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Apoptotic Genes are Differentially Expressed in Aged Gingival Tissue

ABSTRACT

Cellular and molecular changes of the periodontium associated with a higher prevalence of oral diseases (*e.g.*, chronic periodontitis) in aged populations have received little attention. Since impaired apoptosis during aging appears to be related to chronic inflammatory disorders, we hypothesized that the expression of genes associated with apoptotic processes are altered in aged healthy and periodontitis-affected gingival tissue. Ontology analysis of 88 genes related to apoptotic pathways was performed in gingival biopsies of healthy and periodontitis sites from young, adult, and aged non-human primates (*Macaca mulatta*), using the GeneChip® Rhesus Macaque Genome Array. Lower expression of anti-apoptotic and higher expression of pro-apoptotic genes were associated with healthy gingival tissue from young compared with aged animals. Few differences in gene expression were observed in healthy gingival tissue between adult and aged animals. Comparison between healthy and periodontitis gingival tissues showed that the up- or down-regulated apoptotic genes in diseased gingival tissue are different in adults compared with aged animals. These results suggest that apoptotic events normally occurring in gingival tissues could be reduced in aging, and unique aspects of apoptotic pathways are potentially involved in the pathophysiology of periodontal disease in adult *vs*. aged gingival tissues.

KEY WORDS: apoptosis, gingival tissue, gerontology, gene expression, periodontitis.

INTRODUCTION

Although previous studies have suggested a higher prevalence of periodontitis in elderly compared with younger populations (Streckfus *et al*., 1999; Albandar and Tinoco, 2002), it remains unclear if this observation is a natural consequence of aging. Consistent with age being a modifier for the prevalence or severity of periodontal disease, important biological differences in the composition and complexity of the oral microbial ecology, as well as the oral/systemic immune-inflammatory responses during aging, have been described in humans and animal models (Schlegel-Bregenzer *et al*., 1998; Nonnenmacher *et al*., 2001; Ebersole *et al*., 2008). Paradoxically, the reduced immunity in aging is accompanied by chronic inflammation (Back *et al*., 2007), which may reflect a perturbation of the innate immune mechanisms associated with age (Agrawal *et al*., 2007). Recent evidence supports an important role for apoptosis as an essential mechanism that regulates the inflammatory response through the generation of anti-inflammatory signals affecting phagocytes at the site of the infection, originally recruited to clear the infection (Fadok *et al*., 1998; Kim *et al*., 2004). Apoptosis is a mechanism of cell death without inflammation, and is crucial for the maintenance of tissue homeostasis at different stages of life, including embryonic development and normal tissue turnover during adulthood (Vaux and Korsmeyer, 1999). These programmed cell death events appear to occur in a tissue-specific manner with aging (*i.e.*, increased apoptosis in skeletal muscles, neurons, and hepatocytes, and decreased in intestinal epithelial cells) (Holt *et al*., 1998; Higami and Shimokawa, 2000; Marzetti and Leeuwenburgh, 2006). Similar studies addressing the potential role of apoptosis in periodontitis have shown increased expression of apoptotic biomolecules, mainly by neutrophils and mononuclear cells (Gamonal *et al*., 2001), as well as down-regulation of pro-apoptotic molecules in lymphocytic cells associated with chronic adult periodontitis (Lucas *et al*., 2010). Nevertheless, additional reports have not confirmed a significant role for apoptosis in periodontitis (Koulouri *et al*., 1999). Most of these studies evaluated apoptotic changes in periodontitis using gingival biopsies from healthy and periodontitis-affected individuals within a broad age range, and for a limited number of molecules related to apoptosis.

There is limited evidence concerning biological changes of the gingival tissue that may help us better understand the higher prevalence and severity of oral diseases (*e.g.*, periodontal disease) observed in an elderly population. It was recently described that the expression of pro-apoptotic markers related to both the intrinsic (*i.e.*, cytochrome C) and extrinsic (*i.e.*, caspase-3, tumor necrosis factor receptor-associated *via* death domain-TRADD and Bax) apoptotic pathways correlated with periodontitis in gingival biopsies from elderly patients (Das *et al*., 2009), which suggests that age could in fact determine a differential expression of apoptotic molecules within the periodontium (Das *et al*., 2009). With microarray
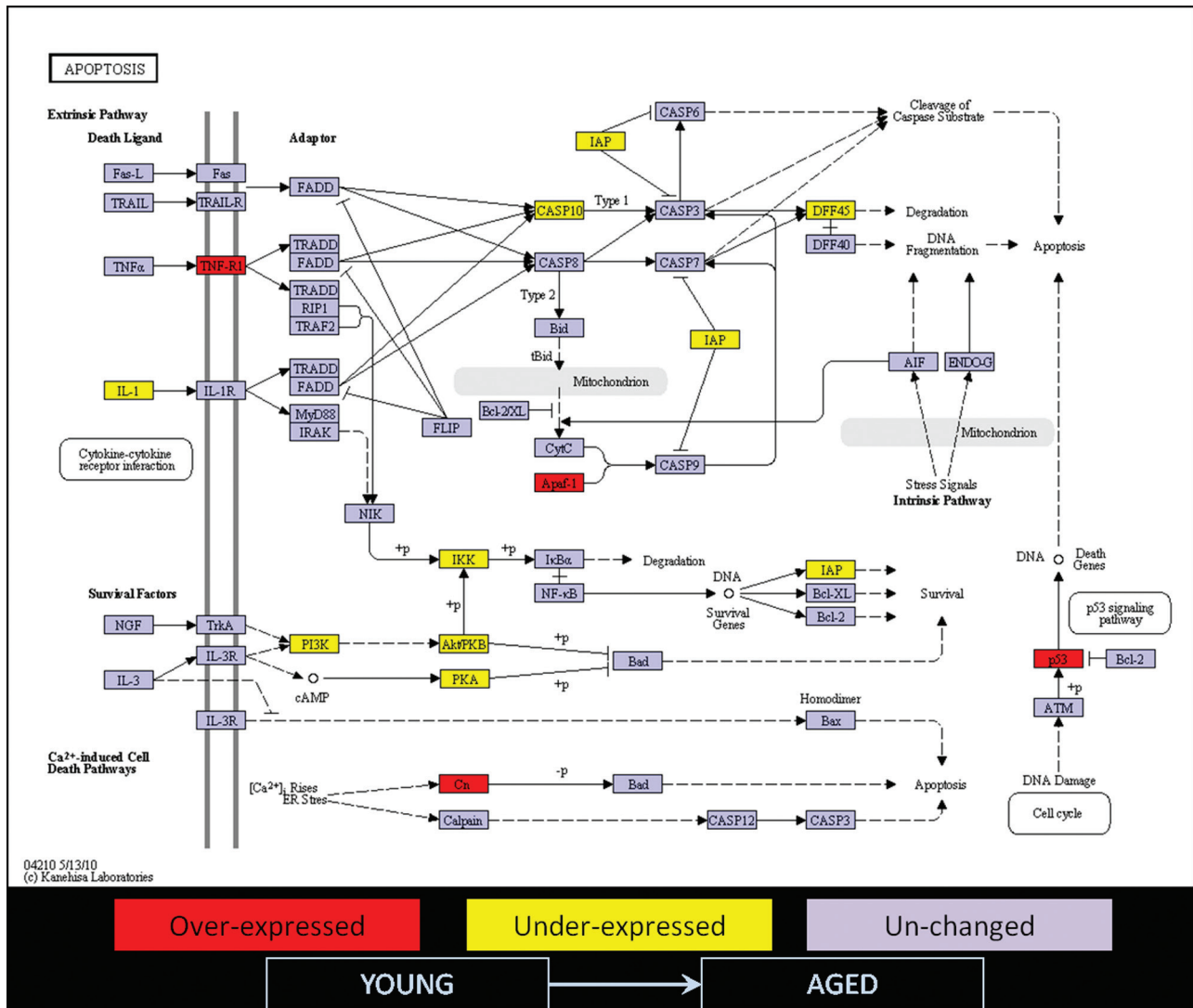
Figure 1. Ontology analysis of apoptotic genes expressed in healthy gingival tissues from young *vs.* aged non-human primates. Comparison of apoptosis gene expression was done between healthy gingival tissues from young *vs.* aged animals as described in MATERIALS & METHODS. Genes shown in red are over-expressed, and genes shown in yellow are under-expressed in young gingival tissue compared with aged gingival tissue. Expression of genes shown in purple was unchanged at $p \leq 0.05$ significance. +p = phosphorylation event; -p = dephosphorylation event.

gene expression technology and a non-human primate model (Struillou *et al.*, 2010), age-related molecular gingival changes associated with the pathogenesis of periodontal disease were evaluated. Specifically, we developed an ontological analysis of apoptotic genes expressed in healthy gingival tissue with regard to age, as well as age-related variations of apoptotic gene expression in healthy compared with periodontitis-affected gingival tissues.

MATERIALS & METHODS

Animals and Diet

Rhesus monkeys (*Macaca mulatta*) (n = 15; 11 females and 4 males) housed at the Caribbean Primate Research Center (CPRC) at Sabana Seca, Puerto Rico, were used in these studies. Healthy animals (3/group) were distributed by age in 3 groups as follows: $\leq$ 3 yrs (young), 12-15 yrs (adult), and 18-22 yrs (aged). Only adult and aged animals (3 additional animals/group) with

periodontitis were used, since periodontitis does not occur in young animals. The non-human primates were typically fed a 20% protein, 5% fat, and 10% fiber commercial monkey diet (diet 8773, Teklad NIB primate diet modified: Harlan Teklad, Madison, WI). The diet was supplemented with fruits and vegetables, and water was provided *ad libitum* in an enclosed corral setting.

Oral Clinical Parameters and Gingival Tissue Sample Collection

Following the protocol approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Puerto Rico, anesthetized animals were examined by a single investigator using a Maryland probe on the facial aspect of the teeth, 2 proximal sites *per* tooth (mesio- and disto-buccal), excluding the canines and 3rd molars. The clinical examination included probing pocket depth (PD) and bleeding on probing (BOP; 0-3 scale) (Ebersole *et al.*, 2008). Clinical characterization for each group
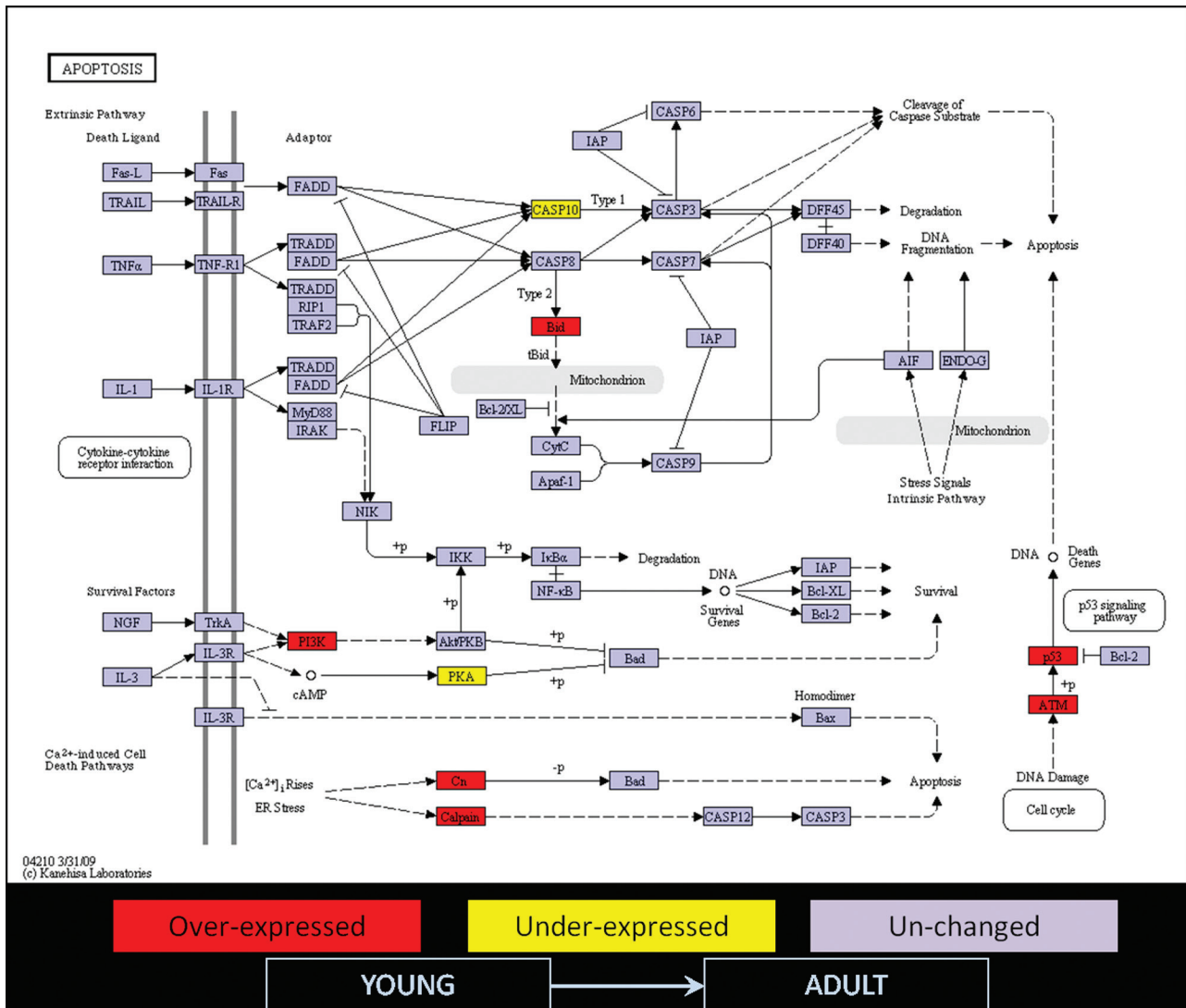
Figure 2. Ontology analysis of apoptotic genes expressed in healthy gingival tissues from young *vs.* adult non-human primates. Comparison of apoptosis gene expression was done between healthy gingival tissues from young *vs.* aged animals as described in MATERIALS & METHODS. Genes shown in red are over-expressed, and genes shown in yellow are under-expressed in young gingival tissue compared with adult gingival tissue. Expression of genes shown in purple was unchanged at $p \leq 0.05$ significance. +p = phosphorylation event; -p = dephosphorylation event.

(n = 3) included: young animals (mean PD < 2.0 mm, mean BOP < 0.5); adult healthy group (mean PD < 2.0 mm, mean BOP < 0.3); adult periodontitis group (mean PD > 3.4 mm, mean BOP > 1.0); aged healthy group (mean PD < 3.0 mm, mean BOP < 1); mean BOP < 1.0); and aged periodontitis group (mean PD > 3.4 mm, mean BOP > 1.5). With a standard gingivectomy technique, 2 adjacent buccal gingival samples from either healthy (PD ≤ 2 mm, no BOP) or periodontitis-affected tissue (PD > 4 mm, BOP ≥ 1) from the premolar/molar region of each animal were taken and handled individually to prepare RNA for microarray analysis.

RNA Extraction, Reverse Transcription, and Gene Chip Hybridizations

Total RNA was isolated from each gingival tissue with Trizol reagent (Invitrogen, Carlsbad, CA, USA). The extracted RNA was cleaned up with the Qiagen RNeasy mini kit (Qiagen, Valencia, CA, USA) and quantified by spectrophotometric

analysis. Equal amounts of RNA were pooled from each sample for an individual animal, followed by a reverse transcription, and hybridization to the GeneChip® Rhesus Macaque Genome Array (Affymetrix, Santa Clara, CA, USA), similar to methods we have described previously (Meka *et al.*, 2010). Probe arrays were scanned with the GeneChip Scanner 3000 for high-resolution scanning and GeneChip Operating Software MAS 5.0.

Data Analysis

The average gene expression for 88 genes associated with apoptosis pathways in the database of the *Kyoto Encyclopedia of Genes and Genomes* (KEGG) (www.genome.jp) (Kanehisa *et al.*, 2010) was compared between different age groups and between healthy and periodontitis gingival tissues by the Student's *t* test. We performed ontology analysis by mapping the statistically differentially expressed genes ($p \leq 0.05$) between age groups into the KEGG pathways as previously described (Demmer *et al.*, 2008). Using a
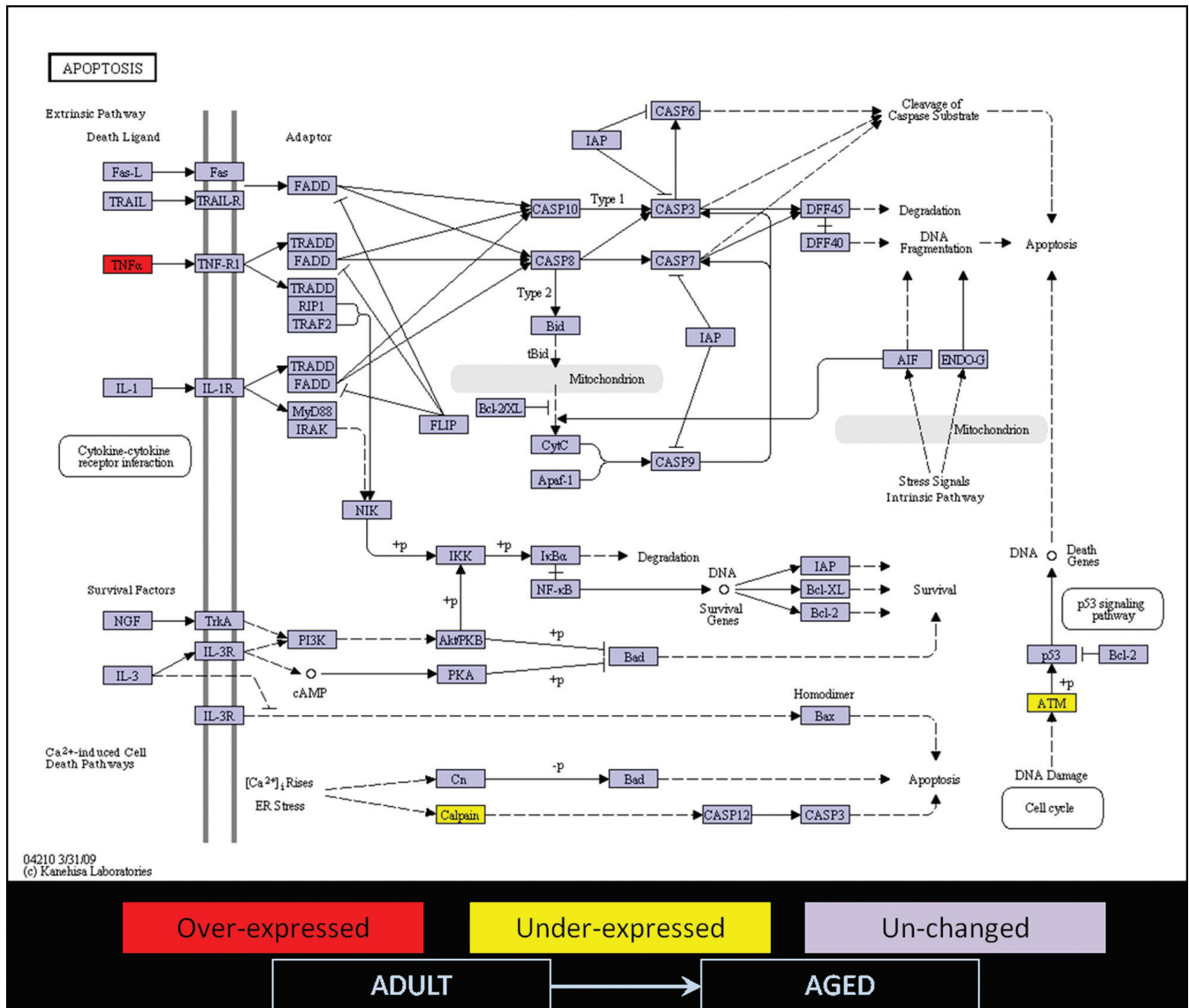
Figure 3. Ontology analysis of apoptotic genes expressed in healthy gingival tissues from adult *vs.* aged non-human primates. Comparison of apoptosis gene expression was done between healthy gingival tissues from young *vs.* aged animals as described in MATERIALS & METHODS. Genes shown in red are over-expressed, and genes shown in yellow are under-expressed in adult gingival tissue compared with aged gingival tissue. Expression of genes shown in purple was unchanged at p ≤ 0.05 significance. +p = phosphorylation event; -p = dephosphorylation event.

p-value cutoff of $p \leq 0.05$, we expected only up to 4 or 5 false-positives by chance out of 88 genes when applying each *t* test.

RESULTS

The expression of 88 genes related to apoptosis pathways in healthy gingival tissue was compared among samples from young, adult, and aged animals. Significant differences in gene expression were found between young and either adult (10 genes) or aged (14 genes) gingival tissues. Ontology analysis showed that the differentially expressed genes in gingival tissue with aging involved both pro- and anti-apoptotic molecules. Specifically, the gingival tissue from young animals exhibited higher expression of pro-apoptotic genes such as tumor necrosis factor receptor 1 (TNFR1), the apoptotic peptidase activating factor 1 (APAF-1), p53, calcium-binding protein P22 (Cn), and Calpain and BH3 interacting domain death

agonist (BID), and lower expression of negative regulators of apoptosis, *e.g.*, survival genes, including baculoviral IAP repeat-containing 2 (IAP), phosphatidylinositol 3-kinase (PI3K), protein kinase A (PKA). and the IkappaB kinase (IKK), when compared with aged and adult gingival tissues (Figs. 1, 2). The PI3K intracellular signal transducer group of biomolecules was expressed at higher levels in young *vs.* adult gingival tissues (Fig. 2), although their expression levels were significantly lower in samples from young animals compared with aged animals (Fig. 1). Lower numbers of statistically differentially expressed genes were found when adult and aged gingival tissues were compared (3 genes) (Fig. 3). Interestingly, the expression of the classic inflammatory molecules TNFR1 and TNFα was up-regulated in gingival tissues from young and adult animals, respectively, compared with aged animals (Figs. 1-3).

Statistically significant differential expression of 12 genes (adult) and 16 genes (aged) was found when healthy gingival

tissues were compared with periodontitis tissues (Table). Strikingly, the genes in the apoptotic pathways either significantly up- or down-regulated in gingival tissue during periodontal disease were unique subsets in samples from adults *vs.* those obtained from aged animals.

DISCUSSION

In this study, we sought to determine the potential age-related changes in the expression of genes involved in apoptosis pathways in healthy and diseased gingival tissue from young, adult, and aged non-human primates (*M. mulatta*). We demonstrated a clear age-related change in the expression of genes in apoptotic pathways in healthy tissues. Additionally, unique subsets of apoptotic genes showed altered expression in health and disease in the adult animals compared with aged animals. Historically, periodontitis in adults and the elderly has been generally considered a similarly chronic immune-inflammatory condition, with the increased risk associated with aging as simply a reflection of the longer-term insult to the periodontium from accumulated oral bacterial biofilms (Schlegel-Bregenzer *et al.*, 1998; Nonnenmacher *et al.*, 2001). However, since we now recognize that the molecular inflammatory fingerprint of gingivitis differs from that of periodontitis (Honda *et al.*, 2006), these findings suggest that tissue destruction in periodontitis may actually have some fundamental differences in mechanism in aged individuals.

    Microarray analyses showed that healthy tissues from aged and adult animals appear to exhibit a lower pro-apoptotic and

Table. Genes are Differentially Expressed in Periodontitis *vs.* Healthy Gingival Tissues from Adult and Aged Non-human Primates

		Adult				Aged			
	Probe #	Healthy	Periodontitis	Fold-change	P Value	Healthy	Periodontitis	Fold-change	P Value
Pro-apoptosis									
BCL2L10	MmugDNA.5988.1.S1_at	159.9	236.4	1.5	0.031	175.2	255.1	1.5	0.123
CAPN1	MmugDNA.2588.1.S1_at	2694.5	3556.5	1.3	0.038	3286.7	3488.1	1.1	0.757
NOD1	Mmu.9460.1.S1_s_at	3240.7	2909.8	0.9	0.029	3031.3	2808.9	0.9	0.226
PPP3CB	Mmu.9989.1.S1_s_at	773.5	718.3	0.9	0.004	821.2	689.9	0.8	0.257
CHP	MmugDNA.2197.1.S1_at	315.1	224.8	0.7	0.017	276.8	318.4	1.2	0.477
TNFRS11B	MmugDNA.5860.1.S1_at	244.3	152.3	0.6	0.044	182.3	188.8	1.0	0.904
APAF1	MmugDNA.37857.1.S1_at	42.6	30.8	0.7	0.617	19.6	45.6	2.3	0.006
CSF2RB	MmugDNA.35543.1.S1_at	3102.3	4439.2	1.4	0.276	2848	4555.4	1.6	0.016
CASP3	MmugDNA.6342.1.S1_at	1008.3	874.5	0.9	0.575	731.2	1132.6	1.5	0.018
DAPK1	MmugDNA.31762.1.S1_at	139.1	170.3	1.2	0.151	140.7	184.3	1.3	0.009
TRAF 3	MmugDNA.10177.1.S1_at	381.0	329.8	0.9	0.405	293.0	388.2	1.3	0.005
CASP1	MmugDNA.31375.1.S1_s_at	563.7	713.4	1.3	0.172	820.8	600.3	0.7	0.002
CASP4	MmugDNA.24199.1.S1_at	2352.5	2854.4	1.2	0.396	3140.9	1997.9	0.6	0.042
CD40 (TNFRSF5)	MmugDNA.20032.1.S1_at	27.5	24.4	0.9	0.849	14.2	5.8	0.4	0.040
Anti-apoptosis									
IGF1R	MmugDNA.12698.1.S1_at	18.1	46.8	2.6	0.047	23.3	10.8	0.5	0.265
PRKX	MmugDNA.3488.1.S1_at	25.7	41.7	1.6	0.048	41.2	42.9	1.0	0.919
NFKB1	MmugDNA.25322.1.S1_at	652.0	799.1	1.2	0.017	760.0	737.2	1.0	0.688
PRKAR1A	MmuSTS.3698.1.S1_at	12146.3	10773.6	0.9	0.036	11708.5	10383.9	0.9	0.213
NOL3	MmugDNA.27369.1.S1_at	401.6	301.6	0.8	0.044	339.1	310.4	0.9	0.481
BNIP2 (II)	MmugDNA.2960.1.S1_at	43.0	18.1	0.4	0.047	14.0	27.1	1.9	0.293
NTRK1	MmugDNA.14382.1.S1_at	23.9	43.3	1.8	0.077	17.2	62.3	3.6	0.027
BNIP3L	MmugDNA.32220.1.S1_at	36.7	50.0	1.4	0.339	40.1	77.3	1.9	0.026
PRKACA	MmuSTS.3696.1.S1_at	590.6	631.1	1.1	0.617	567.4	693.2	1.2	0.014
RELA	MmuSTS.3377.1.S1_at	1360.6	1312.5	1.0	0.694	1295.9	1434.4	1.1	0.032
BNIP2 (I)	MmugDNA.31249.1.S1_at	2217.0	2374.6	1.1	0.537	2347.5	2031.5	0.9	0.022
BCL2L2	MmugDNA.17683.1.S1_at	348.8	319.4	0.9	0.458	353.0	300.5	0.9	0.013
CD2 (cytoplasmic tail)	MmugDNA.32305.1.S1_at	323.6	405.6	1.3	0.213	479.8	372.7	0.8	0.025
PIK3CA	MmuSTS.1135.1.S1_at	1665.3	1696.2	1.0	0.899	1749.8	1395.0	0.8	0.021

The average expression and fold-change for 28 genes related to apoptotic pathways that showed significant differences (p ≤ 0.05) between healthy and periodontitis gingival tissue from either adult or aged animals is shown. Genes were divided as positive (pro-apoptosis) or negative (anti-apoptosis) regulators of programmed cell death. The corresponding probe number IDs for the listed genes were obtained from the database of the Affymetrix Web site with the NetAffx Query software (www.affymetrix.com).

higher anti-apoptotic net gene expression compared with healthy gingival tissues from young animals. These results suggest that apoptotic events that normally occur in the gingival tissue could be reduced in adult/aged individuals compared with young animals. Hypothetically, these physiological changes could involve an adaptation process of the gingival tissue, where the reduced proliferative capacity that is classically observed in the elderly could be compensated for with higher resistance to cell death under stressful environmental conditions associated with the chronic microbial challenge. Similarly, environmental factors as well as aging itself have been associated with the accumulation of gene mutations that could potentially change the ability of the tissues to properly activate specific cell responses as apoptosis (Lee *et al*., 2010).

The relationship of pro- or anti-apoptotic events with susceptibility for periodontal disease remains unclear. Evidence suggests that the majority of apoptotic events appear to be related to immune cells (*i.e.*, neutrophils, monocytes/macrophages, and lymphocytic cells), rather than epithelial cells or fibroblasts, the major structural cells of the periodontium (Sawa *et al*., 1999; Gamonal *et al*., 2003). Hence, with this evidence, along with additional studies that confer an immunomodulatory role on apoptosis (Torchinsky *et al*., 2010), one could hypothesize that the reduced apoptotic responses in aged gingival tissues could involve failure to control the inflammatory response against the chronic bacterial challenge, resulting in dysregulated clearance of the inflammatory infiltrate. In agreement with this hypothesis, Bodineau *et al*. recently showed an increased number of mature dendritic cells in elderly patients with chronic periodontitis (Bodineau *et al*., 2009). Thus, it would be expected that re-establishing the physiological pro-apoptotic mechanisms in aging, specifically within the inflammatory cell population, could reduce the inflammatory response classically observed in periodontal disease, as has been proposed for other chronic inflammatory disorders (Lugering *et al*., 2006). The susceptibility to undergo apoptosis could vary among different cell types (Higami and Shimokawa, 2000). Therefore, the selection of apoptotic markers in future studies of periodontal disease should include both enhancers and inhibitors of apoptosis, using experimental strategies that facilitate discrimination of the net cell types within the gingival tissue that express these apoptotic changes, and more attention to discrimination of patient populations based upon age.

An additional and startling observation was that the genes differentially expressed in periodontitis *vs.* healthy gingival tissues from adult animals were mutually exclusive to those genes expressed in tissues from aged animals. These results are consistent with a potential role of apoptosis in the pathogenesis of periodontal disease, as has been previously suggested (Gamonal *et al*., 2001; Lucas *et al*., 2010), although further functional studies involving these genes will be required for full evaluation of their role in the complex processes needed to maintain homeostasis in the periodontium. Variations in gene expression associated with periodontitis included both over-expression- and down-regulation of genes that are in pathways to enhance or inhibit apoptosis. Although these findings clearly show gene expression profiles related to regulation of apoptotic events during periodontitis, the net results of altered expression of specific enhancers or inhibitors of apoptosis may change throughout the disease process, emphasizing the need for further prospective controlled studies of the initiation and progression of periodontal lesions.

Changes in the expression of classic inflammatory molecules such as TNFR1 that was observed in gingival tissues from adult but not aged animals, and the significantly increased expression of APAF1 in diseased gingival tissue from aged but not adult animals, are examples reinforcing the fact that the apoptotic mechanisms contributing to periodontitis in aged individuals differ from those in adults. Similarly, there was an age-related expression of the critical enzyme PI3K with fluctuating variations among young, adult, and aged gingival tissues. Importantly, PI3K-dependent resistance to apoptosis during *Porphyromonas gingivalis* infection has been shown in oral epithelial cells as a tissue invasion mechanism (Yilmaz *et al*., 2004). Thus, higher apoptosis resistance in aged gingival tissue, related to increased levels of PI3K, may increase the likelihood for periodontopathogenic tissue infection and further onset of periodontitis. Additional results indicated that, among the genes highly expressed in diseased gingival tissues, there were molecules that have not been classically associated with periodontitis, such as IGF1R (insulin growth factor type 1 receptor) in adult gingival tissue, and APAF1 and NTRK1 (neurotrophic tyrosine kinase receptor type 1) in the aged gingival tissues. Future studies addressing the potential role of these molecules in periodontitis appear warranted.

Age-associated diseases (*e.g.,* cardiovascular disease, cancer, arthritis, osteoporosis, type 2 diabetes, Alzheimer's disease) increase rapidly with aging and are distinguished from the aging process, since all humans age, but not all experience all age-associated diseases. Periodontitis has often been considered a disease of aging, rather than an age-associated disease, being expressed as more continuous undermining of the integrity of the periodontium that accumulates in response to the noxious microbial challenge. Our findings suggest that periodontitis may more accurately reflect an age-associated disease, with somewhat unique/distinct molecular characteristics, as reflected by the gingival apoptotic events inferred by this study.

In general, we have shown that: (i) the net expression of apoptotic genes involved in the homeostasis of the healthy gingival tissue appears to change with age toward a more apoptosis-resistant phenotype, and (ii) the apoptotic pathway gene profile expressed in periodontitis tissues differs in aged when compared with adult gingival tissues. Besides the expression of apoptosis-associated genes, post-transcriptional and post-translational events (*e.g.*, sumoylation, microRNAs, phosphorylation, and ubiquitination) also regulate apoptotic events (Huen and Chen, 2008). Therefore, further investigations to confirm the gingival apoptotic outcomes during health and periodontitis related to age are necessary. These observations will need to be replicated in clinical studies with humans, and could help to clarify inconsistent findings concerning apoptosis in periodontal disease in humans, which normally included populations with a broad range of ages. A better understanding of the age-associated molecular changes of the periodontium and how those variations correlate with oral health or disease is required, since differences in underlying molecular mechanisms of this biologic process in aged individuals could have an effect on more specific therapies to benefit this rapidly expanding subgroup of the US population.

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