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Comparative Analysis of Gingival Tissue Antigen Presentation Pathways in Aging and Periodontitis

O.A. Gonzalez^{1,*}, M.J. Novak¹, S. Kirakodu¹, L. Orraca⁴, K.C. Chen², A. Strom-berg³, J. Gonzalez-Martinez⁵, and J. L. Ebersole¹

¹Center for Oral Health Research, College of Dentistry, University of Kentucky, Lexington, KY

²Microarray Core Facility, College of Medicine, University of Kentucky, Lexington, KY

³Department of Statistics, College of Arts & Sciences, University of Kentucky, Lexington, KY

⁴School of Dental Medicine, University of Puerto Rico, San Juan, PR

⁵Caribbean Primate Research Center, University of Puerto Rico, Sebaná Seca, PR

Abstract

Aim—Gingival tissues of periodontitis lesions contribute to local elevations in mediators, including both specific T cell and antibody immune responses to oral bacterial antigens. Thus, antigen processing and presentation activities must exist in these tissues to link antigen-presenting cells with adaptive immunity. We hypothesized that alterations in the transcriptome of antigen processing and presentation genes occur in aging gingival tissues and that periodontitis enhances these differences reflecting tissues less capable of immune resistance to oral pathogens.

Materials and Methods—Rhesus monkeys (n=34) from 3–23 years of age were examined. A buccal gingival sample from healthy or periodontitis sites were obtained, total RNA isolated, and microarray analysis was used to describe the transcriptome.

Results—The results demonstrated increased transcription of genes related to the MHC class II and negative regulation of NK cells with aging in healthy gingival tissues. In contrast, both adult and aging periodontitis tissues showed decreased transcription of genes for MHC class II antigens, coincident with up-regulation of MHC class I-associated genes.

Conclusion—These transcriptional changes suggest a response of healthy aging tissues through the class II pathway (*i.e.*, endocytosed antigens) and altered responses in periodontitis that could reflect host-associated self-antigens or targeting cytosolic intra-cellular microbial pathogens.

Keywords

Periodontitis; aging; antigen presentation; adaptive immunity

INTRODUCTION

Periodontitis is a chronic infection of the oral cavity with pathogenic biofilms triggering a persistent inflammatory response in gingival tissues that lead to loss of function of the periodontium and consequently loss of teeth (Jakubovics and Kolenbrander, 2010, Armitage

*Corresponding author: Octavio A. Gonzalez, Complete Address: Center for Oral Health Research, College of Dentistry, University of Kentucky, 1095 VA Drive. HSRB 414 Lexington, KY 40536-0305. Phone: (859)-323-0125. Fax number: (859)-257-6566. octavio.gonzalez@uky.edu.

Conflict of interest

The authors declare that there are no conflicts of interest related to this study.

and Cullinan, 2010). Historically, a range of immune and non-immune cell responses to oral bacteria *in vitro* and in gingival crevicular fluid of periodontitis have been described (Belibasakis and Guggenheim, 2011, Yin et al., 2010, Peyyala et al., 2012, Bodet et al., 2006, Kinane and Bartold, 2007). More recently, numerous investigations have emphasized the importance of the innate immune system in oral mucosal tissues, producing an array of biomolecules to maintain homeostasis (DeSantis et al., 2006). Nevertheless, the apparent inability of innate immunity and the inflammatory response to control oral infections results in the generation of more specific adaptive immune responses (Hayman et al., 2011, Ebersole, 2003a).

Both local and systemic immune responses result from periodontal infections, and are composed of antigen specific T cells and antibody of diverse isotypes and subclasses (Ebersole, 2003b). Various studies have documented that the phenotype and function of T cells in the periodontium reflect the types of antigens inducing the local responses and contribute to communicating with osteogenic processes leading to a potential control of the bone resorptive processes (Vernal et al., 2006, Kawai et al., 2006). Additionally, elevated levels of antibodies are detected to bacteria considered to be pathogens in oral biofilms (Hayman et al., 2011, Ramseier et al., 2009, Kinane and Bartold, 2007, Takeuchi et al., 2006). The breadth of adaptive immune responses, coupled with the detection and proposed role of professional antigen presenting cells (APCs), macrophages (Ku et al., 2011, Artese et al., 2011, Ren et al., 2009) and den-dritic cells (Jotwani et al., 2001, Cutler and Jotwani, 2006) supports that local antigen uptake, processing, and presentation must occur and play a role in control of periodontal infections.

Existing epidemiological data demonstrate increases in the prevalence and severity of periodontitis with aging in the presence of altered immune responses that may contribute to both protection and tissue destructive processes (Huttner et al., 2009). The accepted paradigm from these observations is that the disease in aging represents an accumulation of noxious challenge over time linked with more general disruptions in the integrity of the periodontal tissues (Hajishengallis, 2010, Gonzalez et al., 2011, Ebersole et al., 2008b, Ebersole et al., 2008a). However, substantial literature from other models of infection has demonstrated significant age-associated increases in susceptibility to infections. These observations have identified decreases in the capacity of older individuals to produce specific antibody (Frasca et al., 2011), and alterations in T cell activation profiles that could affect antibody levels/functions (Ebersole et al., 2008b, McArthur et al., 1995, Haynes and Swain, 2012).

Various aspects of human periodontal disease may be assessed in animal models that possess similar oral structures to the human periodontium (Graves et al., 2012, Oz and Puleo, 2011, Struillou et al., 2010, Yoshinari et al., 2006, Persson, 2005, Hardham et al., 2005, Ebersole et al., 2002, Assuma et al., 1998, Persson et al., 1994, Schou et al., 1993, Persson et al., 1993, Dreyer et al., 1986). These animal models of periodontal bone loss also include extensive studies in nonhuman primates (Roberts et al., 2004, Ebersole et al., 2002, Ebersole et al., 2000a, Schou et al., 1993, Holt et al., 1988, Ebersole et al., 1999, Moritz et al., 1998, Beem et al., 1991), in which significant bone loss results from ligature-induced disease, enable the examination of microbiological, immunological, and clinical features of periodontal disease and its prevention and treatment, and provide data supporting disease related to infection by *P. gingivalis* (Holt et al., 1988) similar to humans. It is clear that the primate model has provided the essential bridge for understanding the interaction of selected members of the subgingival microbiota with the host, particularly as reflected by the longitudinal alterations, which occur in the clinical and microbiological progression of ligature-induced periodontitis similar to the human periodontal experience (Madden and Caton, 1994, Persson et al., 1993). We and others have shown that characteristics of the

inflammatory response and systemic humoral immune responses that accompany ligature-induced periodontitis in nonhuman primates parallel those observed in human periodontitis (Ebersole et al., 2010, Ebersole et al., 2009, Ebersole et al., 2008b, Ebersole et al., 2002, Persson et al., 1994). Soluble receptors to IL-1 and TNF significantly inhibited recruitment of inflammatory cells in close proximity to bone, reduced osteoclast formation, and reduced bone loss in ligature-induced periodontitis in a nonhuman primate animal model (Assuma et al., 1998, Delima et al., 2001). Thus, we hypothesized that nonhuman primates could be employed to delineate alterations in the transcriptome of antigen processing and presentation genes that occur in aging gingival tissues and that periodontitis would enhance these differences reflecting tissues less capable of immune resistance to oral pathogens.

MATERIALS AND METHODS

Oral clinical evaluation

Rhesus monkeys (*Macaca mulatta*) (n=34; 14 females and 20 males) housed at the Caribbean Primate Research Center (CPRC) at Sabana Seca, Puerto Rico, were used in these studies. Animals were selected by age based on the following criteria: <3 years (young; n=5), 3–7 years (adolescent; n=5), 12–16 years (adult; n=12) and 18–23 years (aged; n=12). Nonhuman primates were fed a 20% protein, 5% fat, and 10% fiber commercial monkey diet (diet 8773, Teklad NIB primate diet modified: Harlan Teklad). The diet was supplemented with fruits and vegetables, and water was provided ad libitum in an enclosed corral setting.

A protocol approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Puerto Rico, enabled anesthetized animals to be examined for clinical measures of periodontal including probing pocket depth (PD), and bleeding on probing (BOP) (Ebersole et al., 2008a), including measures on 2 interproximal sites per tooth (mesio- and disto-buccal), excluding the canines and 3rd molars.

Tissue sampling and gene expression microarray analysis

A buccal gingival sample from either a healthy or periodontitis-affected site from the premolar/molar maxillary region of each animal was taken using a standard gingivectomy technique, and maintained frozen in RNAlater solution. Total RNA from each gingival tissue was isolated using a standard procedure as we have described, and submitted to the microarray core to assess RNA quality and analyze the transcriptome using the GeneChip® Rhesus Macaque Genome Array (Affymetrix) (Meka et al., Gonzalez et al., 2011).

qPCR Analysis

Based upon the microarray outcomes we selected 5 genes and performed a qPCR analysis using a standard technique in our laboratory employing a Roche 480 LightCycler. The oral tissue from *M. mulatta* was stored in RNAlater (Invitrogen, IN) in –80°C. Oral tissue was removed from RNAlater solution and homogenized in TRIZOL reagent (Invitrogen, IN) and RNA was isolated following vendors instruction. RNA was cleaned up using RNeasy Mini kit (Qiagen), and subsequently quantified using Nanodrop 1000 (Thermo Scientific). RNA from 4 different animals was pooled from each group and a total of 1 µg was used for each cDNA synthesis reaction. The first strand cDNA synthesis was carried out using 2.5 µM anchored-oligo (dT)₁₈ primer, 1X transcriptor RT reaction buffer, 20 units of 40 U/µl RNase inhibitor, 1 mM of deoxynucleotide mix and 10 units of reverse transcriptase (Roche, IN) to a final reaction volume of 20 µl. The reaction mixture was incubated 30 min at 55°C and the reverse transcriptase was inactivated by incubating 5 min at 85°C.

qPCR primers were designed using software PrimerQuest at Integrated DNA Technologies website (www.idtdna.com) and were synthesized by Integrated DNA Technologies, Inc (Coralville, IA). Primers were prepared for PSME2 (forward - CCACCCAAGGATGATGAGATG; reverse - CAGGGACAGGACTTTCTCATTC), IF130 (forward - GAGAAGAGGAGTGCAAACCTCAA; reverse - TCTCCATGTCGTCAAACCTCTTC), HLA-DPB1 (forward - TTTCTACCCAGGCAGCATTC; reverse - TGGAAGGTCCAGTCTCCATTA), HLA-DRA (forward - TCTCCCAGAGACTACAGAGAAC; reverse - CGCACACCCTTGATGATGA), CTSS (forward - GATGCGAGTCATCCTTCTTTCT; reverse - CACCATAGCCAACCACAAGTA) and GAPDH (forward - GGTGTGAACCATGAGAAGTATGA; reverse - GAGTCCTTCCACGATACCAAAG) genes. The cDNA was diluted 1:4 and used as a template for the real-time PCR analysis using a LightCycler 480 (Roche, IN). qPCR was performed in a total volume of 20 μ l containing 2 μ l of 10X LightCycler 480 SYBR Green I Master (Roche, IN), 0.5 μ M each of forward and reverse primers, and 2 or 4 μ l of the diluted cDNA. Real-time qPCR was carried out with an initial incubation at 95°C for 5 min followed by 45 cycles consisting of denaturing at 95°C for 10 s, annealing at 60°C for 5 s followed by amplification at 72°C for 5 s.

After amplification, a melting curve analysis was done to determine the specificity of the PCR products by incubating the products for 15 s at 55°C, and then increasing the temperature to 95°C at a ramp rate of 0.1°C/s. Melting curve profiles was used to identify and genotype PCR products. Relative quantification analysis was performed with LightCycler 480 software (Roche, IN). The concentration ratios for the target genes were calculated by normalizing to the housekeeping gene GAPDH. The level of message was determined and those levels compared across the RNA samples prepared from each of the healthy groups and the 2 periodontitis groups.

Data Analysis

Normalization of values across the chips was accomplished through signal intensity standardization across each chip using Affymetrix PLIER algorithm. The Ge-neChip® Rhesus Macaque Genome Array contained matched and mismatched pairs allowing the MAS 5 algorithm to be used. For each gene we first determined differences in expression across the groups using ANOVA (version 9.3, SAS Inc., Cary, NC). The healthy aged tissues were then compared to the other age groups, or compared with health versus periodontitis tissues in adults or aged animals using a *t*-test and accepting a *p*-value 0.05 for significance. Because of the cost of these types of nonhuman primate experiments and availability of primates of the various ages, we did not have sufficient samples to identify if the relationship between age and gene expression could be treated using a linear model, thus the subjects were classified and ANOVA was used for analysis. The choice of Least Significant Difference for multiple comparisons (ANOVA followed by *t*-tests) provided maximum power given our necessarily small sample sizes. We did determine a correlation with aging in healthy tissues or periodontitis tissues using a Spearman Rank correlation analysis that was fit to the gene expression by age. A *p*-value 0.05 was used to evaluate the significance of the correlation. Genes whose expression showed significant correlation with age were mapped into the Kyoto Encyclopedia of Genes and Genomes (KEGG) antigen processing and presentation pathway (www.genome.jp) to develop an ontology analysis. JMP (version 10.0, SAS Inc., Cary, NC) was used to create metagenes independently of group classification using principal components based on the correlation matrix. The plots are of the first two PCA scores across the healthy tissues and comparing healthy to periodontitis tissues within the two age groups. The variability is explained by each of the

scores indicated on the plots. The data has been uploaded to <http://www.ncbi.nlm.nih.gov/geo/info/submission.html>.

RESULTS

Gene Expression Profiles in Aging Healthy Gingival Tissues

Table 1 lists the genes examined in the antigen presentation and processing pathways. Table 2 identifies those genes that were significantly altered in aging healthy gingival tissues compared to other groups. The results demonstrated that the majority of alterations were observed in genes related to the Major Histocompatibility Complex class II (MHC-II) pathway, although selected NK cell genes were up-regulated in the healthy aging gingival tissues. Table 2 also provides an overview of the genes significantly correlated with age in healthy tissues. As was noted with the up-regulation of the MHC-II pathway genes, the majority of the correlated genes were in this pathway. Interestingly, this analytic approach also demonstrated a negative correlation with a primary MHC class I (MHC-I) antigen, MAMU-A, the heat shock protein, HSP90, and NFYA, a nuclear transcription factor.

Gene Expression Profiles in Periodontitis Tissues

Table 3 summarizes the variations in gene expression comparing healthy and periodontitis tissues. In adults MHC-I and MHC-II pathway genes were up-regulated, while HSP90 were down-regulated. Similar patterns were observed in periodontitis tissues from aged animals, with HSP70 down-regulation. Additionally various NK antigens were up-regulated in periodontitis tissues. Table 3 also summarizes the gene expression profiles that correlated with age in periodontitis tissues. While a number of these correlated genes overlapped with those noted in healthy tissues, *i.e.* aging effect, there were a number of genes in both MHC-I and II pathways that were significantly related to age, but only in the diseased tissues. Negative correlations were again noted with selected heat shock proteins and an NFY transcription factor.

The results of a Principal Components Analysis for the groups depicted in Figure 1 demonstrate that the transcriptome of aged healthy tissues was somewhat distinct from the other age groups with over 42% of the variation among the age groups described by these gene patterns. Additionally, the periodontitis tissues from the aged animals demonstrated a distinctive profile of genes expressed compared to healthy adult or aged tissues with 44.7% of the variation between disease and healthy tissues accounted for by the differential gene expression. Table 4 identifies those genes that were primary determinants in the PC analysis, showing representation of both the class I and class II pathways in discriminating among the healthy age groups and periodontitis tissues. We subsequently performed a PC Analysis on the animals, based upon clinical presentation of the gingival tissue for extent of bleeding on probing and mouth mean pocket depths, rather than stratified based on age. While the clinical characteristics increased with elevated bleeding and pocket depths with age and in those animals identified with naturally acquired periodontitis, the results demonstrated a much less robust grouping of the individual animals when age was omitted from consideration in this analysis (data not shown).

From the list of altered gene expression, we selected 5 genes (PSME2, IF130, HLA-DRB1, HLA-DRA, CTSS) for qPCR validation of the microarray analysis. Table 5 provides the results of this comparison identifying similar directions for the altered gene expression, although the magnitude varied between these 2 independent analyses. Only 3/15 comparisons appeared to be at variance between the methods. Correlation analyses demonstrated that 11/15 of the comparisons showed a positive correlation of >0.5 .

Figure 2 provides a schematic of antigen processing and presentation pathways for both the MHC-I and II antigens as well as T cell interactions with the resulting antigenic moieties that have been processed by the APCs. Highlighted within the figure are those genes whose expression was altered with aging in healthy gingival tissues. Figures 3A & B provide a schematic of the gene expression profiles in the antigen presentation and processing pathways that were affected by periodontitis in adult or aged animals. In both age groups, periodontitis was reflected by up-regulation of genes within the MHC-I pathway, with fewer increases in gene expression for NK cell antigens compared to healthy tissues. Additionally, both HSP70 and HSP90 gene expression was down-regulated with disease. The aged periodontitis tissues continued to demonstrate up-regulation of genes within the MHC-II processing compartment (MIIC)/class II vesicle (CIIV), albeit this was coincident with a lower increase in expression of MHC-II antigens for presentation processes.

DISCUSSION

Evidence from humans and nonhuman primate models of periodontitis has demonstrated an array of immune responses in the local gingival tissues. Innate immune mechanisms are crucial for maintaining homeostasis, while alterations in the microbial challenge to these tissues results in an acute inflammatory response (Cobb et al., 2009, Van Dyke and Sheilesh, 2005, Kinane and Bartold, 2007, Preshaw et al., 2004). The existing paradigm is that this acute response is incapable of removing the noxious microbial challenge resulting in a chronic or persistent inflammatory response with dys-regulated control of tissue destructive host mediators, enzymes, and cytokines. However, it has also been demonstrated that an adaptive immune response is taking place in these local tissues reflected by local and systemic sensitization of T cells and antibody specific for oral bacteria (Ebersole, 2003a, Ebersole et al., 2000b). Antibody development, antigenic specificity, response to therapy, and the impact of vaccination with select periodontopathogens on resistance to periodontitis have all supported the role of the adaptive immune response in the host-bacterial interactions that occur within the subgingival sulci (Graves et al., 2012, Schou et al., 1993). However, investigations of adaptive immune responses in other infectious diseases have suggested that these responses may wane with aging, albeit, this varies with antigen and pre-existing immune responses (Frasca and Blomberg, 2011, McElhaney, 2011, Grubeck-Loebenstien et al., 2009).

The principal finding in aging healthy gingival tissues in this study was significant increases in gene expression in the MHC-II pathway. Beyond a general up-regulation of MAMU-DM, -DO, -DP, -DQ and -DR genes, numerous other genes crucial for the pathway function were also up-regulated. Presentation of antigen and engagement of TCR on CD4⁺ T cells in the context of MHC-II molecules require processing of native proteins into short peptide fragments. These processes in professional APCs involve antigen denaturation via unfolding coupled with proteolysis. IFI30 (GILT; gamma-interferon-inducible lysosomal thiol reductase) can reduce protein disulfide bonds to facilitate unfolding of protein antigens for degradation and processing (Hastings and Cresswell, 2011). This endosomal processing of exogenous antigens integrates the resulting peptides with the MHC-II antigens in the context of the MIIC/CIIV compartment in the cells (Stern et al., 2006).

Besides the MHC-II heterodimers with isoforms that can bind and present different antigens to T cells, CD74 (HLA-DR antigens-associated invariant chain (Ii)) codes for a chaperone protein that associates with MHC-II and regulates antigen presentation in the endoplasmic reticulum (ER) and in endocytic vesicles (Beswick and Reyes, 2009). It stabilizes peptide-free MHC-II heterodimers and directs transport of the complex from the ER to the endosomal/lysosomal system for processing and binding of antigenic peptides to MHC-II (Shachar and Haran, 2011). Further enzymatic processing of the antigen for interaction with

the MHC-II is accomplished by a stepwise activity of pro-teinasases including a cysteine proteinase with small leupeptin-induced peptides (SLIP), which also initiate the release of Ii chain from MHC-II (Cresswell et al., 1990). The MHC-II proteins are exported from the ER in a vesicle, which is directed by the Ii-chain allowing fusion with a late endosome containing the endocytosed, and degraded proteins. This heterodimer is cleaved by cathepsin S (CTSS), a member of the peptidase C1 family of lysosomal cysteine proteases, leaving only a small fragment of the Ii-chain (CD74) called CLIP that continues to block peptide binding to the MHC-II protein. MAMU-DM (*e.g.*, MAMU-DMB) is a molecular chaperone that works in lysosomes and endosomes and binds to MHC-II, releasing CLIP and replacing it with an antigenic peptide from the endosome (Jensen et al., 1999, Zavasnik-Bergant and Turk, 2006). The stable MHC-II-exogenous antigen peptide is then presented on the cell surface. Of particular interest is that all of these components are up-regulated in aging healthy gingival tissues.

A corollary to this up-regulation of the exogenous antigen presentation pathway was a down-regulation of MHC-I pathway genes in aging healthy tissues compared to other groups. Interestingly, β_2 -microglobulin (B2M) and ERp57 (PDIA3) were increased in the aging healthy tissues. B2M is a serum protein found in association with the MHC-I heavy chain on the surface of cells. B2M knockout mice lose surface expression of MHC-I and show altered stability of the peptide binding groove for antigen presentation (Cooper et al., 2007). This lack of functional surface MHC-I minimizes the development of CD8⁺ T cells and as a consequence reduce cytotoxic T cell activities. The PDIA3 gene results in an ER protein that interacts with lectin chaperones and is a portion of the protein loading complex for MHC-I proteins in the endogenous pathway (Garbi et al., 2007). Current information suggests that complexes of PDIA3 and the lectins mediate protein folding by promoting formation of disulfide bonds. However, while the expression of the genes for proteins important in interfacing with the MHC-I proteins for antigen expression was elevated, the lower level of MHC-I antigens would be expected to negate the overall function of this antigen processing and presentation pathway in the aging healthy tissues. Additionally, the heat shock protein, HSP90, related to proteasome processing of cytosolic antigens was also decreased. This is a member of the heat shock protein family, which is up-regulated in response to stress, *e.g.*, heat shock. These are among the most highly expressed cellular proteins across all species. HSP90 assists in protein folding, aids in protein degradation, and transport of proteins (Tsan and Gao, 2009).

Finally, within the patterns of aging effects in healthy gingival tissues, nuclear factors that have a role in both MHC-I and MHC-II functions were significantly increased. The CIITA (class II, major histocompatibility complex, transactivator), which is up-regulated in aging tissues, is a transcriptional co-activator that translocates to the nucleus and acts as an essential positive regulator of MHC-II gene transcription (Drozina et al., 2005). Mutations in this gene severely compromise the immune system and increase susceptibility to rheumatoid arthritis and multiple sclerosis (Friese et al., 2005). Consistent with the alterations in pathway genes identified in the aging healthy tissues, the NFYB (nuclear transcription factor Y subunit beta) gene is down-regulated with aging. It is a member of a heterotrimeric transcription factors composed of three components, NFYA, NFYB and NFYC, that interacts with the SP1 transcription factor (Jabrane-Ferrat et al., 2002). The NFY complex stimulates the transcription of various genes, such as type 1 collagen, albumin and β -actin genes. Thus, NFY decreased expression parallels aging-related down regulation of MHC-I genes.

A crucial aspect for a successful antigen processing and presentation requires the capacity of the APCs to interact with and stimulate the functions of distinct T cell populations. The data showed no changes in genes related to interactions with CD4⁺ helper T cells and a minimal

effect on CD8⁺ T cells in healthy aging tissues. However, substantive increases in expression of genes related to NK cell interactions with APCs were observed. Natural killer (NK) cells are lymphocytes that can lyse abnormal/infected cells without previous specific antigen activation. They can also regulate specific humoral and cellular immunity. The aging tissues showed up-regulation of an array of genes for KIRs (killer cell immunoglobulin-like receptors). The ligands for these receptors (*e.g.* KIR3DL3, KIR3DP1, etc), are subsets of MHC-I proteins, which contribute to regulation of immune responses (Parham et al., 2012, Thielens et al., 2012). More specifically, increased expression of the KIR2D4 receptor that binds to HLA-C alleles and inhibits the activity of NK cells was seen in healthy aging gingival tissues (Jamil and Khakoo, 2011). Also altered were levels of the genes for KLRC1 that is a member of the killer cell lectin-like receptor family preferentially expressed by NK cells (Middleton et al., 2002). These proteins form a complex with KLRD1 (CD94) related to binding of MHC class I HLA-E molecules in NK and some cytotoxic T-cells resulting in negative regulation through the inhibitory CD94 receptor (Pegram et al., 2011). KLRC3 acts in a similar fashion through binding of HLA-Bw4 allele and inhibiting the activity of NK cells. Thus, it appears that the perspective related to increased levels of gene expression for NK cells in the aging healthy gingival tissues were related to down-regulating the function of these cells in the tissues.

Comparison of the findings with healthy gingival tissues demonstrated quite distinctive differences in gene expression profiles for antigen processing and presentation in periodontitis tissues. We noted that HSP90 and HSP70 gene expression were down-regulated in adult and aged periodontitis tissues. As described above, these are ubiquitously expressed chaperones up-regulated with cell stress and are critical for protein folding requirements of the cell (Chen et al., 2007, Udono, 2012). It would be expected that the noxious microbial challenge to the gingival tissues during periodontitis would be detected as a cellular stress. Thus, the lack of HSP responses under the conditions of periodontitis would be predicted to be a risk for the cells and tissues.

Also observed in the periodontitis tissues from both adults and aged animals was a seeming transition from up-regulation of the MHC-II pathway in health, to the MHC-I pathway in periodontitis. In particular, the aging periodontitis tissues presented a minimal effect on all the molecules in the MHC-II pathway. In adults, the expression of multiple genes in the MIIC/CIIV compartment of this pathway were up-regulated; however, little effect on the various members of the HLA class II antigen family was observed. In contrast, aging periodontitis tissues had up-regulation of the IFN γ gene, a pleiotropic cytokine, which plays a central role in promoting innate and adaptive mechanisms of host defense. IFN γ is secreted by T_H1 cells, CD8⁺ T cells, and NK cells and has antiviral, immunoregulatory, and anti-tumor capabilities. Related specifically to these studies, IFN γ stimulates normal cells to increase expression of MHC-I antigens through induction of antigen processing genes related to proteasome activities in APCs. It has also been suggested that this molecule can directly up-regulate MHC-I heavy chains and β 2-microglobulin, as part of the antigen-presentation complex (Zhou, 2009). In addition, in the adult periodontitis tissues, TAPBP (TAP-associated glycoprotein, tapasin) was significantly up-regulated (Lampton et al., 2008). The resulting glycoprotein is present in the lumen of the (ER) and mediates interaction between newly-assembled MHC-I molecules and the transporter associated with antigen processing (TAP), which is required for the transport of antigenic peptides across the ER membrane. Thus, taken together these findings support that antigen processing and presentation pathway activities in naturally occurring periodontitis in adults and aged animals are generally similar, and appear to primarily engage MHC-I-based processes. This could infer that during periodontitis cytosolic antigens processed through the proteasome and ER compartments may predominate in the tissues. These could be host-associated self-antigens, enabling some features of localized autoimmune reactions, or more likely

indicating immune responses that are targeting intracellular microbes, *e.g.* bacteria, viruses. This interpretation is consistent with numerous reports emphasizing the importance of cell invasion by periodontal pathogens, *e.g.* *P. gingivalis* and *A. actinomycetemcomitans* that exacerbate local inflammatory responses and tissue destruction.

A study by Papapanou and colleagues (Papapanou et al., 2009) evaluated gene expression patterns in human gingival tissues related to colonization by various oral bacteria. The results demonstrated that genes represented in the Gene Ontology database that were the most overrepresented GO group, was antigen processing and presentation related to *A. actinomycetemcomitans*, *P. gingivalis*, *T. forsythia*, and *T. denti-cola*. Stoecklin-Wasmer *et al.* (Stoecklin-Wasmer et al., 2012) identified difference expression of microRNAs in disease and healthy gingival tissues from humans. As in the previous study, GO analysis of the gene families most impacted by altered by specific miRNAs were related to immune cell trafficking of immune system development and function. Most recently Davanian *et al.* (Davanian et al., 2012) demonstrated a highly significant up-regulation of genes related to immune system processes in periodontitis gingival tissues of humans. In contrast, a report evaluating gene expression in tissues from experimental gingivitis, identified numerous immune response gene that were altered, although there appeared to be a predilection for inflammatory response genes rather than those associated with antigen processing and presentation (Offenbacher et al., 2009). Consequently, the alterations that we observed in naturally-occurring periodontitis lesions in these nonhuman primates demonstrate changes in this immune response pathway similar to those reported in humans and supports and important role for adaptive immune reactions in the local tissues within the context of this chronic disease.

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CLINICAL RELEVANCE

Scientific rationale for the study

The cellular and molecular mechanisms involved in higher prevalence of periodontitis with aging remain unclear. Antigen processing and presentation is a central immune mechanism involved in maintaining health at mucosal surfaces including the periodontium.

Principal findings

Age-related increased activities of antigen processing and presentation pathways of extracellular antigens in gingival tissues were associated with periodontal health, whereas pathways of intracellular antigens handling were specifically up-regulated with periodontitis.

Practical implications

Elucidation of potential mechanisms involved in down-regulation of MHC-II-related genes with periodontal disease could provide potential new therapeutic targets.

Figure 1. Principal Components Analysis of antigen presentation and processing pathway genes (**A**) across the age groups of healthy gingival tissues and (**B**) periodontitis tissues of adult and aged animals. Each point denotes the positional values for an individual animal. The red circle highlights the clustering of the animals (aged) or periodontitis.

Gene Expression in Aging Healthy Gingival Tissues

Figure 2.

KEGG pathway presentation of gene expression alterations in antigen processing and presentation pathways in aging healthy gingival tissues compared to tissues from the other groups of younger animals. Red denotes significantly up-regulated and correlated with aging. Yellow denotes significantly up-regulated and correlated with aging with minimal fold-difference. Green denotes down-regulation or negatively correlated with aging. Expression of genes shown in blue was unchanged at $p < 0.05$ significance.

Fig. 3A

Gene Expression in Adult Periodontitis Tissues

Fig. 3B

Gene Expression in Aged Periodontitis Tissues

Figure 3.

KEGG pathway presentation of gene expression alterations in antigen processing and presentation pathways in (A) adult or (B) aged periodontitis gingival tissues compared to tissues from healthy adult and aged animals respectively. Red denotes significantly up-regulated and correlated with aging. Green denotes down-regulation or negatively correlated with aging. Orange denotes fewer genes within the family that were significantly changed. Expression of genes shown in blue was unchanged at p 0.05 significance.

Table 1

Genes evaluated in the antigen processing and presentation pathways with their corresponding Probe Identification numbers.

GENE ID	GENE NAME	AFFYMETRIX PROBE
IFNG (KO: K04687)	interferon-gamma	MmugDNA.41414.1.S1_at
TNF (KO: K03156)	tumor necrosis factor	Mmu.14298.1.S1_at
PSME1 (KO: K06696)	Hypothetical protein LOC700644	MmugDNA.21695.1.S1_at
PSME2 (KO: K06697)	proteasome activator subunit 2 (PA28 beta)	MmugDNA.22991.1.S1_at
HSPA1A (KO: K03283)	similar to Heat shock 70 kDa protein 1 (HSP70.1) (HSP70-1/HSP70-2)	MmuSTS.602.1.S1_at
HSPA1B (KO: K03283)	heat shock 70kDa protein 1B	MmuSTS.603.1.S1_at
HSPA6 (KO: K03283)	similar to heat shock 70kDa protein 6 (HSP70B)	MmugDNA.25285.1.S1_at
HSPA8 (KO: K03283)	Heat shock 70kDa protein 8	MmugDNA.25572.1.S1_at
HSPA4 (KO: K09489)	heat shock 70kDa protein 4	MmugDNA.42100.1.S1_at
HSP90AA1 (KO: K04079)	Heat shock protein 90kDa alpha (cytosolic), class A member 1	MmuSTS.4086.1.S1_at
HSP90AB1 (KO: K04079)	heat shock 90kDa protein 1, beta	MmugDNA.32274.1.S1_at
MAMU-A (KO: K06751)	major histocompatibility complex, class I, A	Mmu.2935.4.S1_x_at
MAMU-B (KO: K06751)	major histocompatibility complex, class I, B	Mmu.2177.1.S1_x_at
MAMU-E (KO: K06751)	major histocompatibility complex, class I, E	MmunewRS.790.1.S1_at
MAMU-F (KO: K06751)	major histocompatibility complex, class I, F	MmugDNA.1042.1.S1_s_at
MAMU-G (KO: K06751)	major histocompatibility complex, class I, G	Mmu.2935.3.S1_s_at
B2M (KO: K08055)	beta-2-microglobulin	MmugDNA.5628.1.S1_at
PDIA3 (KO: K08056)	protein disulfide isomerase family A, member 3	MmugDNA.2124.1.S1_at
TAPBP (KO: K08058)	TAP binding protein (tapasin)	MmugDNA.10000.1.S1_at
TAP1 (KO: K05653)	transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)	MmugDNA.1081.1.S1_at
TAP2 (KO: K05654)	antigen peptide transporter 2-like	MmugDNA.3066.1.S1_at
CD8A (KO: K06458)	similar to T-cell surface glycoprotein CD8 alpha chain precursor (T-lymphocyte differentiation antigen T8/Leu-2) (CD8a antigen)	MmugDNA.5711.1.S1_at
CD8B (KO: K06459)	similar to T-cell surface glycoprotein CD8 beta chain precursor (CD8b antigen)	MmugDNA.16367.1.S1_at
KIR3DL (KO: K07980)	killer immunoglobulin-like receptor KIR3DL (CD158k antigen)	Mmu.7460.14.S1_s_at
KIR2DL4 (KO: K07981)	killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, 4	Mmu.7460.2.S1_x_at
KLRC1 (KO: K06541)	killer cell lectin-like receptor subfamily C, member 1	Mmu.1906.10.S1_s_at
KLRC3 (KO: K06541)	killer cell lectin-like receptor subfamily C, member 3	Mmu.1906.7.S1_at
KLRD1 (KO: K06516)	killer cell lectin-like receptor subfamily D, member 1	MmugDNA.17475.1.S1_s_at
IFI30 (KO: K08059)	similar to Gamma-interferon-inducible lysosomal thiol reductase precursor (Gamma- interferon-inducible protein IP-30)	MmunewRS.536.1.S1_at
LGMN (KO: K01369)	legumain	Mmu.2523.1.S1_at
MAMU-DMB (KO: K06752)	similar to HLA class II histocompatibility antigen, DM beta chain precursor (MHC class II antigen DMB)	MmugDNA.18015.1.S1_at
MAMU-DOA (KO: K06752)	similar to HLA class II histocompatibility antigen, DO alpha chain precursor (MHC class II antigen DOA) (MHC DZ alpha) (MHC DN-alpha)	MmugDNA.36614.1.S1_s_at

GENE ID	GENE NAME	AFFYMETRIX PROBE
MAMU-DOB (KO: K06752)	similar to HLA class II histocompatibility antigen, DO beta chain precursor (MHC class II antigen DOB)	MmuSTS.84.1.S1_at
MAMU-DPA1 (KO: K06752)	major histocompatibility complex, class II, DP alpha	MmugDNA.37154.1.S1_at
MAMU-DPB1 (KO: K06752)	major histocompatibility complex, class II, DP beta	MmugDNA.18601.1.S1_at
MAMU-DQA1 (KO: K06752)	major histocompatibility complex, class II, DQ alpha 1	MmugDNA.15798.1.S1_s_at
MAMU-DQB1 (KO: K06752)	similar to HLA class II histocompatibility antigen, DQ(1) beta chain precursor (DC-3 beta chain)	MmuSTS.86.1.S1_at
MAMU-DRA (KO: K06752)	major histocompatibility complex, class II, DR alpha	MmugDNA.1046.1.S1_s_at
MAMU-DRB1-4 (KO: K06752)	similar to HLA class II histocompatibility antigen, DRB1-4 beta chain precursor (MHC class I antigen DRB1*4) (DR-4) (DR4)	MmunewRS.436.1.S1_s_at
CD74 (KO: K06505)	CD74 molecule, major histocompatibility complex, class II invariant chain	Mmu.9241.2.S1_at
CTSS (KO: K01368)	similar to Cathepsin S	MmuSTS.3988.1.S1_at
CD4 (KO: K06454)	CD4 molecule	Mmu.15035.1.S1_at
CIITA (KO: K08060)	class II, major histocompatibility complex, transactivator	MmugDNA.20.1.S1_at
RFX5 (KO: K08061)	regulatory factor X, 5	MmuSTS.3763.1.S1_at
RFXANK (KO: K08062)	similar to regulatory factor X-associated ankyrin-containing protein isoform a	MmugDNA.24504.1.S1_at
RFXAP (KO: K08063)	regulatory factor X-associated protein	MmugDNA.10869.1.S1_at
CREB1 (KO: K05870)	cAMP responsive element binding protein 1	MmuSTS.3958.1.S1_at
NFYA (KO: K08064)	similar to Nuclear transcription factor Y subunit alpha (Nuclear transcription factor Y subunit A) (NF-YA) (CAAT-box DNA-binding protein sub- unit A)	MmugDNA.24571.1.S1_at
NFYB (KO: K08065)	nuclear transcription factor Y, beta	MmugDNA.37609.1.S1_at

Table 2

Differences in gene expression of aging healthy gingival tissues compared to younger animals (negative fold-difference denotes decrease in aging tissues), and correlations in gene expression with aging.

Gene ID	Fold Difference	<i>P-value</i>	Correlation (R)	<i>P-value</i>
B2M	1.09	0.023	0.4010	0.05
CD8A			0.4470	0.032
CD74	1.35	0.044	0.5870	0.003
CIITA	1.53	0.102		
CREB1	-1.18	0.019		
CTSS	1.48	0.007	0.5950	0.003
HSPA1B	-1.11	0.050		
HSP90AB1	-1.19	0.039	-0.4420	0.035
HLA-A			-0.747	<0.001
HLA-DMB	1.44	0.015		
HLA-DOB	1.51	0.008	0.5200	0.010
HLA-DPB1			0.5980	0.003
HLA-DQB1			0.5160	0.012
HLA-DRA	1.44	0.013		
HLA-DRB1-4	1.48	0.043		
IFI30	1.39	0.017		
KLRC1	1.52	0.06		
KLRC3	1.86	0.022		
KLRD1	1.41	0.015	0.64705	<0.001
NFYA	-1.19	0.022	-0.4330	0.039
PSME2	1.21	0.008		
PDIA3	1.26	0.030		

Table 3

Differences in gene expression between healthy and periodontitis gingival tissues in adult and aged animals (negative fold-difference denotes decrease in periodontitis tissues), and correlations with age in periodontitis tissues.

Gene ID	Adult		Aged		Correlation (R)	P-value
	Fold Difference	P-value	Fold Difference	P-value		
CD8A			1.43	0.045		
CD74						
CTSS	1.50	0.017	1.62	0.017	0.5660	0.06
HSPA1B			-1.16	0.004	-0.5750	0.06
HSP90AA1	-1.43	0.06			-0.6350	0.032
HLA-A	1.47	0.034	1.49	0.047		
HLA-F			1.51	0.05	0.6800	0.019
HLA-DMB					0.6030	0.047
HLA-DOB	1.63	0.038				
HLA-DPB1					0.5620	0.06
HLA-DRB1-4	1.54	0.05			0.6030	0.047
IFI30(GILT)	1.33	0.020			0.6800	0.019
IFN γ					0.7210	0.011
KLRC1			7.23	0.05		
KLRC3	2.22	0.015				
KLRD1	2.31	0.06	1.42	0.023		
NFYA			1.29	0.005		
NFYB			1.33	0.030	-0.5800	0.05
PSME2					0.5620	0.06
PDIA3					0.7950	0.002
REFANK			1.20	0.024		
TAPBP	1.44	0.022	1.56	0.06	0.6030	0.047

Table 4

Discriminatory gene expression derived from Principal Component Analysis and relationship to antigen processing and presentation classes. The + symbols denote the antigen presentation pathway of genes that contribute to stratifying the aged or periodontitis tissue profiles.

	Healthy		Periodontitis	
	MHC Class I	MHC Class II	MHC Class I	MHC Class II
CD74(li)		+		
CD8A	+			
CIITA	+	+		
CTSS		+		+
HLA-A	+			
HLA-B			+	
HLA-F	+			
HLA-DMB		+		
HLA-DOA		+		+
HLA-DPA1		+		+
HLA-DPB1		+		+
HLA-DRA		+		
HSPA1B			+	
HSPA8			+	
IFI30(GILT)				+
KLRC3	+			
KLRD1	+			

Table 5

Comparison of gene expression profiles using qPCR and microarray analyses. Values represent fold-difference compared to Adulty Healthy tissue message levels. Five samples were assessed for each group and the asterisk (*) signifies a correlation of >0.5.

GENE ID	AGED HEALTHY	ADULT PERIODONTITIS	AGED PERIODONTITIS
PSME2			
qPCR	2.06*	0.55	2.12*
GeneChip	1.76	1.12	1.84
IF130			
qPCR	2.74*	2.85*	1.74*
GeneChip	1.91	2.02	1.82
HLA-DPB1			
qPCR	1.95*	3.81	8.59*
GeneChip	1.63	1.25	2.53
HLA-DRA			
qPCR	1.56*	1.87*	1.18*
GeneChip	1.30	1.52	1.22
CTSS			
qPCR	1.53*	0.79	0.82
GeneChip	1.33	1.50	2.02