µl FCS-free media, assembled in the 24-well plate. Media supplemented with FCS (700µl) were added to the lower compartment of each well. Cells were incubated at 37°C and 5% CO₂ for 12hr. Non-migrated cells in the upper compartment of the membrane were removed using a cotton swab; cells on the lower surface of the membrane were fixed with 4% paraformaldehyde for 15min followed by Giemsa staining (Sigma, UK) (30). Cell counts were performed on 4 random fields obtained using phase contrast microscopy. All experiments were performed in triplicate.

Statistical analysis

Normal distribution of data was first checked using Statistical Package for the Social Sciences (SPSS) (version 21, IBM, USA) to decide which statistical tests to be applied for analysis. Results were expressed as mean ± standard deviation. Analyses were performed using one-way ANOVA followed by Tukey's post-hoc test between and within multiple groups to compare differences. Statistically significant results were considered for P values < 0.05.

RESULTS

Effect of periodontal pathogens on oral keratinocyte growth rate and viability

During the experimental period of 10 days, the number (Fig.1A) and viability (Fig.1B) of oral keratinocytes cells exposed to heat-killed *F. nucleatum*, *P. gingivalis*, and 20µg/ml *E. coli* LPS, did not show any significant differences at most of time points when compared with cultures in media only. However, a significant decrease ($P < 0.05$) in percentage of viable cells was observed at days 7 and 8 only in association with exposure to *F. nucleatum* and *P. gingivalis*, respectively.

Expression of EMT-related markers in oral keratinocyte cultures stimulated with bacterial components

Exposure of oral keratinocytes to heat-killed periodontal pathogens and *E. coli* LPS for 8 days resulted in altered expression of key molecules, consistent with EMT-related changes. PCR data demonstrated that

the transcription of E-cadherin in stimulated cultures was significantly downregulated ($P < 0.001$) in comparison with controls. This was associated with significant upregulation ($P < 0.001$) of mesenchymal markers (vimentin, N-cadherin, Snail-1, and MMP2). The highest upregulation of mesenchymal associated markers at day 8 was associated with cells stimulated with *F. nucleatum*. In addition, the level of TLR-4 expression was also significantly ($P < 0.001$) increased in stimulated cultures relative to unstimulated controls (Fig.2A, B).

Consistent with these findings, the analysis of immunofluorescence images (Fig.3A) showed an increase in the percentage of epithelial cells staining positively for vimentin in response to bacterial exposure ($P < 0.05$) after 8-days culture (Fig.3B). This was also associated with the decrease of E-cadherin expression on the cell membrane of stimulated cultures compared with controls. In addition, vimentin and E-cadherin were co-expressed in some fibroblast-like cells (Fig.3A).

Integrity of oral epithelial cultures stimulated with bacteria

The analysis of Snail ICC images showed that the number of Snail-positive epithelial cells treated with bacterial components was higher compared with unstimulated controls (Fig.4A). Snail activation during EMT is considered to be a major event responsible for suppressing E-cadherin transcription with subsequent loss of cellular adhesion. ICC images (Fig.4B) showed increased nuclear and/or cytoplasmic activity of Snail when compared with unstimulated cultures. Stimulated cultures, at day 8, (Fig.4C) had increased Snail activity associated with dispersion of epithelial cells. In contrast, unstimulated control cultures retained their integrity and cells were tightly packed in a typical cobblestone pattern. Further confirmation of potential loss of epithelial barrier function was obtained from the TEER experiments. Resistance of epithelial monolayers to the passage of an electric current was significantly decreased ($P < 0.05$) in cultures exposed to bacteria compared with unstimulated controls (Fig.4D). A decrease in electric impedance began at day 5, following exposure to heat-killed *F. nucleatum*, *P. gingivalis* and *E. coli* LPS, and continued until day 7 which suggested that epithelial integrity was compromised by exposure to these bacteria or *E. coli* LPS.

Effect of bacterial components on migratory ability of oral keratinocytes

EMT is typically accompanied by an increased migratory activity of the cells. This feature was assessed in oral keratinocytes, stimulated with bacteria for 8 days, using a transwell migration assay which showed that the migration rate of epithelial cells was significantly increased ($P < 0.001$) (Fig.5A). The migratory ability was further investigated using the scratch wound assay on confluent monolayers also treated

with heat-killed bacteria and *E. coli* LPS. For each group, scratch wounds were performed multiple times in order to generate sufficient samples with the same initial gap width (~450µm) (Fig.5B). This avoided differences in closure rate due to variations of wound widths. Measurement, at 12 and 24hr, did not show any significant differences between stimulated and control groups in the rate of gap closure. However, after 36hr, stimulated cultures showed a greater rate of gap closure ($P<0.05$) in comparison with unstimulated controls (Fig. 5C)

DISCUSSION

EMT is a process that has been proposed to be involved (to varying degrees) in embryogenesis, cancer metastasis, and impaired healing of injured epithelium (9). The EMT process comprises a sequence of cellular events; starting with loss of apico-basal polarity, architectural cytoskeletal changes, loss of cell-cell and cell-extracellular matrix attachment, simultaneous downregulation of epithelial markers with upregulation of mesenchymal markers and finally acquisition of a migratory-phenotype (18). In the current study, we investigated the possibility of periodontal pathogens being able to trigger such EMT in primary oral epithelial cells. The study hypothesis was based on the presence of potential EMT-predisposing factors such as Gram-negative bacteria and inflammatory cytokines in the pocket microenvironment (14, 31, 32). This may interfere with the integrity of the pocket lining that represents an impenetrable physical barrier against bacteria in the dental biofilm, hence participating in disease progression.

In the current study, whole dead periodontal bacteria (*F. nucleatum* and *P. gingivalis*) were used to stimulate epithelial cells. The rationale for using dead bacteria was that the established periodontal pocket contains a relatively static biofilm with a number of dead bacteria. In vitro viable bacteria would multiply in the cell growth media over the experimental course resulting in changes in the bacterial host cell ratio, infection of the cell monolayer and production of metabolic byproducts that would compromise cell viability. It is well reported in the literature that whole dead bacteria are able to stimulate an inflammatory response in gingival epithelial cells (7). Whole dead bacteria contains several important virulence factors such as LPS, known to be released after death of bacteria, and bacterial DNA. Further, LPS is a well-characterised component of Gram-negative bacteria responsible for inducing an intense inflammatory response during periodontitis (33). Before investigating EMT-related features, a preliminary experiment was carried out on our model to investigate any harmful effect of bacterial components used on the oral keratinocytes. Data indicated that the concentration of bacterial stimuli used had limited inhibitory or toxic effect on keratinocyte cultures, which may interfere with the

results of this study. However, a transient decrease in viability was noted at day 7 (FN) and day 8 (PG). Cell viability was restored on days 9 & 10.. The precise reason for this temporary fluctuation in epithelial cell viability at these time points is not clear; however, this could be due to exhaustion of media nutrients prior to media change on day 8 which resulted in restoration of % cell viability. Another possible explanation is the increases in bacteria utilized during the experiment (increased numbers of bacteria introduced at each media change to reflect the increasing cell numbers) which could have compromised viability due to toxic effects of bacterial components, bacteria could have developed some level of tolerance resulting in improvements in cell viability.. Stimulation of primary oral keratinocytes with periodontal bacteria resulted in upregulation of TLR-4 expression that is responsible for recognition of LPS. Similar results have been reported in a previous study, which showed upregulation of TLR-4 in oral keratinocytes cultures following 24hr incubation with heat-inactivated *P. gingivalis* and *F. nucleatum* (26). TLR-4 acts as transducer for LPS-associated signaling, and activation of this pathway results in increased production of inflammatory cytokines (34), which are potentially involved in EMT-induction. This notion is supported by previous studies, which indicated that LPS is a potent EMT stimulus (35, 36). In addition, treatment of oral gingival epithelium with *E. coli*-LPS resulted in downregulation of E-cadherin expression (16). For this reason, *E. coli*-LPS was used as a positive control in our EMT studies. Various types of cellular junctional complexes including tight junctions, desmosomes, and adherence junctions (AJ) maintain the coherence of the epithelium (37). Calcium-dependent AJ is the most abundant epithelial junctions formed mainly by smaller structural units, such as E-cadherin, which also plays a crucial role in the formation of other cellular junctions (38, 39). Furthermore, E-cadherin complexes with the cytokeratin structural proteins of the epithelial cell cytoskeleton, thus forming a continuous network between the epithelial cells that add further support to the cellular attachment and hence generates a relatively strong physical barrier (40). Therefore, downregulation of E-cadherin expression is considered a major driver of EMT induction *in vivo* and *in vitro* (9). Results presented here demonstrated decreased E-cadherin transcription in response to stimulation with periodontal pathogens. This finding was in agreement with results from previous studies which indicated downregulation of E-cadherin expression in the inflamed periodontal tissue *in vivo* (16, 41) and *in vitro* (42, 43), and this was mostly attributed to Gram-negative anaerobic bacteria such as *P. gingivalis*. Additionally, permeability of the epithelial monolayer, in tissue culture inserts, increased in association with downregulation of E-cadherin expression in response to *P. gingivalis*-LPS exposure, which suggested loss of E-cadherin-mediated cellular junction (16). These data are also consistent with our findings that showed decrease in resistance of stimulated oral epithelial monolayers

to the passage of an electric current. Further support for this was obtained from the ICC images, which showed dispersion of stimulated epithelial cells in comparison with unstimulated cultures that maintained a classical cobblestone appearance. The increased percentage of cells with positive Snail-1 nuclear activity, as determined by ICC, was observed following stimulation of primary keratinocytes cultures with periodontal pathogens. Notably this transcriptional factor plays a pivotal role in EMT (19). The reason for increased nuclear activity of Snail-1 was not known but was likely due to signaling directly related to TLR-4 activation or in response to alternative signaling pathways triggered by inflammatory cytokines. Nevertheless, increased Snail-1 expression is a known marker of EMT-induction (19). Notably the increased Snail-1 activity could provide a possible mechanism for the downregulation of E-cadherin, which is in agreement with the fact that Snail-1 binds to the motif sites of E-cadherin and consequently decreases the expression of this adhesive protein. This molecular change will result in the subsequent disruption of cellular attachment essential for EMT programming (19, 20).

According to the definition of EMT, one of the major criteria used to identify this process is cadherin switching from an epithelial to a mesenchymal-like phenotype (9, 10), which was indicated by increased expression of mesenchymal N-cadherin with simultaneous downregulation of epithelial E-cadherin. Furthermore, upregulation of MMP2 was also noted during the experimental period. This enzyme is highly expressed during periodontitis and is involved in the breakdown of the basement membrane and local tissue destruction (5, 6). This is also regarded as an essential step required by EMT to facilitate migration of cells to the underlying connective tissue (9, 10). In addition, the acquisition of a migratory-phenotype, usually observed at the later stages of the EMT process, is reported in several studies and is regarded as a key indicator of EMT-associated cell plasticity (9). Notably the treatment of epithelial cultures with periodontal pathogens resulted in an increased rate of closure of scratch wounds inflicted on confluent epithelial monolayers. This was further confirmed by the transwell migration assay, which showed increased numbers of migrated cells through the membrane after bacterial stimulation. The increased cell mobility was possibly due to a decrease in cohesiveness of epithelial cells as a consequence of downregulation of E-cadherin associated with upregulation of vimentin expression, which is involved in formation, and maturation of cellular protrusion (38). This explanation is supported by findings of previous studies which indicated that increased locomotion of epithelial cells was associated with overexpression of vimentin and decreased E-cadherin expression (21, 22).

Previous studies were limited in investigating the effect of periodontal pathogens or their virulence factors on E-cadherin expression. Our study further explored other aspects of EMT in a periodontitis

model that further highlighted the role of Gram-negative bacteria in compromising the integrity of the epithelial barrier potentially by altering epithelial-phenotype. Our study has now indicated molecular, functional, and structural changes, representative of EMT, in primary oral keratinocytes in response to Gram-negative periodontal pathogens. These findings may implicate EMT in the pathogenesis of periodontitis. Further work is needed however to investigate the role of other periodontal pathogens, particularly ones from the red complex group, in inducing EMT. In addition, tissue samples collected from patients with varying severities of periodontitis are required to demonstrate the clinical importance of EMT in periodontitis. Furthermore, this phenotypic shifting could also occur due to shared risk factors for both EMT and periodontitis that include smoking, micronutrient availability, and a genetically determined aberrant immune response. Ultimately these data could explain the predisposition of certain individuals to EMT and hence their susceptibility to progressive periodontal disease.

ACKNOWLEDGEMENTS

This work was sponsored by Iraqi Ministry of Higher Education and Scientific Research. The authors report no conflicts of interest related to this study.

REFERENCES

- (1) Armitage GC. Development of a classification system for periodontal diseases and conditions. *Ann Periodontol.* 1999;4:1-6.
- (2) Martin JA, Page RC, Loeb CF, Levi PA, Jr. Tooth loss in 776 treated periodontal patients. *J Periodontol.* 2010;81:244-250.
- (3) Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL, Jr. Microbial complexes in subgingival plaque. *J Clin Periodontol.* 1998;25:134-144.
- (4) Offenbacher S. Periodontal diseases: pathogenesis. *Ann Periodontol.* 1996;1:821-878.
- (5) Gursoy UK, Kononen E, Uitto VJ. Stimulation of epithelial cell matrix metalloproteinase (MMP-2, -9, -13) and interleukin-8 secretion by fusobacteria. *Oral Microbiol Immunol.* 2008;23:432-434.
- (6) Grayson R, Douglas CWI, Heath J, Rawlinson A, Evans GS. Activation of human matrix metalloproteinase 2 by gingival crevicular fluid and Porphyromonas gingivalis. *J Clin Periodontol.* 2003;30:542-550.
- (7) Page RC, Offenbacher S, Schroeder HE, Seymour GJ, Kornman KS. Advances in the pathogenesis of periodontitis: summary of developments, clinical implications and future directions. *Periodontol 2000.* 1997;14:216-248.
- (8) Ji S, Choi YS, Choi Y. Bacterial invasion and persistence: critical events in the pathogenesis of periodontitis? *J Periodontal Res.* 2015;50:570-585.

- (9) Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. *J Clin Invest.* 2009;119:1420.
- (10) Lamouille S, Xu J, Derynck R. Molecular mechanisms of epithelial–mesenchymal transition. *Nat Rev Mol Cell Biol.* 2014;15:178-196.
- (11) Shen HJ, Sun YH, Zhang SJ, et al. Cigarette smoke-induced alveolar epithelial-mesenchymal transition is mediated by Rac1 activation. *Biochim Biophys Acta.* 2014;1840:1838-1849.
- (12) Bose SK, Meyer K, Di Bisceglie AM, Ray RB, Ray R. Hepatitis C Virus induces epithelial-mesenchymal transition in primary human hepatocytes. *J Virol.* 2012;86:13621-13628.
- (13) Scharl M, Huber N, Lang S, Fürst A, Jehle E, Rogler G. Hallmarks of epithelial to mesenchymal transition are detectable in Crohn’s disease associated intestinal fibrosis. *Clin Transl Med.* 2015; 4: 1.
- (14) Hofman P, Vouret-Craviari V. Microbes-induced EMT at the crossroad of inflammation and cancer. *Gut Microbes.* 2012;3:176-185.
- (15) Nagarakanti S, Ramya S, Babu P, Arun KV, Sudarsan S. Differential Expression of E-Cadherin and Cytokeratin 19 and Net Proliferative Rate of Gingival Keratinocytes in Oral Epithelium in Periodontal Health and Disease. *J Periodontol.* 2007;78:2197-2202.
- (16) Abe-Yutori M, Chikazawa T, Shibasaki K, Murakami S. Decreased expression of E-cadherin by *Porphyromonas gingivalis*-lipopolysaccharide attenuates epithelial barrier function. *J Periodontal Res.* 2017;52:42-50.
- (17) Fujita T, Firth JD, Kittaka M, Ekuni D, Kurihara H, Putnins EE. Loss of claudin-1 in lipopolysaccharide-treated periodontal epithelium. *J Periodontal Res.* 2012;47:222-227.
- (18) Zeisberg M, Neilson EG. Biomarkers for epithelial-mesenchymal transitions. *J Clin Invest.* 2009;119:1429-1437.
- (19) Wu Y, Zhou BP. Snail: More than EMT. *Cell Adh Migr.* 2010;4:199-203.
- (20) Peinado H, Ballestar E, Esteller M, Cano A. Snail Mediates E-Cadherin Repression by the Recruitment of the Sin3A/Histone Deacetylase 1 (HDAC1)/HDAC2 Complex. *Mol Cell Biol.* 2004;24:306-319.
- (21) Lee M-Y, Chou C-Y, Tang M-J, Shen M-R. Epithelial-Mesenchymal Transition in Cervical Cancer: Correlation with Tumor Progression, Epidermal Growth Factor Receptor Overexpression, and Snail Up-Regulation. *Proc Am Assoc Cancer Res.* 2008;14:4743-4750.
- (22) Myong N-H. Loss of E-cadherin and Acquisition of Vimentin in Epithelial-Mesenchymal Transition are Noble Indicators of Uterine Cervix Cancer Progression. *Korean J Pathol.* 2012;46:341-348.
- (23) Jing YY, Han ZP, Sun K, et al. Toll-like receptor 4 signaling promotes epithelial-mesenchymal transition in human hepatocellular carcinoma induced by lipopolysaccharide. *BMC Med.* 2012;10:98.
- (24) Ngo H-K-C, Lee HG, Piao J-Y, et al. *Helicobacter pylori* induces Snail expression through ROS-mediated activation of Erk and inactivation of GSK-3 β in human gastric cancer cells. *Mol Carcinog.* 2016;55:2236-2246.

- (25) Dierickx K, Pauwels M, Van Eldere J, Cassiman JJ, Van Steenberghe D, Quirynen M. Viability of cultured periodontal pocket epithelium cells and Porphyromonas gingivalis association. *J Clin Periodontol*. 2002;29:987-996.
- (26) Milward MR, Chapple ILC, Wright HJ, Millard JL, Matthews JB, Cooper PR. Differential activation of NF- κ B and gene expression in oral epithelial cells by periodontal pathogens. *Clin Exp Immunol*. 2007;148:307-324.
- (27) Abdulkareem AA. Potential involvement of epithelial-mesenchymal transition in the pathogenesis of periodontitis. University of Birmingham, 2017. 258pp. Ph.D.
- (28) Sume SS, Kantarci A, Lee A, Hasturk H, Trackman PC. Epithelial to Mesenchymal Transition in Gingival Overgrowth. *Am J Pathol*. 2010;177:208-218.
- (29) Rasband W. ImageJ, U. S. Maryland, USA: National Institutes of Health, Bethesda.
- (30) Schaeffer D, Somarelli JA, Hanna G, Palmer GM, Garcia-Blanco MA. Cellular Migration and Invasion Uncoupled: Increased Migration Is Not an Inexorable Consequence of Epithelial-to-Mesenchymal Transition. *Mol Cell Biol*. 2014;34:3486-3499.
- (31) Wang H, Wang H-S, Zhou B-H, et al. Epithelial–Mesenchymal Transition (EMT) Induced by TNF- α Requires AKT/GSK-3 β -Mediated Stabilization of Snail in Colorectal Cancer. *PLoS ONE*. 2013;8:e56664.
- (32) Kao S-H, Wu K-J, Lee W-H. Hypoxia, Epithelial-Mesenchymal Transition, and TET-Mediated Epigenetic Changes. *J Clin Med*. 2016;5:24.
- (33) Dumitrescu AL, Abd El-Aleem S, Morales-Aza B, Donaldson LF. A model of periodontitis in the rat: effect of lipopolysaccharide on bone resorption, osteoclast activity, and local peptidergic innervation. *J Clin Periodontol*. 2004;31:596-603.
- (34) Lu Y-C, Yeh W-C, Ohashi PS. LPS/TLR4 signal transduction pathway. *Cytokine*. 2008;42:145-151.
- (35) Li H, Li Y, Liu D, Liu J. LPS promotes epithelial–mesenchymal transition and activation of TLR4/JNK signaling. *Tumour Biol*. 2014;35:10429-10435.
- (36) Zhao L, Yang R, Cheng L, Wang M, Jiang Y, Wang S. LPS-Induced Epithelial-Mesenchymal Transition of Intrahepatic Biliary Epithelial Cells. *J Surg Res*. 2011;171:819-825.
- (37) Baum B, Georgiou M. Dynamics of adherens junctions in epithelial establishment, maintenance, and remodeling. *J Cell Biol*. 2011;192:907-917.
- (38) Tunggal JA, Helfrich I, Schmitz A, et al. E-cadherin is essential for in vivo epidermal barrier function by regulating tight junctions. *EMBO J*. 2005;24:1146-1156.
- (39) Nagafuchi A. Molecular architecture of adherens junctions. *Curr Opin Cell Biol*. 2001;13:600-603.
- (40) Tian X, Liu Z, Niu B, et al. E-Cadherin/beta-Catenin Complex and the Epithelial Barrier. *J Biomed Biotechnol*. 2011:567305.
- (41) Ye P, Chapple C, Kumar R, Hunter N. Expression patterns of E-cadherin, involucrin, and connexin gap junction proteins in the lining epithelia of inflamed gingiva. *J Pathol*. 2000;192:58-66.

- (42) Katz J, Sambandam V, Wu JH, Michalek SM, Balkovetz DF. Characterization of Porphyromonas gingivalis-Induced Degradation of Epithelial Cell Junctional Complexes. *Infect Immun*. 2000;68:1441-1449.
- (43) Katz J, Yang Q-B, Zhang P, et al. Hydrolysis of Epithelial Junctional Proteins by Porphyromonas gingivalis Gingipains. *Infect Immun*. 2002;70:2512-2518.