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ABSTRACT

Diabetes impairs the resolution of periodontal inflammation. We explored pathways altered by inflammation in the diabetic periodontium by using ligatures to induce periodontitis in type-2 diabetic Goto-Kakizaki rats. Ligatures were removed after 7 days, and rats were then treated with TNF inhibitor (pegsunercept) or vehicle alone and euthanized 4 days later. RNA was extracted from periodontal tissue, examined by mRNA profiling, and further analyzed by functional criteria. We found that 1,754 genes were significantly up-regulated and 1,243 were down-regulated by pegsunercept ($p < 0.05$). Functional analysis revealed up-regulation of neuron-associated and retina-associated gene clusters as well as those related to cell activity and signaling. Others were down-regulated by TNF inhibition and included genes associated with host defense, apoptosis, cell signaling and activity, and coagulation/hemostasis/complement. For selected genes, findings with microarray and rt-PCR agreed. *PPAR*-α was investigated further by immunohistochemistry due to its anti-inflammatory function and was found to be up-regulated in the gingiva during the resolution of periodontal inflammation and suppressed by diabetes. The results indicate that diabetes-enhanced inflammation both up- and down-regulates genes involved in cellular activity and cell signaling, while it predominantly up-regulates genes involved in the host response, apoptosis, and coagulation/homeostasis/complement and down-regulates mRNA levels of neuron, retina, and energy/metabolism-associated genes.

KEY WORDS: DAVID, diabetes, GSEA, inflammation, microarray, periodontal disease(s).

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INTRODUCTION

Type 2 diabetes is a metabolic disorder with insulin resistance and decreased β-cell function. It causes a number of complications that involve the retina, kidneys, nervous system, wound healing, and cardiovascular disease. Dysregulation of inflammatory pathways contributes to the development and progression of diabetic complications. Enhanced pro-inflammatory cytokine levels have been found in several diabetic complications, such as nephropathy, which exhibits high levels of *TNF*-α in the kidneys and the urine of diabetic patients (Wu, Chen *et al*., 2010).

Dysregulation of TNF is linked to diabetic complications in animal studies. By the use of inhibitors, it has been shown that diabetes-enhanced TNF contributes to impaired wound healing (Goren *et al*., 2007). Anti-TNF treatment of dermal wounds in type 2 diabetic mice attenuates inflammation, improves re-epithelialization, and restores insulin signaling in wounded tissue (Siqueira *et al*., 2010). TNF inhibition reduces fibroblast apoptosis and enhances fibroblast proliferation in diabetic wounds to enhance healing (Siqueira *et al.*, 2010). Moreover, it reduces endothelial cell death in the early stages of diabetic retinopathy (Behl *et al*., 2009) and prevents blood-retinal barrier breakdown (Huang *et al.*, 2011). In diabetic nephropathy, anti-TNF treatment reduces sodium retention, renal hypertrophy, and urinary albumin (DiPetrillo *et al*., 2003). Other less specific treatments with a component of anti-TNF activity have improved experimental diabetic neuropathy in rats (Chauhan *et al.*, 2012) and limited peripheral neuropathy progression (Sagara *et al.*, 1996). In humans, TNF blockers reduce macular thickness and increase visual acuity in patients with severe diabetic macular edema (Sfikakis *et al*., 2005).

Diabetes increases the risk and severity of periodontal disease (Mealey *et al*., 2006). The levels of *TNF*-α and other inflammatory mediators are elevated in diseased periodontal tissues (Benakanakere and Kinane, 2012). These mediators stimulate the production of enzymes that break down connective tissue and induce bone resorption. Diabetes partly aggravates periodontitis by reducing the capacity to down-regulate inflammation (Graves *et al*., 2011). Peripheral blood monocytes in individuals with diabetes produce elevated levels of *TNF*-α and other inflammatory mediators compared with levels found in non-diabetic control individuals (Gacka *et al.*, 2010). In animal studies, diabetes alters the host response to bacterial challenge through prolonged *TNF*-α expression (Naguib *et al*., 2004). Diabetes-enhanced inflammation also increases cellular apoptosis and decreases proliferation,

reducing the potential repair of damaged periodontal tissue (Liu *et al*., 2006).

To further investigate the impact of diabetes on the periodontium, we carried out mRNA profiling. We focused on the resolution of periodontitis by treatment of diabetic animals with a TNF inhibitor, pegsunercept, following the cessation of experimental periodontitis. The results indicate that several biological pathways—particularly those involving host defense, cell signaling, and apoptosis—are elevated by prolonged inflammation in diabetic animals, while other pathways—including neuron-associated genes, cell activities such as proliferation and migration, and retina-associated genes—are predominantly down-regulated.

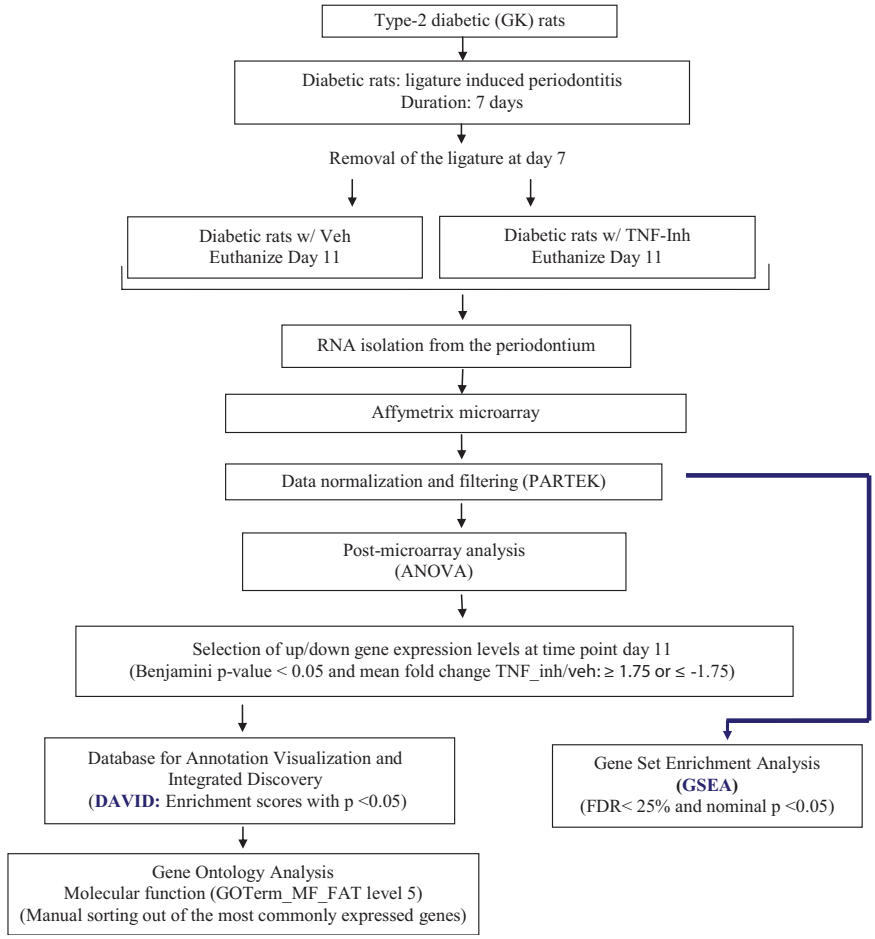

Figure 1. Diagram of the approach used for functional analysis of genes regulated by high levels of TNF in the diabetic periodontium. RNA was isolated from diabetic rats treated with vehicle alone or pegsunercept during the resolution of periodontal inflammation and subjected to mRNA profiling. The distribution of the functional gene clusters was determined by Database for Annotation, Visualization and Integrated Discovery (DAVID) and also by gene set enrichment analysis. The approach identified gene clusters that are up- or down-regulated when TNF is inhibited with pegsunercept in diabetic animals and provides insight as to which gene sets are specifically modulated by diabetes-enhanced inflammation.

MATERIALS & METHODS

Animals

Type-2 diabetic male Goto-Kakizaki (GK) rats were originally obtained by selective breeding of Wistar (WT) rats. Normoglycemic WT rats were used as a control, and both GK and WT rats were purchased from Charles River Laboratories (Wilmington, MA, USA). To induce periodontitis, we placed ligatures around the maxillary 2nd molars after rats were hyperglycemic for 3 to 4 wks (Liu *et al*., 2006; Pacios *et al*., 2012). After 7 days, ligatures were removed to allow for resolution of periodontal inflammation. GK rats were treated with the TNF-specific inhibitor pegsunercept, given only once (provided by Amgen, Thousand Oaks, CA, USA) by intraperitoneal injection (4 mg/kg) immediately after ligatures were removed. Control GK rat counterparts were treated with vehicle alone (phosphate-buffered saline, PBS) (Fig. 1). At day 11, 4 GK rats treated with TNF inhibitor and 5 GK rats with vehicle alone were euthanized for microarray analysis. Glycemic levels of GK rats were 436 ± 32 mg/dL, and those of GK rats treated with TNF inhibitor were 452 ± 48 mg/dL at the time of death. All animal procedures were approved by the Institutional Animal Care and Use Committee, and the experiments conformed to ARRIVE guidelines.

mRNA Profiling

We extracted total RNA from the dissected periodontal tissue and determined mRNA levels of 26,214 genes using Affymetrix rat Exon gene 1.0ST array by the Path BioResource, University of Pennsylvania. Partek software was used to standardize and normalize fluorescence intensity. For selected genes, relative mRNA was assessed by real-time PCR (rt-PCR) with primers and probe sets from Applied Biosystems (Foster City, CA, USA) or Roche Applied Science (Indianapolis, IN, USA) (Appendix Table 1). Each value represents the mean of 3 distinct experiments normalized to ribosomal protein L32.

Immunohistochemistry (IHC)

TNF-α contributes to the tissue destruction induced by periodontal bacteria. Periodontal specimens were prepared according to the approach used by Garlet *et al.* (2007).

In total, 30 GK rats and 22 WT rats were analyzed with IHC. Periodontal tissue was fixed for 2 days with cold 4% paraformaldehyde at 4°C and decalcified in EDTA; 5-μm paraffin sections were prepared. IHC or immunofluorescence with an antibody specific to peroxisome-proliferator-activated receptor-alpha (*PPAR*-α) (Ab8934, Cambridge, MA, USA) was performed with antigen retrieval (0.01 M citrate) and tyramide signal amplification (Perkin-Elmer, Waltham, MA, USA). Intensity and number of positive cells (Alblowi *et al.*, 2009) of

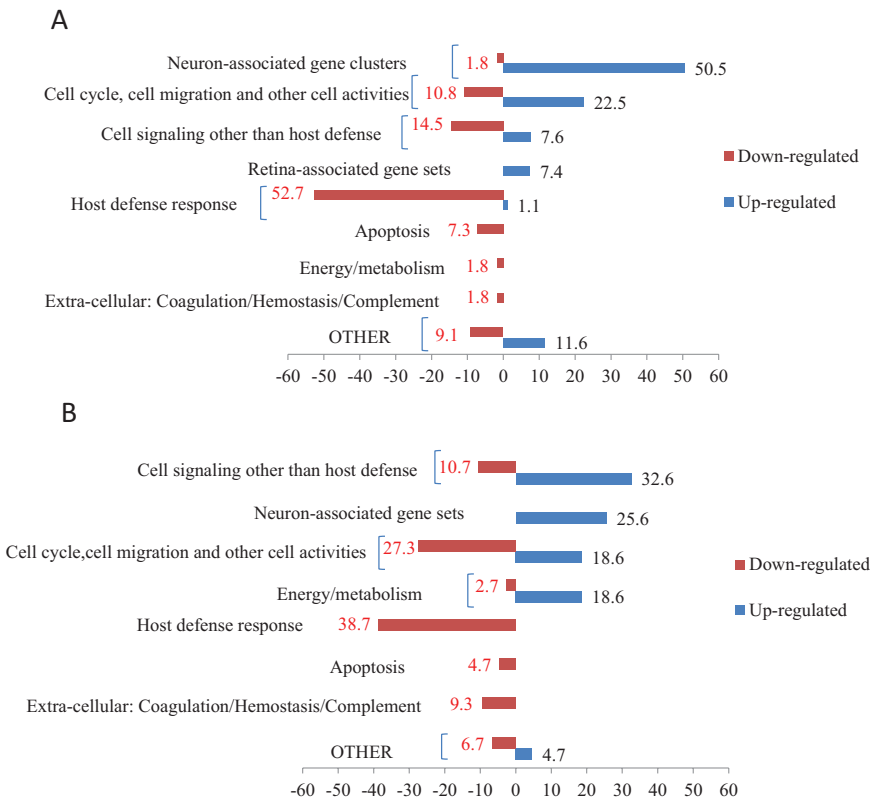

Figure 2. Functional distribution of gene clusters up- or down-regulated by TNF inhibition in diabetic specimens. **(A)** Functional distribution of gene clusters up- or down-regulated as determined by DAVID. RNA was isolated from diabetic rats treated with vehicle alone or pegsunercept during the resolution of periodontal inflammation and subjected to mRNA profiling. Bars represent the distribution of the functional gene clusters determined by Database for Annotation, Visualization and Integrated Discovery (DAVID). Numbers represent the percentage that each category contributed to the total number of up- or down-regulated gene clusters. Gene clusters that are up-regulated when TNF is inhibited with pegsunercept are shown as positive numbers and those that are down-regulated as negative. Only functional annotation clusters with enrichment scores $\geq$ 1.5 and biological functional ontologies with adjusted $p < 0.05$ were selected and are displayed in the Fig. **(B)** Functional distribution of significantly up- or down-regulated gene sets as determined by GSEA. mRNA profiling data described in Fig. 1 were further analyzed by gene set enrichment analysis (GSEA). Only gene sets with FDR < 25% and nominal p value < 0.05 were considered significant and are displayed in the Fig.

the anti-*PPAR*-α immunostaining were blindly quantified, confirmed by a second observer, at 600x magnification according to the following scale: 0, no positive staining in the field; 1, 1 to 10% cells immunopositive with light immunostaining; 2, 11 to 25% cells immunopositive with light to moderate immunostaining; 3, 26 to 40% cells immunopositive with moderate immunostaining; 4, 41 to 60% cells positive with heavy immunostaining; and 5, 61% or more cells immunopositive with dark immunostaining.

Bioinformatics and Statistical Analysis

Genes with mRNA levels 1.75-fold higher or lower in the experimental (pegsunercept-treated) *vs*. control (vehicle-treated) group and with adjusted $p < 0.05$ were identified (Galindo *et al*., 2009). Database for Annotation, Visualization and Integrated Discovery (DAVID) was used for functional analysis with an enrichment score with a cut-off point of 1.5. Microarray data were also examined by Gene Set Enrichment analysis (GSEA) software and the Molecular Signature Database (MSig DB), with a threshold of false-discovery rate (FDR) < 25% and $p < 0.05$ (Fig. 1). With the rt-PCR and immunohistochemical analyses, we used an unpaired *t* test and one-way analysis of variance (ANOVA) to compare 2 unpaired groups or 3 or more independent groups at a particular time-point, respectively. If the result from the ANOVA test was significant, planned multiple comparisons between groups were performed with the contrast method. Statistical significance was set at a level of 5%.

RESULTS

Diabetes causes prolonged and elevated inflammation during the resolution of periodontitis (Liu *et al*., 2006; Pacios *et al*., 2012). We examined the differential mRNAs expression levels in diabetic rats treated with a TNF-specific inhibitor compared with levels in rats treated with vehicle alone. Microarray analysis identified 2,997 up-regulated and 1,243 down-regulated genes in the experimental group that exhibited at least a 1.75-fold change in mRNA levels and an adjusted p value < 0.05. The top 500 up-regulated and down-regulated genes are given in Appendix Table 2. These genes were further examined by DAVID (Fig. 2A), which identified 82 up-regulated and 55 down-regulated gene annotation clusters. The most frequently up-regulated functional subgroups were: neuron-associated genes (51%); genes associated with cell activities (cell cycle, cell migration, endocytosis, cytoskeleton organization, cell adhesion, etc.) (27%); cell signaling other than host defense (8%); and retina-associated genes (7%). Down-regulated functional groups involved: host immune defense (53%); cell signaling other than host defense (15%); cell activities (11%); apoptosis or cell death (7%); energy or metabolism (2%); and coagulation, hemostasis, or wound healing (2%).

Genes from each cluster described above were manually sorted based on their molecular function, as described by the Gene Ontology database terminology (GOTERM_MF_FAT level 5) (Appendix Table 3). Genes up-regulated by TNF inhibition participated in inter- or intracellular signaling, ion binding, and membrane transporter activity. Molecular terms most commonly observed in the down-regulated subgroups included intercellular signaling, particularly inflammation such as immunoglobulin, cytokine, and chemokine receptor activity.

Table. Fold Increase in mRNA Levels of Selected Genes Determined by Microarray and rt-PCR

Gene	Fold Increase TNF Inhibitor/ Veh (Microarray)	Fold Increase TNF Inhibitor/ Veh (rt-PCR)	Relative mRNA Level
MAP2	3.57	9.38	0.0009
SYP	24.66	416.05	0.0003
SNCA	5.46	27.54	0.14
STXBP1	6.97	31.55	0.26
NEFL	16.26	160.05	0.004
NPY	1.94	5.53	0.91
TAC1	3.04	255.11	0.34
PPAR-α	7.3	19.94	0.000011

RNA was isolated from rats treated with TNF inhibitor compared with vehicle alone, and the relative mRNA level of each gene was assessed by real-time PCR or by microarray. The relative mRNA level of each gene was based upon real-time PCR results from GK rats treated with vehicle relative to the mRNA level of a housekeeping gene, ribosomal protein L32.

Using the other approach by GSEA to examine functional changes, we found that 86 gene sets were up-regulated in diabetic rats treated with TNF-inhibitor, and 150 sets were down-regulated (Appendix Table 4, Fig. 2B). Up-regulated gene sets included cell signaling other than host defense (33%), neuronal function (26%), energy or metabolism (19%), and cellular activity such as transmembrane transport, cell cycle, and cell migration (19%). Down-regulated gene sets included: inflammatory host defense response (38%); cell cycle, cell migration, and other cell activities (27%); cell signaling other than host defense (11%); extracellular processes such as coagulation or hemostasis (9%); apoptosis (5%); and gene sets related to energy/metabolism (3%).

Since neuronal genes as a group were particularly affected by TNF inhibition, we examined them in more depth using rt-PCR. Among the up-regulated neuronal genes, microtubule-associated protein 2 (*MAP2*), synaptophysin *(SYP)*, synuclein-alpha (*SNCA*), syntaxin-binding protein 1 (*STXBP1*), neurofilament light polypeptide (*NEFL*), neuropeptide Y (*NPY*), and tachykinin, precursor 1 (*TAC1*), were further validated (Table). The overall pattern between the groups was similar, although the magnitude of the difference was typically underestimated in the microarray data. The mRNA levels of GK rats treated with vehicle relative to that of *L32* are given in the Table as well. Generally, the expression levels of these genes were 0 to 91% of the level of *L32*, which represents low to moderate mRNA levels.

Because of the potential importance of *PPAR*-α in the dysregulation of inflammation, *PPAR*-α protein level was examined by IHC (Fig. 3; Appendix Fig.). In the gingival connective tissue, the number of cells with *PPAR*-α protein was low at baseline (day 0) and when periodontitis was induced (day 7) in normoglycemic and diabetic rats. When periodontitis was resolved by the removal of ligatures, there was a 3-fold increase in the relative expression of *PPAR*-α in normoglycemic rats on day 11 ($p < 0.05$). In contrast, no change was observed in the diabetic animals ($p > 0.05$). However, when diabetic rats were treated with TNF-inhibitor, there was a significant increase ($p < 0.05$), so that the diabetic rats with TNF-inhibitor behaved similarly to the normal animals. Similar trends were observed in the periodontal ligament (PDL).

DISCUSSION

The resolution of inflammation following periodontitis is an active process that involves lipoxins and resolvins and a reduction in pro-inflammatory cytokine expression (Kayal *et al*., 2010; Hasturk *et al.*, 2012). Over-expression of *TNF*-α prevents down-regulation of inflammation in the periodontium and prolonged high levels of apoptosis (Graves *et al*., 2006; Pacios *et al*., 2012). Our previous findings showed that the largest effect of the TNF-inhibitor in the diabetic group was during the resolution of inflammation (Pacios *et al.*, 2012). Therefore, the microarray analysis was focused on day 11. Our findings suggest the presence of biologically important pathways significantly affected by inflammation in diabetic animals during the resolution of periodontitis. Diabetes-enhanced inflammation decreases mRNA levels of genes associated with neurons, cell signaling, and cell activities such as migration. In contrast, diabetes-enhanced inflammation stimulates the host response, inter- or intracellular cell signaling, extracellular processes including coagulation, or hemostasis, apoptosis, and energy/metabolism-related pathways.

Several neuron-associated genes were up-regulated when TNF was inhibited in the diabetic group. Many of these genes are expressed by cells other than neurons, including fibroblasts (*SNCA, NEFL*), epithelial cells (*STXBP1, TAC1*), keratinocytes *(TAC1)*, monocytes (*SNCA*), neutrophils (*TAC1*), endothelial cells (*TAC1*), osteoclasts (*TAC1*), or osteoblasts (*TAC1, NPY*) (Wu, Orozco *et al.*, 2009). Moreover, inflammation modulates the expression levels of several of them: *NEFL, TAC1, NPY*, and *SNCA*. Many of these genes affect functions that are important in the periodontium. For example, *NPY* and substance P, a *TAC1* gene product, can enhance osteoblast differentiation (Goto *et al.*, 2007; Franquinho *et al.*, 2010). Thus, mRNA levels of neuron-associated genes are affected by diabetes-enhanced inflammation, which may have important ramifications in the periodontium.

We found that an approximately 1.75 greater number of genes were up-regulated by the TNF inhibitor than were down-regulated. Similar differences have been noted by other investigators (Paiva *et al*., 2012). Findings from DAVID and GSEA showed consistencies in identifying up-regulated gene clusters when inflammation is inhibited, including clusters associated

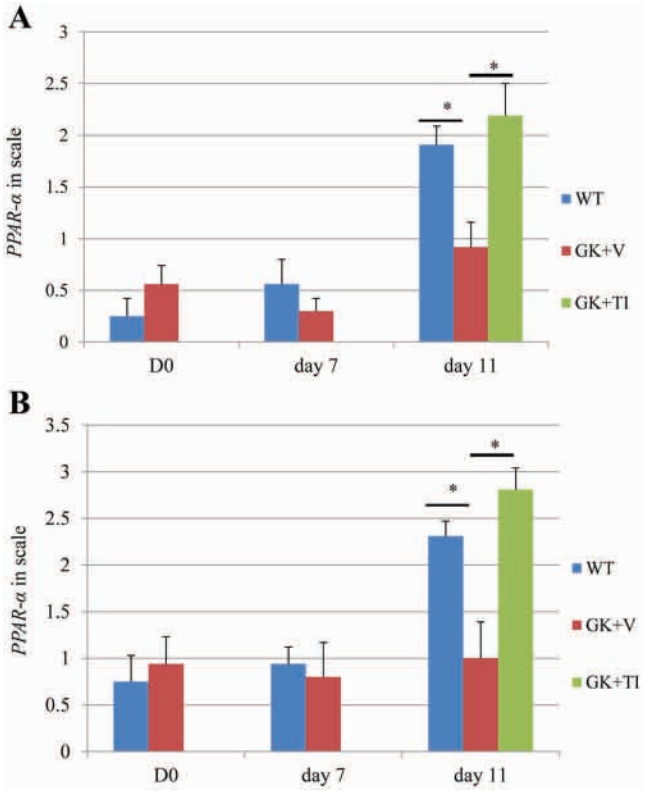

Figure 3. Up-regulation of *PPAR*-α protein levels in periodontal tissues is reduced by diabetes during the resolution phase of inflammation. Periodontal tissue samples at days 0, 7, and 11 were obtained from normal wild-type rats (blue bars), diabetic rats treated with vehicle (red bars), and diabetic rats treated with TNF-inhibitor (green bars). Immunohistochemistry with antibody specific for *PPAR*-α was carried out and analyzed according to the following scale: 0, no positive staining in the field; 1, 1 to 10% immunopositive cells with light immunostaining; 2, 11 to 25% immunopositive with moderate immunostaining; 3, 26 to 40% immunopositive with moderate immunostaining; 4, 41 to 60% immunopositive with dark immunostaining; and 5, 61% or higher immunopositive with dark immunostaining. **(A)** Immunostaining in gingival connective tissue. **(B)** Immunostaining in periodontal ligament space. Data are mean ± SEM. *Significant difference ($p < 0.05$). Each group at each time-point included from 6 to 8 animals. WT, Wistar rats; GK + V, GK rats treated with vehicle only; GK+TI, GK rats treated with TNF-specific inhibitor.

with neuron, cell cycle, cell migration, and other cellular activities. They also identified down-regulated clusters related to host inflammatory responses, apoptosis, and extracellular processes such as coagulation and hemostasis. In contrast, the 2 methods showed some dissimilarities: DAVID showed clusters associated with cell signaling other than host defense and with energy/metabolism to be predominantly or slightly down-regulated by TNF inhibition, while GSEA showed the corresponding clusters to be predominantly or slightly up-regulated. These differences may be related to the slight difference in database sources for gene set/cluster annotations.

PPAR-α is a target of fibrates for hypolipidemic drugs. *PPAR*-α was selected for further study because of its potential function in promoting down-regulation of inflammation (van Bilsen and van Nieuwenhoven, 2010). It is expressed in certain tissues, including the periodontium (Offenbacher *et al*., 2009; Belfort *et al.*, 2010). We found that *PPAR*-α protein was expressed in the gingiva and PDL of normal rats during the resolution, but not in diabetic rats. However, when diabetic rats were treated with a TNF-inhibitor, the expression of *PPAR*-α, at both the protein and mRNA levels, was significantly enhanced. Interestingly, mRNA *PPAR*-α levels are up-regulated during the resolution of experimental gingivitis in healthy individuals (Offenbacher *et al.*, 2009). *PPAR*-α inhibits TNF activity and suppresses activation of the pro-inflammatory transcription factors nuclear factor kappa-light-chain-enhancer of activated B-cells (*NF*-κ*B*) and activator protein 1 (*AP-1*) activity (Delerive *et al*., 1999). Thus, *PPAR*-α may participate in the resolution of periodontitis through its anti-inflammatory properties, but under diabetic conditions its up-regulation is suppressed.

Other studies have examined mRNA profiling of experimental gingivitis in healthy humans (Offenbacher *et al*., 2009). They noted the down-regulation of immune response genes during gingivitis resolution, similar to the down-regulation in the diabetic group treated with TNF-inhibitor. Proteomic analysis of the gingival fluid samples has been examined in human specimens of acute gingivitis (Grant *et al*., 2010). A high level of proteins associated with inflammatory response was reported during the induction, which decreased during resolution. Two other studies found an increase in neuron-associated genes when inflammation was induced (Offenbacher *et al*., 2009; Grant *et al*., 2010), in contrast to our results, in which the mRNA levels of neuron-associated genes increased when inflammation was inhibited. This difference may be due to the fact that we examined a diabetic animal model while the others examined healthy humans. Alternatively, the specific neuron-associated genes in the 3 studies are different. Moreover, many of the neuron-associated genes in one of the reports exhibited only a slight decrease in mRNA levels during the resolution of gingivitis (Offenbacher *et al*., 2009).

Prolonged inflammation in diabetes has a broad effect in up-regulating the expression of host response genes and apoptotic genes in the periodontium. This is consistent with findings that diabetes leads to dysregulation of cytokine networks: There is an up-regulation of pro-inflammatory and pro-apoptotic pathways *in vivo* in diabetic fracture-healing (Kayal *et al*., 2010). The results presented here indicate that diabetes-enhanced inflammation both up- and down-regulates genes involved in cellular activity and cell signaling, while it predominantly up-regulates genes involved in the host response, apoptosis, and coagulation/homeostasis/complement and down-regulates mRNA levels of neuron, retina, and energy/metabolism-associated genes.

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