Effects of aging and bacterial factors on periodontal disease - in vitro and in vivo studies on the oral epithelial tissue integrity and host microbiomes

Abstract

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Abstract

Introduction:

Gingival tissue shows progressive alterations with aging. It is of great importance to know if these histopathological or clinical alterations in oral cavity with age is associated with physiological or pathological conditions. *Porphyromonas gingivalis* (*P. gingivalis*) colonization in aging subject is another important parameter which has a determinant role in modifying age related periodontal changes. Intracellular junctional molecules maintain the integrity of tissue including gingival tissue. However, the relationship between normal aging and *P gingivalis* superinfected aging with intercellular junctional molecules have not been studied thus far. The purpose of this study is to demonstrate the three-way relationship between junctional molecules, aging and *P. gingivalis* exposure.

Materials and Methods:

A. In vitro studies

i. Establishment of gingival senescence model

Primary human gingival epithelial progenitor (HGEPp) cells were cultured. On confluency, cells were treated with different concentrations of hydrogen peroxide. To determine an optimum concentration, cell vitality, and senescence-associated beta-galactosidase (SA-β-gal) staining was performed. After determining an optimum concentration, mRNA and protein expression of known senescence markers (p16, p21, and p53) were performed. Cell cycle assay was performed to determine the phase where senescence cells were arrested. Based on these results, optimum concentration of hydrogen peroxide was determined to induce senescence in gingival epithelial cells.

ii. Expression of intracellular molecules

HGEPp cells were cultured and treated with optimum concentration (determined as 400 μM) of hydrogen peroxide. Untreated cells were used as control. Both control and senescence-induced cells were further treated with 1 μgmL^{-1} *Pg* Lipopolysaccharide (*Pg* LPS).

Analysis of mRNA level of intracellular molecules

Total RNA was extracted from cells using TRIzol according to the manufacturer's instructions (Invitrogen) and reverse transcription was performed. The relative mRNA expression of tight junction molecules such as Claudin-1, Claudin-2 and E-cadherin were measured by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Data were analyzed using the ΔΔCt method.

Analysis of protein level of intracellular molecules

Cells were homogenized in lysis buffer (EzRIPA Lysis kit). 15 μg of protein was loaded into sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore). Membranes were blocked with 5% skimmed milk and then incubated with primary and secondary antibody solutions. β-actin was used as loading control. The bands were visualized using a chemiluminescent (Millipore).

iii. Transepithelial resistance and flux permeability

Cells in four groups {(Controls(young), 400 μM (old), controls with Pg LPS and old with Pg LPS treated)} were seeded at a density of 50,000 cells/ml in upper well of transwell insert. An epithelial voltmeter with STX electrodes (EVOM; Sarasota, FL) was used to measure the monolayers for transepithelial electrical resistance (TER). For

electrical measurements, two electrodes are used, one electrode placed in the upper compartment and the other in the lower compartment. The ohmic resistance was determined by applying a direct current (DC) voltage to the electrodes. The ohmic resistance is calculated based on Ohm's law. Paracellular permeability was measured using fluorescein isothiocyanate-conjugated albumin (FITC-albumin). Once the cultured cell monolayers had reached confluency, media in the top compartments were replaced with cell media mixed with FITC-albumin. The transwells were then placed in an incubator for two hours. Liquid samples can be seen after two hours of incubation, from which 100µl of solution was taken from basolateral well and transferred to 96 well fluorescent reader plate. The corresponding fluorescent intensities of all samples were measured at 485 excitation/528 emission.

B. In vivo studies

i. Oral administration of *P.gingivalis* to young and old mice:

C57BL/6J mice at 4 weeks (young) and at 76 weeks (old) of age were purchased and maintained under pathogen-free condition. Mice were divided into 4 groups, young group (n=16), old group (n=15). Each of these young and old groups were divided further into two groups. Young mice as controls (n=8), young mice inoculated with *P. gingivalis* (n=8), old mice (n=8) and old mice inoculated with *P gingivalis* (n=8). *P. gingivalis* ATCC 33277 was anaerobically grown on a blood agar for 5 days, and then a single colony was inoculated and incubated in a broth. Bacterial cells were collected by centrifugation, and suspended in phosphate-buffered saline mixed with 2% carboxymethylcellulose (CMC) to achieve a concentration of $1x10^9$ CFU/ml. *P. gingivalis* was inoculated to the oral cavity of mice through a feeding needle. As a

control, the young and old groups were sham-administered with 2% CMC without *P. gingivalis*. The 2% CMC with/without *P. gingivalis* was repeatedly administered thrice a week for 5 weeks. At 30 days after last inoculation, oral swab/saliva was collected first and mice were then killed to collect intestinal stool, gingiva and alveolar bone.

ii. Analysis of alveolar bone loss:

Hemisected mandible was fixed in 10% neutral buffered formalin for 24 hours, stored in 30% hydrogen peroxide for another 24 hours at room temperature to debride gingival tissue attachment and stained with 0.1% methylene blue to be viewed under digital microscope. Alveolar bone loss was determined by the total length measurement between the cementoenamel junction and the alveolar bone crest (ABC). The ABC distance was measured using Image J software.

iii. Analysis of mRNA and protein level of intracellular molecules

Total RNA was extracted from cells using TRIzol according to the manufacturer's instructions and reverse transcription was performed. The mRNA level was evaluated using qRT-PCR.

Gingival tissues were fixed in 10% formalin for immunohistochemistry to demonstrate the intracellular proteins using western blotting.

iv. Metagenomic analysis of oral and intestinal microbiome

Bacterial DNA was extracted from oral swab and intestinal stool. Metagenomic analysis was performed using 16S rRNA sequencing with next generation sequencer (Illumina). V3-V4 regions of bacterial 16S rRNA was targeted using region specific primers during

Amplicon PCR. Pooled samples were quantified fluorometrically, cleaned up and normalized. The normalized samples were sequenced on the Illumina MiSeq sequencer. Bacterial taxonomy, and alpha and beta diversities were analyzed using Quantitative Insights into Microbial Ecology 2 (QIIM2). Analysis of composition of microbes (ANCOM) was used to compare the composition of microbes among thousands of taxa.

Results

A. In-vitro studies

The relative mRNA expression of tight junction molecules such as Claudin-1 and Claudin-2 were significantly upregulated in *Pg LPS* treated groups as compared to untreated groups. In contrary, the expression of E-cadherin was significantly reduced in *Pg LPS* treated groups as compared to untreated groups.

There was significant reduction in transepithelial resistance in old cells as compared to controls. *Pg LPS* treated groups showed further reduction in transepithelial resistance as compared to untreated groups. Flux permeability was significantly higher in *Pg LPS* treated groups as compared to untreated groups.

B. In vivo studies

Quantitative analysis of alveolar bone revealed that both young and old mice administered with *P. gingivalis* exhibited bone loss severer than the sham-administered mice. The expression of Claudin-1 and Claudin-2 was significantly higher in *P.gingivalis* infected old group as compared to that of non-infected young and old groups. In contrast, E-cadherin expression was significantly lower in *P.gingivalis* treated group as compared to un-treated groups at mRNA expression level. Immunohistochemistry

showed concurrence with the mRNA expression level.

The metagenomic study showed significant effect of oral administration of *P.gingivalis* on oral and intestinal microbiome in both young and old groups. The alpha diversity (Shanon index) in the oral microbiome was significantly altered by administration of *P. gingivalis* in both young and old mice as compared to their controls. Also, the beta diversity (unweighted UniFrac) in the oral microbiome was significantly altered by administration of *P. gingivalis* in both young and old mice as compared to its control. In intestinal microbiome, significant alterations in beta diversity were seen between each group. The ANCOM analysis showed that bacteria such as *Bacteroides, Akkermansia* and *Alistipes* at genus level and *Coribacteriaceae, Clostridaceae* and *Ruminococcaceae* at family level were significantly altered between each group.

Conclusion:

In accordance with previous reports, our study also showed the altered expression of gingival intracellular molecules and oral and intestinal microbiome in old mice as compared to that of young mice. In addition, our study for the first time showed that the expression of epithelial barrier molecules is remarkably altered in LPS treated senescence-induced cells *in vitro* and *in vivo* as compared to all other groups. In addition, alpha and beta diversity of gut microbiome is also more pronounced in *P gingivalis* inoculated old mice as compared to young, *P gingivalis* inoculated young mice and old mice. We therefore concluded that the presence of periodontopathogenic bacteria will intensify epithelial degradation in old cells as compared to young cells. These finding may be helpful in understanding age related periodontal destruction in relation to *P. gingivalis* infection.