EFFECT OF RAGE-LIGAND INTERACTIONS ON GINGIVAL EPITHELIAL CELLS IN THE ABSENCE AND PRESENCE OF HYPERGLYCEMIC CONDITIONS

By

JAMIE AMIR

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To my parents
ACKNOWLEDGMENTS

I thank my research mentor Shannon Wallet, without whom none of this would have been possible. I also would like to thank all of the faculty at the University of Florida, who have provided me with an outstanding education in the field of Periodontics. Finally, I must also thank all my fellow residents, who have made my journey thoroughly enjoyable, entertaining, and educational.
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Abstract of Thesis Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Master of Science

EFFECT OF RAGE-LIGAND INTERACTIONS ON GINGIVAL EPITHELIAL CELLS IN THE ABSENCE AND PRESENCE OF HYPERGLYCEMIC CONDITIONS

By

Jamie Amir

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Diabetes mellitus is a heterogenous group of disorders leading to abnormal sugar, protein and fat metabolism. Hyperglycemia is a characteristic feature of diabetes, stemming from a deficiency of insulin production and/or insulin resistance. The pathophysiology of diabetic complications is understood to involve the direct effect of hyperglycemia leading to glycation and subsequent dysfunction of body proteins and lipids, as well as the cellular effects that these irreversibly glycated lipo-protiens, the Advanced Glycation End products (AGE’s), exert through their receptor, RAGE (Receptor for Advanced Glycation End products). Activation of RAGE leads to both an upregulation in the inflammatory response, as well as upregulation in expression of RAGE itself.

Periodontal disease is an inflammatory disease in which the supporting structures of the teeth are destroyed, leading to eventual tooth loss if untreated. Diabetes and periodontal disease share an intimate relationship, with uncontrolled diabetics having a 2.8–3.4 times increased likelihood of having periodontal disease.

Gingival epithelial cells (GECs) of the periodontium are the first line of defense from bacterial pathogens, expressing a variety of innate immune receptors, including RAGE. The purpose of our investigation was to determine the effect that stimulation of innate immune
receptors would have on the production of inflammatory cytokines and expression of innate immune receptors including RAGE on GECs. We also wanted to investigate the effect simulated hyperglycemic conditions would have on the mentioned scenarios.

To accomplish this goal, we stimulated GEC’s with AGE, lipopolysaccharide from *P. gingivalis*, individually, and in combination with sRAGE, both in normoglycemic and hyperglycemic conditions. The Luminex-100 system was used to measure cytokine production, while real time polymerase chain reaction (qPCR) was used to measure expression of RAGE, TLR2, and TLR4 mRNA..

Our results demonstrated that stimulation of GEC with AGE leads to a pro-inflammatory cytokine response, as well as increased RAGE expression, leading to a hyper-inflammatory cellular state. This effect was amplified in the presence of glucose. We were also able to determine that ligation of RAGE in combination with TLR2 induced an exacerbated inflammatory response, over riding an anti-inflammatory response induced by TLR2 ligation alone. The presence of glucose amplified all pro-inflammatory responses while inhibiting any anti-inflammatory responses.
CHAPTER 1
INTRODUCTION

Diabetes is a metabolic disease, leading to abnormal fat, sugar, and protein metabolism. Diabetes can be separated into 2 types, where Type I diabetes (T1D) is cellular mediated autoimmune destruction of the insulin producing β-cells of the pancreas resulting in life-long dependence on exogenous insulin and Type II diabetes (T2D) manifests as insulin resistance whereby utilization of endogenously produced insulin is altered at the target cells (1). The pathophysiology of diabetic complications is understood to involve the direct effect of hyperglycemia leading to glycation and subsequent dysfunction of body proteins and lipids, as well as the cellular effects that these irreversibly glycated lipo-proteins, the Advanced Glycation End products (AGEs), exert through their receptor, RAGE (Receptor for Advanced Glycation End products). Activation of RAGE leads to both an upregulation in the inflammatory response, as well as upregulation in expression of RAGE itself. This ‘snowball’ effect helps to explain the sustained and exaggerated inflammatory response to injury and infection seen in uncontrolled diabetics (2).

Periodontal disease is a host modulated bacterial infection affecting the supporting structures of the teeth, leading to eventual tooth loss if untreated. It can be divided into chronic and aggressive types, based on the severity and speed of periodontal destruction. The effects of periodontal disease can eventually lead to tooth loss, by causing alveolar bone loss and destruction of the periodontal ligament, which attaches the tooth to the bone. (3) Periodontitis is sometimes considered to be the sixth complication of diabetes, as uncontrolled diabetics have a 2.8–3.4 increased odds of having periodontal disease compared to healthy controls (3, 4). The severity of periodontitis in these patients in closely correlated to their level of glycemic control.
The relationship between periodontal disease and diabetes is also seen in reverse, as treatment of periodontal disease results in improvement in glycemic control (3).

Gingival epithelial cells (GECs) of the periodontium are the first line of defense from bacterial pathogens, expressing a variety of innate immune receptors, including RAGE. The purpose of our investigation was to determine the effect that stimulation with AGE alone, in combination with a common bacterial ligand, or in combination with a soluble form of RAGE (sRAGE), would have on the production of inflammatory cytokines and expression of innate immune receptors including RAGE in GECs. We also wanted to investigate the effect simulated hyperglycemic conditions would have on the mentioned scenarios.

To accomplish this goal, we stimulated plates of GECs with AGE, lipopolysaccharide from *P. gingivalis*, individually, and in combination with sRAGE, both in normo-glycemic and hyperglycemic conditions. The Luminex-100 system to measure cytokine production, then real time polymerase chain reaction (qPCR) was used to measure expression of RAGE, TLR2, and TLR4, the latter two being innate immune receptors.

Our results demonstrated that stimulation of GEC with AGE leads to a pro-inflammatory cytokine response, as well as increased RAGE expression, leading to a hyper-inflammatory cellular state. This effect was amplified in the presence of glucose. We were also able to determine that ligation of RAGE in combination with TLR2 induced an exacerbated inflammatory response, over riding an anti-inflammatory response induced by TLR2 ligation alone. The presence of glucose amplified all pro-inflammatory responses while inhibiting any anti-inflammatory responses.
**Hypothesis:** AGE-RAGE interactions enhance the intrinsic hyper-reactivity of diabotogenic gingival epithelial cells.
Periodontal Health

The periodontal (pert = around, odontos = tooth) tissues are the tissues that allow for attachment of the tooth to the underlying bone tissues of the jaw. They comprise the gingiva (G), the periodontal ligament (PDL), the root cementum (RC) and the alveolar bone (AB). The teeth are the only structures of the body that are exposed to both the internal and external environment, thus the periodontal tissues also function to maintain the integrity of the masticatory mucosa of the oral cavity.

Healthy periodontal tissues allow for functional tooth movement during mastication while maintaining the position of the teeth in relation to the surrounding anatomy. They also have a proprioceptive function that allows for feedback to the muscles of mastication, preventing damage to teeth through excessive muscular force during mastication. The PDL comprises connective tissue fibers that attach the entire root of the tooth to the AB. The PDL in health is protected by a layer of epithelium. This epithelial layer provides a physical barrier, protecting the underlying tissues from bacterial and physical insult. The gingiva thus attaches to the necks of the teeth, just below the demarcation of the enamel and cementum, the cemento-enamel junction (CEJ). The portion of the gingiva that attaches to the tooth is known as the junctional epithelium (JE). The gingiva continues over the alveolar bone, and terminates at the mucogingival junction, at which point the tissues become alveolar mucosa.

In health, the visible periodontal tissues, the gingiva, appear light pink, have a stippled appearance, and have a firm consistency. Clinically, a periodontal probe can be used to assess the health of the tissues by periodontal probing. Healthy periodontal tissues do not bleed on probing, and the probe will not penetrate into the periodontal connective tissues. The gingival sulcus, a
A small crevice present in health typically measures 2–3 mm in height, and is covered by sulcular epithelium.

**Periodontal Disease**

Periodontal diseases are inflammatory processes that affect the periodontal tissues around the teeth. Bacterial plaque accumulation on the tooth surface leads to inflammation of the marginal tissues, known as gingivitis. Clinically, gingivitis results in tissue edema, loss of gingival stippling, and an increase in vascularity which leads to reddening of the gingiva. A common clinical sign of gingivitis is bleeding upon probing (5). Bacterial plaque consists of over 300 identifiable species of bacteria, bounded together in a polysaccharide matrix. Bacterial plaque is considered a *biofilm*, a term which describes the buildup in biological systems of bacteria on non-shedding or any other hard material. Biofilms normally occur in systems exposed to moisture containing planktonic bacteria, the mouth being a perfect example of this (6). If left untreated, gingivitis can progress to periodontitis, which is characterized by infection by predominantly gram-negative organisms in the plaque biofilm, destruction of PDL, RC and AB, eventually resulting in tooth loss (7). Diagnosis of periodontal disease is based on radiographic evidence of alveolar bone loss and clinical evidence of attachment loss (8). The clinical examination typically involves a combination of periodontal probing and measurement of gingival recession. Healthy periodontal tissues will typically measure 2–3 mm in depth, corresponding to the height of the gingival sulcus. The margin of the gingiva in health is above the CEJ. By determining the position of the depth of the periodontal pocket in relation to the CEJ, the amount of attachment loss can be determined (8). Periodontal diseases are classified as either aggressive or chronic, based on the speed of progression of attachment loss. They are also classified as either generalized or localized, generalized affecting >30% of sites. Chronic periodontitis is further divided into categories based on severity, with early chronic periodontitis
classified as 1–2 mm attachment loss, moderate as 3–4 mm attachment loss, and severe 5 mm or more of attachment loss (8).

The prevalence of periodontal disease varies among the adult population depending on severity and extent, however, up to 80% of the American population is affected by this disease at some level (3). Although plaque bacteria must be present for periodontal disease to occur, a susceptible host is also required (9). The host response is determined primarily by genetic, environmental and acquired factors. The purpose of the host response is protection; however, an abnormal host immune response could result in exacerbated tissue destruction. Such an abnormal inflammatory response, known as a ‘hyper-inflammatory trait’, has been linked to diabetes (10, 11). Here, there is an increased susceptibility to infection which is more inflammatory in nature, associated with an exaggerated secretion of innate inflammatory mediators and systemic markers of inflammation, implicating this process in the pathology associated with chronic disease processes such as periodontitis.

**Diabetes**

Diabetes mellitus affects over 21 million Americans, including >9% of the adult population (12). Classification of diabetes is based upon the patho-physiology of each form of the disease (1). Type I diabetes (T1D) is cellularly mediated autoimmune destruction of the insulin producing β-cells of the pancreas resulting in life-long dependence on exogenous insulin. Type II diabetes (T2D) manifests as insulin resistance whereby utilization of endogenously produced insulin is altered at the target cells (1). Both forms are metabolic diseases that lead to abnormal fat, sugar and protein metabolism. The resultant hyperglycemia ultimately induces diverse system pathologies (3).

Hyperglycemic conditions found in diabetic patients lead to exaggerated oxidative stress caused by increased oxidation of sugars, non-saturated fats and glycated proteins leading to
glucose auto-oxidation and decreased antioxidant levels (1). Hyperglycemia is a strong risk
factor in the development of secondary diabetic complications including the potential to alter the
periodontal environment (13). Secondary complications are consequences of indirect and direct
acellular damage caused by prolonged periods of high glucose concentrations (14).
Hyperglycemia affects cells indirectly due to the production of advance glycated end products
(AGE) (15), which are discussed below. Studies have shown that hyperglycemia causes impaired
host defense against pathogens, prolonged inflammatory response, microvascular alterations,
impaired bone formation and repair, and impaired wound healing in the periodontium (16-20).
Hyperglycemia, caused by diabetes, increases glucose concentration in the gingival crevicular
fluid altering the salivary environment in the periodontal pockets (21-24) leading to cellular and
molecular alteration in the periodontium (3).

**Periodontal Disease and Diabetes**

Much research has shown the close correlation between periodontal disease and diabetes
mellitus (4, 25-30). For instance, a meta-analysis using 3,500 diabetic adults concluded that the
majority of past studies found more severe periodontal disease in diabetic patients than in adults
without diabetes, confirming a significant association between periodontal disease and diabetes
(31). Also, a 2-year longitudinal study demonstrated that diabetic subjects had a significantly
increased risk for alveolar bone loss compared to non-diabetic individuals, with an odds ratio of
4.2. Among those patients, poorly controlled diabetics had an odds ratio of 11.4 compared to 2.2
of well controlled diabetics (30). The strength of evidence on the relationship between diabetes
and periodontal disease have led some to suggest that periodontal disease should be listed among
the “classic” complications of diabetes (4).
Just as diabetes contributes to increased incidence and severity of periodontal disease, periodontal disease can have a significant impact on the metabolic state of diabetics (3). For instance, in a 2-year longitudinal trial, diabetic subjects with severe periodontitis at baseline had a six-fold increased risk of worsening glycemic control compared to diabetic subjects without periodontitis (32). In addition, 82% of diabetic patients with severe periodontitis experienced the onset of one or more diabetic complications such as major cardiovascular, cerebrovascular or peripheral vascular events compared to only 21% of diabetic subjects without periodontitis (33). These and other studies support the notion that the presence of periodontal disease in diabetic patients may increase insulin resistance and contribute to worsening of the diabetic state and diabetic complications (32-39), although the mechanism of how this occurs is still unclear. Importantly, studies have demonstrated that mechanical periodontal treatment can improve the level of metabolic control in patients with diabetes (40-42). Therefore, appropriate treatment of secondary periodontitis in diabetic individuals is imperative to long term health.

**Innate Immunity And Inflammation**

Immunology provides the primary system shared by all multicellular organisms and provides the first line of defense in response to bacterial insult. In mammals, the innate immune response leads to production and secretion of pro-inflammatory cytokines from the nucleus of a wide variety of immune cells, including macrophages, monocytes, T cells, B cells, and gingival epithelial cells. The mechanism through which bacteria are recognized by the immune system involves the recognition of pathogen associated molecular patterns (PAMPs) by cellular pattern recognizing receptors (PRRs), the mammalian Toll-like receptors (TLRs). TLRs recognize various PAMPs such as the cell wall components of bacteria and fungi, and single and double-strand RNA from viruses. 11 different TLRs have been identified in humans. The
relatively small number of TLRs is able to recognize a wide variety of PAMPs through homo- and heterodimerization. Activation of TLRs helps to guide the adaptive immune response through activation of innate immune cells, more specifically antigen presenting dendritic cells. Activation of TLRs by interaction of a pathogen in immature dendritic cells leads to expression of co-stimulatory molecules, chemokines, and cytokines, all of which are important for the priming and activation of T-cells. The stimulation of different TLRs in dendritic cells will stimulate them to elicit distinct T-cell responses. For instance, ligand activation of TLR4 and TLR5, which are stimulated by *E. coli* lipopolysaccharide (LPS) and flagellin, respectively, induce a Th1 response. In contrast, stimulation of TLR4 by *P. gingivalis* LPS causes induction of a T helper type 2 response (43).

Cells of the innate immune system such as Langerhans cells, macrophages, dendritic cells and neutrophils are present in low numbers in healthy periodontal tissues. As well as these immune cells, cells of the periodontium also express TLRs to varying degrees. Bacterial ligand activation of TLRs in periodontal tissues can lead to the excessive production of pro-inflammatory mediators, which can result in tissue destruction.

Infection is a metabolic stressor, resulting in an increased demand for insulin, glucose and lipids (44-46) as well as the systemic challenge of pyrogenic cytokines such as IL-1β, TNF α and IL-6 (47-50). TNF α and IL-1β promote glycogenolysis and impaired uptake by cells in the periphery (48, 51-55). In addition, they induce insulin resistance by inhibiting the insulin receptor tyrosine kinase and other signaling proteins, further increasing the physiological demand for insulin secretion (47, 56-58). Therefore, infectious challenge can induce or worsen a metabolic diabetic state. Since periodontitis consists of inflammatory components which result in the production of a similar cytokine profile, this chronic disease process could serve as a
stimulus to a systemic-based inflammatory response resulting in a metabolic stressor in diabetic patients. Thus, periodontitis may enhance insulin resistance and impair insulin secretion leading to increased morbidity associated with diabetic complications. A clearer understanding of interactions between periodontal disease and diabetes should lead to the development of better primary prevention strategies to reduce complications and contribute to better glycemic control in individuals with diabetes.

In diabetics the ‘hyper-responsive’ phenotype has primarily been described in monocytes and macrophages of the innate immune system. It has been demonstrated that the monocytic hyper-responsiveness to bacterial antigens results in increased production of pro-inflammatory cytokines and mediators which induce tissue destruction, attachment loss and bone loss (59-61). In addition, AGEs that bind to macrophage and/or monocyte receptors induce a hyper-responsive cellular state resulting in increased secretion of pro-inflammatory cytokines including IL-12, insulin-like growth factor, and TNFα (62, 63). Interestingly, gingival epithelial cells (GEC) have many of the same immunological characteristics as monocytes, macrophages and dendritic cells (DC) and are one of the first barriers against oral bacteria in the periodontal tissues, yet their role in the hyper-responsiveness trait has yet to be elucidated.

**AGE/RAGE/Hyperglycemia**

Advanced Glycation End Products (AGE’s) are a heterogenous class of molecules produced from the glycation of proteins, lipids and nucleic acids (64). They can be produced exogenously through the Maillard reaction, also known as the browning reaction in cooking, and involves heating of sugars, lipids and proteins (65). Intestinal absorption of exogenous AGE’s is low, although human studies have shown elevated plasma AGE levels within 2 hours after an AGE rich meal (66). Endogenous AGE are formed as a result of exposure of proteins and lipids in the body to reducing sugars such as glucose, leading to, through a series of complex molecular
reactions, the formation of AGE (65). Accumulation of AGE in body tissues increases with age and at an accelerated rate in diabetes, due to the hyperglycemic conditions seen in diabetic patients, which leads to increased glycation of proteins (64). The presence of AGE results in a significant alteration of normal tissue structure and cellular composition. Covalent cross-linking of proteins is the primary outcome of AGE formation. Formation of AGE is time dependent, so long lived proteins such as collagen are usually affected, leading to increased stiffness of the protein matrix, abnormal barrier function and trapping of macromolecules such as low density lipoproteins (65). Histologic studies have demonstrated a correlation between the accumulation of AGE and increasing aortic stiffness, as well as increased accumulation of AGE in the kidneys of diabetic subjects (67-70). AGE are implicated in a wide range of diabetic complications, including atherosclerosis, diabetic neuropathy, nephropathy and microvascular disease (65). As well as having direct effects on tissue proteins, they also produce effects through activation of cellular receptors involved in innate immunity.

The receptor for AGE (RAGE) is a member of the immunoglobulin superfamily of receptors. It is capable of binding a large variety of ligands, including AGE’s, amyloid β peptide (Aβ), β sheet fibrils, the S100/calgranulins family of pro-inflammatory cytokines, and amphoterin (2). RAGE is expressed on a wide variety of cells, including endothelial cells, smooth muscle cells, monocytes, macrophages and neurons (71). Stimulation of cells bearing RAGE, results in a wide variety of cellular responses. In vivo and in vitro studies have demonstrated that in macrophages, endothelial cells, and vascular smooth muscle cells, activation of RAGE results in increased release of pro-inflammatory factors and increased chemotaxis. Increased translocation of NF-κB is a key pathway leading to this pro-inflammatory response (64). In contrast activation of RAGE in neuronal cells ultimately results in programmed
cell death (72). The biology of RAGE is unusual in that ligand stimulation results in an increased expression of the receptor, rather than down-regulation which is normally seen in receptor-ligand interaction. Since endogenous clearance of AGE is poor, the sustained ligand-receptor interaction and subsequent upregulation of RAGE expression results in a pathologic increase in cellular inflammatory response. Thus upon stimulation by pro-inflammatory ligands such as bacterial lipopolysaccharide (LPS) and tumor necrosis factor-α, a hyper-inflammatory reaction occurs, leading to enhanced cellular activation and tissue injury (2). This concept may help to explain the increased severity and incidence of periodontal disease in diabetic patients.

**Gingival Epithelial Cells**

The gingiva is the part of the oral mucosa that covers the supporting bone of the teeth, the alveolar bone. It is comprised of connective tissue covered by a layer of keratinized, stratified, squamous epithelium. The cells comprising this epithelium are known as gingival epithelial cells (GEC’s). It has long been determined that oral epithelium play an important role in the innate immune response by producing anti-microbial peptides and chemokines which recruit neutrophils (73, 74). More recently it has been shown that oral epithelium express several of the innate immune receptors for bacterial components, which are also found on monocytes, macrophages and DC (75-78). In addition, GECs can phagocytize and digest extracellular debris, erythrocytes, as well as microorganisms such as *Candida albicans*, *Mycobacterium leprae* and *Actinobacillus actinomycetemcomitans* (79-83), although the question of whether GECs can present exogenously acquired bacterial antigen to T cells is still under investigation (74). These similarities to the functions of immune cells, known to be responsible for hyper-responsiveness in diabetic patients, warrant investigation into the role GEC may play in the initiation of the over active inflammatory process.
Expression of innate immune receptors on GEC including toll-like receptor TLR4 and TLR2 along with NOD1 and NOD2 in healthy oral epithelial tissues has been demonstrated (76). Interestingly, there is significantly higher expression of these receptors in diseased gingival epithelial tissues. In addition, GEC respond differently to TLR stimulation than other mucosal epithelium(76). For instance, GEC do not secrete IL-8, monocyte chemo-attractant protein -1 (MCP-1), granulocyte colony stimulating factor (GCSF), granulocyte macrophage colony-stimulating factor (GM-CSF), or vascular endothelial growth factor (VEGF) upon stimulation with bacterial components unlike colonic epithelium. Therefore, it can be hypothesized that these cells are partially desensitized to avoid tissue destruction by excessive innate immune responses to bacterial stimulus. This is similar to dendritic cell (DC) responses to apoptotic self-cells (76, 77). Immature DC which encounter apoptotic cells exhibit an impaired capacity to stimulate T lymphocytes as a mechanism of self-tolerance (84, 85). This tolerogenic phenotype of DC is long-lasting and relatively stable to the point that lipopolysaccharide (LPS) treatment of such DC are unable to overcome the tolerance (86). Therefore, activation of DC carrying the remnants of self-antigen is halted, preventing auto-immune activation. It is entirely plausible that the hyper-reactive state associated with diabetes results in a lack of immune tolerance in the GEC resulting in over activation of the immune response and exaggerated periodontal tissue destruction. This lack of immune tolerance, analogous to loss of tolerance to β cell antigens (T1D) or insulin receptor (T2D), would then perpetuate a metabolic stressor and promote further glycogenolysis and insulin resistance as discussed above.
CHAPTER 3
MATERIAL AND METHODS

Gingival Epithelial Cell Culturing And Stimulation

Human gingival oral keratinocytes (HOK), a line of primary gingival epithelial cells (GEC) were grown to 90% confluency, after which 2.5 x 10⁴ total cells per well were plated in 2 6-well plates and allowed to adhere overnight. Some wells received 1μg/ml of soluble RAGE (sRAGE) (R & D) and were allowed to incubate for 30 minutes. After which some wells received 15 mM of glucose and allowed to incubate for 30 minutes. Cells were then stimulated with 1μg/mL of N-carboxyl(methyl-lysine) (CML) (Fisher) and/or 1 μg/mL of LPS from Porphyromonas gingivalis (ultra pure TLR2 ligand) (InVivogen). They were allowed to incubate for 24 hours after which supernatants were harvested and cells were placed in Trizol and frozen at -80C.

Cytokine Detection/Luminex

A cytokine detection kit was used to measure cytokine production. A filter plate was wet with 25 uL of Beadlyte® Cytokine Assay Buffer per well, vortexed, then a vacuum was applied to the bottom of the plate. The bottom of the plate was then dabbed on a paper towel to remove excess moisture. 50 uL of standard or sample of harvested supernatants was then added to the appropriate well. The Beadlyte® Human 22-plex Multi-Cytokine Beads were vortexed at high speed for 15 second, then sonicated for an additional 15 seconds using a microbead sonicator bath. 25 uL of bead solution was then added to each well. The plate was covered and vortexed briefly before being incubated for 2 hours in the dark at room temperature on a plate shaker. After incubation was complete, a vacuum manifold was applied to the bottom of the filter plate to remove liquid. 50 uL of the Beadlyte® Cytokine Assay Buffer was then added to each well, vortexed and vacuumed. This last wash step was repeated twice, blotting excess liquid in
between using a paper towel. 75 uL of the Beadlyte® Cytokine Assay Buffer was then added and the plate was vortexed at low speed. At this point, 25 uL of Beadlyte® Human 22-plex Multi-Cytokine, biotin was added to each well. The plate was covered and again vortexed at low speed. The plate was then incubated in the dark at room temperature on a plate shaker. Streptavidin-Phycoerythin was diluted in a 1:12.5 ratio with Beadlyte® Cytokine Assay Buffer. 25 uL of the diluted solution was added to each well. The plate was covered, vortexed at low speed, and then incubated for 30 minutes in the dark on a plate shaker. 25 uL of Beadlyte® Stop Solution was then added to each well, the plate was vortexed gently, then left for 5 minutes in the dark at room temperature. The plate was then vacuumed to remove excess liquid, each well was resuspended in 125 uL of sheath fluid, the plate was vortexed at low speed and placed on plate shaker for 1 minute. The results were acquired on a Luminex 100 system, while Beadview Software, a standard curve and 5-parameter logistics was used to analyze the data.

**RNA Harvesting**

The plate of the cells with Trizol added to them were firstly allowed to thaw under a fume hood, after which the entire volume of each sample (~700ul) was drawn up and transferred to individual 1.5 mL eppendorph tubes, labeled appropriately and allowed to incubate at room temperature for 5 minutes. 200 uL of chloroform was then added to each tube, after which each tube was vigorously shaken by hand for 15 seconds. The tubes were left to incubate for 15 minutes at room temperature and then centrifuged for 15 minutes at 4C at 12,000g. 500 uL of isopropynol was then added to the resulting supernatant in a new tube and left to incubate at -80C overnight. After which, samples were centrifuged at 4C for 10 minutes at 12,000g. The supernatant was then decanted and 1000 uL of 75% EtOH was added to each tube, vortexed for 15 seconds, and centrifuged at 4C for 5 minutes at 7500g. The supernatant was again decanted and allowed the tubes were allowed to air dry for 15 minutes. Finally, 20 uL of RNase/DNase
free H$_2$O was added to each tube which were then incubated at 65C for 1 hour. Resultant RNA was stored at -20C until Reverse Transcription (RT) could be performed.

**Reverse Transcription (RT) And Real Time PCR (qPCR)**

To create cDNA for qPCR, a master mix consisting of 5x buffer (Invitrogen), 10mM DTTs (Invitrogen), dNTPs (Invitrogen), reverse transcriptase (Invitrogen), oligo nucleotides (Invitrogen) and RNase/DNase free H$_2$O was added to 5 uL of RNA and placed in thermocycler (BioRad) conditions of 40C for 40 minutes, 70C for 15 minutes, and held at 4C. 10 uL of cDNA were placed into individual wells of a 96-well polypropylene RNase/DNase-free plate (USA Scientific) along with a standard 2x Cyber Green qPCR mater mix (BioRad) including 100nM of qPCR primers specific for either GAPDH: 5' TCC ACC ACC CTG TTG CTG TA 3' (reverse), 5' ACC ACA GTC CAT GCC ATC AC 3' (forward) (Integrated DNA Technologies, Coralville, IA); hRAGE: 5' GGA CTT CAC AGG TCA GGG TTA C 3' (reverse), 5' GAC TCT TAG CTG GCA CTT GGA T 3' (forward) (Integrated DNA Technologies, Coralville, IA); TLR-2 RT$^2$ qPCR primers, (SABiosciences, Frederick, MD) and TLR-4 RT$^2$ qPCR primers (SABiosciences) using thermocycler conditions of 95C for 4 minutes, 94C for 30 secs, 55C for 30 secs , 72C for 30sec, (40cycles), 56C for 1 minute, 72C for 5 minutes, and then held at 4C. Standard curves that were generated from serial dilutions of each gene of were used to calculate mRNA transcript copy number. Each gene was detected in independent real-time PCR reactions. Data are expressed as a copy number normalized GAPDH content. The normalized mRNA copy number for a gene was determined by: [raw transcript copy number derived from standard curve] [GAPDH corrective ratio]. The GAPDH corrective ratio was calculated as [lowest GAPDH copy number within sample set]/[GAPDH copy number for cell of interest].
CHAPTER 4
RESULTS

TLR2 Activation Results In An Anti-Inflammatory Response

In order to determine the innate immune response of gingival epithelial cells, we stimulated human oral keratinocytes (HOKs) with TLR2 ligand (LPS from \textit{P. gingivalis}). After which we measured the cytokine production 24 hours post stimulation in the supernatant. What we found was that levels of the pro-inflammatory cytokines GMCSF, IFNγ, IL12, IL6, MIP1α and TNFα generally showed no difference from unstimulated samples, in that no pro-inflammatory cytokines were produced (Figs 4-1, 4-3, 4-4, 4-6, 4-7, 4-8). On the other hand, the anti-inflammatory cytokines IL4 and IL-10 showed marked production with stimulation of TLR2 (Figs 4-2, 4-5). This signified an overall anti-inflammatory response. In order to determine the effects a hyperglycemic state might affect the TLR2 ligation, we added 15 mM of glucose to the samples described above. With the addition of glucose to the TLR2 ligand, both an increase in pro-inflammatory cytokines, and a decrease in the production of anti-inflammatory cytokines was seen (Figs 4-1 through 4-8).

Stimulation With AGE Results In A Pro-Inflammatory Response

To determine the effect of AGEs and hyperglycemic conditions on the innate immune response of HOKs, we also stimulated the cells with CML with and without glucose present. Addition of CML to the sample resulted in a significant increase in pro-inflammatory cytokine production such as IL-12 and TNFα versus the unstimulated group (Figs 4-1, 4-3, 4-4, 4-6, 4-7, 4-8), which was blocked in the presence of soluble RAGE, indicating the response was a result of AGE-RAGE interactions. Addition of both CML and glucose resulted in a substantially larger increase in pro-inflammatory cytokine production versus CML alone (Figs 4-1, 4-3, 4-4, 4-6, 4-7, 4-8). Addition of sRAGE again blocked the production of pro-inflammatory cytokines when
both CML and glucose were present (Figs 4-1, 4-3, 4-4, 4-6, 4-7, 4-8). Adding CML had no effect versus the unstimulated sample on the production of anti-inflammatory cytokines, with or without glucose being present (Figs 4-2, 4-5). These data suggest that AGE-RAGE interactions result in a pro-inflammatory response, which is exacerbated in the presence of glucose.

**Stimulation With AGE And TLR2 Ligand With And Without Glucose**

Under biological conditions, multiple innate immune receptors are engaged. To determine the affect of multiple interactions on GEC innate immune responses, we stimulated the samples with both TLR2 ligand and CML, again with and without glucose. Co-stimulation with CML and TLR2 ligand produced an increase in pro-inflammatory cytokine production versus unstimulated and TLR2 ligand alone (Figs 4-1, 4-4, 4-6, 4-7, 4-8). Additionally, adding both CML and glucose to TLR2 ligand stimulation resulted in an additive effect, producing significantly higher levels of pro-inflammatory cytokines such as GMCSF and TNFα and lower levels of anti-inflammatory cytokines IL-4 and IL-10 (Figs 4-1 through 4-8). The exception to this was with IFNγ, where co-stimulation with CML and TLR2 ligand resulted in cytokine production equal to the TLR2 ligand alone (Fig 4-3). However, adding glucose this co-stimulation resulted again in significantly increased INFγ production versus CML and TLR2 ligand alone (Fig 4-3). These data indicate that AGE-RAGE interaction is dominant over the signaling events of TLR2 ligation.

**TLR2 And TLR4 Expression Is Affected By TLR2 Ligation But Not AGE Stimulation**

To further understand the effect of TLR 2 ligation and AGE on the inflammatory response, we looked at expression of the TLR2, TLR4 and RAGE in HOKs when stimulated with *P. gingivalis* LPS and CML, with and without the presence of glucose and soluble RAGE. Compared to the unstimulated control, stimulation with *P.g.* LPS induced increased expression of the TLR2 receptor, while stimulation with CML had no effect on TLR2 expression (Fig 4-9).
Stimulation with a combination of *P. g.* LPS and CML resulted in a similar increase in TLR2 expression as *P. g.* LPS alone. Interestingly, addition of soluble RAGE slightly decreased TLR2 expression in the presence of *P. g.* LPS, although this effect just approached statistical significance (Fig 4-9).

TLR4 expression reacted very similarly to TLR2 expression when HOK cells were stimulated with *P. g.* LPS, CML, *P. g.* LPS+CML, with and without sRAGE (Fig 4-10). *P. g.* LPS stimulation resulted in upregulation of TLR4 expression, while stimulation with CML had no effect compared to the unstimulated control (Fig 4-10).

**RAGE Expression Is Affected By AGE Stimulation as Well as TLR2 Ligation**

Expression of RAGE was upregulated by stimulation with CML; as expected, this effect was blocked by sRAGE (Fig 4-11). Stimulation with *P. g.* LPS and *P. g.* LPS + CML significantly increased expression of RAGE. Addition of sRAGE to *P. g.* LPS showed no difference in RAGE expression compared to stimulation with *P. g.* LPS alone (Fig 4-11). These data are interesting in the fact that TLR ligation resulted in the upregulation of RAGE expression, while RAGE ligation had no affect on the expression of TLR2 or TLR4 in the absence of hyperglycemic conditions.

**Addition Of Glucose Increases The Expression Of TLR2, TLR4 And RAGE Regardless Of Receptor Ligation**

When looking at expression of TLR2, TLR4 and RAGE, stimulation with *P. g.* LPS and CML produced a dramatically larger upregulation of these genes in the presence of glucose (Figs 4-9, 4-10, 4-11). All increases in gene expression seen with ligand stimulation in normoglycemic conditions were amplified. This enhanced upregulation was greater for TLR4 and RAGE expression than TLR2 expression, with the change in TLR2 ligation in the presence of glucose versus normoglycemic conditions approaching statistical significance (Figs 4-9, 4-10, 4-11). Although stimulation with CML had no effect compared to unstimulated control on expression...
of TLR4 in normoglycemic conditions, increasing the concentration of glucose to levels seen in hyperglycemic patients resulted in a 10-fold increase in TLR4 expression, while the same was not true of TLR2 expression (Figs 4-9, 4-10). sRAGE blocked gene expression when induced with CML, but not when induced with \textit{P.g.} LPS indicating that both AGE-RAGE interaction and TLR2 ligation were responsible for the upregulation of receptors seen (Figs 4-9, 4-10, 4-11).
Figure 4-1. Production of granulocyte macrophage colony stimulating factor (GMCSF). HOK cells with and without addition of glucose were stimulated or unstimulated as depicted. Concentration of cytokine determined by Luminex. * p value <0.001 CML vs. control, # p value < 0.024 P.g. LPS + CML vs. P.g. LPS and CML, + p value <0.001 CML + sRAGE vs. CML, ^ p value < 0.001 P.g. LPS + glucose vs. P.g. LPS, ζ p value <0.001 CML + glucose vs. CML, ξ p value < 0.001 P.g. LPS + CML + glucose vs. P.g. LPS + CML.
Figure 4-2. Production of interleukin-10 (IL-10). HOK cells with and without addition of glucose were stimulated or unstimulated as depicted. Concentration of cytokine determined by Luminex. * p value < 0.01 P.g. LPS vs. control, # p value < 0.01 P.g. LPS + CML vs. P.g. LPS, † p value = 0.043 P.g. LPS + sRAGE vs. P.g. LPS, ^ p value = 0.021 P.g. LPS + glucose vs. P.g. LPS, ζ p value = 0.145 CML + glucose vs. CML, ξ p value = 0.056 P.g. LPS + CML + glucose vs. P.g. LPS + CML.
Figure 4-3. Production of interferon-γ (IFN-γ). HOK cells with and without addition of glucose were stimulated or unstimulated as depicted. Concentration of cytokine determined by Luminex. * p value < 0.001 * P.g. LPS vs. control, # p value < 0.001 P.g. LPS + CML vs. CML, + p value < 0.001 CML + sRAGE vs. CML, ζ p value < 0.001 P.g. LPS + glucose vs. P.g. LPS, ζ p value = 0.051 CML + glucose vs. CML, ζ p value = 0.245 P.g. LPS + CML + glucose vs. P.g. LPS + CML.
Figure 4-4. Production of interleukin-12 (IL-12). HOK cells with and without addition of glucose were stimulated or unstimulated as depicted. Concentration of cytokine determined by Luminex. * p value <0.001 CML vs. control, # p value < 0.001 P.g. LPS + CML vs. P.g. LPS, ^ p value < 0.001 CML + sRAGE vs. CML, ζ p value < 0.001 P.g. LPS + glucose vs. P.g. LPS, ξ p value < 0.001 CML + glucose vs. CML, η p value < 0.001 P.g. LPS + CML + glucose vs. P.g. LPS + CML.
Figure 4-5. Production of interleukin-4 (IL-4). HOK cells with and without addition of glucose were stimulated or unstimulated as depicted. Concentration of cytokine determined by Luminex. * p value < 0.01 P.g. LPS vs. control, # p value < 0.01 P.g. LPS + CML vs. P.g. LPS, + p value = 0.031 P.g. LPS + sRAGE vs. P.g. LPS, ^ p value = 0.021 P.g. LPS + glucose vs. P.g. LPS, ζ p value = 0.145 CML + glucose vs. CML, ξ p value = 0.013 P.g. LPS + CML + glucose vs. P.g. LPS + CML.
Figure 4-6. Production of interleukin-6 (IL-6). HOK cells with and without addition of glucose were stimulated or unstimulated as depicted. Concentration of cytokine determined by Luminex. * p value < 0.001 CML vs. control, # p value < 0.001 P.g. LPS + CML vs. P.g. LPS, + p value < 0.001 CML + sRAGE vs. CML, ^ p value < 0.001 P.g. LPS + glucose vs. P.g. LPS, ζ p value = 0.034 CML + glucose vs. CML.
Figure 4-7. Production of macrophage inflammatory protein-1α (MIP1-α). HOK cells with and without addition of glucose were stimulated or unstimulated as depicted. Concentration of cytokine determined by Luminex. * p value = 0.023 CML vs. control, # p value = 0.004 P.g. LPS + CML vs. P.g. LPS, + p value = 0.025 CML + sRAGE vs. CML, ^ p value < 0.001 P.g. LPS + glucose vs. P.g. LPS, ζ p value = 0.032 CML + glucose vs. CML, ξ p value = 0.021 P.g. LPS +CML+ glucose vs. P.g. LPS + CML.
Figure 4-8. Production of tumor necrosis factor-α (TNF-α). HOK cells with and without addition of glucose were stimulated or unstimulated as depicted. Concentration of cytokine determined by Luminex. * p value < 0.001 CML vs. control, # p value < 0.001 P.g. LPS + CML vs. P.g. LPS, + p value = 0.027 CML + sRAGE vs. CML, ^ p value < 0.001 P.g. LPS + glucose vs. P.g. LPS, ζ p value = 0.033 CML + glucose vs. CML, ξ p value = 0.037 P.g. LPS + CML + glucose vs. P.g. LPS + CML.
Figure 4-9. Toll-like receptor 2 (TLR2) expression was measured using qPCR in normoglycemic and hyperglycemic conditions while unstimulated and stimulated as depicted. * p value = 0.05 P.g. LPS vs. control, # p value = 0.01 P.g. LPS + CML vs. control, ^ p value = 0.06 P.g. LPS vs. P.g. LPS + sRAGE, + p value = 0.01 P.g. LPS vs. P.g. LPS + glucose, ζ p value = 0.06 P.g. LPS + CML vs. P.g. LPS + CML + glucose, ξ p value = 0.001 P.g. LPS + sRAGE vs. P.g. LPS + sRAGE + glucose.
Figure 4-10. Toll-like receptor 4 (TLR4) expression was measured using qPCR in normoglycemic and hyperglycemic conditions while unstimulated and stimulated as depicted. * p value < 0.0001 P.g. LPS vs. control, # p value = 0.002 P.g. LPS + CML vs. control, + p value < 0.0001 P.g. LPS vs. P.g. LPS + glucose, ζ p value < 0.0001 P.g. LPS + CML vs. P.g. LPS + CML + glucose, ^ p value < 0.0001 CML vs. CML + glucose.
Figure 4-11. Receptor for advanced glycation end-products (RAGE) expression was measured using qPCR in normoglycemic and hyperglycemic conditions while unstimulated and stimulated as depicted. ^ p value = 0.006 CML vs. control, * p value < 0.0001 P.g. LPS vs. control, # p value = 0.0014 P.g. LPS + CML vs. control, + p value = 0.008 CML vs. CML + sRAGE, ζ p value < 0.0001 CML vs. CML + glucose, ξ p value = 0.0005 P.g. LPS vs. P.g. LPS + glucose, δ p value < 0.0001 P.g. LPS + CML vs. P.g. LPS + CML + glucose.
The relationship between advanced glycation products and periodontal disease is multifactorial. The direct effects of irreversible glycation on body proteins may in part explain the increased severity and prevalence of periodontitis seen in uncontrolled diabetics. It seems that the more complex, and certainly, more interesting effects happen at the cellular level.

When the receptor for AGE is ligated, the resultant effect is pro-inflammatory. An interesting outcome of RAGE ligation is increased expression of RAGE, resulting in a cascade type response, where stimulation of the receptor results in more receptor being produced, and thus greater cellular effects when stimulated. Repeated stimulation of the receptor therefore results in a much greater pro-inflammatory response than single stimulation (2). Our findings indicate that stimulation of other toll-like receptors, like TLR2, also results in a marked increase in RAGE expression (Fig. 4-11). This effect is greatly amplified in hyperglycemic conditions.

Diabetes affects the bodies ability to process glucose, either because of destruction of insulin producing B cells in the pancreas, or because of insulin resistance. Insulin causes muscle, liver and fat cells to take up glucose from the blood. A lack of insulin or insulin resistance therefore results in elevated blood glucose levels, or hyperglycemia. Hyperglycemic conditions lead to increased formation of advanced glycation end products in body tissues (87). Our findings have shown that this combination of hyperglycemic conditions and presence of AGES results in a cumulative effect on pro-inflammatory cytokine production in gingival epithelial cells, as well as increased expression of RAGE (Figures 4-1, 4-3, 4-4, 4-6, 4-7, 4-8, 4-11). This ‘double whammy’ effect helps to explain the increased incidence and severity of periodontitis seen in uncontrolled diabetics (3). Explaining how hyperglycemic conditions increase the pro-inflammatory response requires further investigation.
Ligation of TLR2 and TLR 4 (data not shown), also upregulates expression of RAGE, as well as the upregulation of their own receptor (Figures 4-9, 4-11). This seems to indicate the RAGE may function in part as an integral part of the innate immune response to help mediate the response to bacterial invasion. Stimulation with AGE does not upregulate TLR2 and TLR 4 expression, as these receptors are very specific for bacterial ligands. RAGE is a multi-ligand receptor, whose ligands are well documented (2). Some of the known endogenous ligands include S100/calgranulins. S100/calgranulins are a family of pro-inflammatory cytokines expressed by inflammatory cells. They are normally expressed intra-cellularly, where they are associated with calcium binding, cellular motility, and phagocytosis. They are also able to be released in the extracellular environment where they can bind to RAGE. Since RAGE ligation results in a pro-inflammatory response, release of S100/calgranulin by inflammatory cells and subsequent ligation of RAGE present in other cells may be a method of sustaining and communicating the need for a pro-inflammatory response in tissues experiencing infection or injury. Another endogenous ligand shown to bind RAGE is amphoterin, a polypeptide involved in neuronal development (2). Rauvala et al. showed that amphoterin mediates neurite outgrowth. Investigation by Hori et al. lead to the finding that RAGE is the receptor that mediates the effects of amphoterin by showing that selective inhibition of neurite outgrowth occurs in the presence of sRAGE or anti-RAGE IgG. Amphoterin has also been noted to be highly expressed in migrating cells, such as tumors (88). Several studies have shown that blockade of RAGE reduces tumor growth and metastases in animal models (89). Thus, RAGE has several functions and is an integral part of the innate immune response.

Our data confirm the findings that the presence of soluble rage blocks RAGE ligation, as expression of TLR2, TLR4 and RAGE is the same as control when stimulated with CML, with
or without hyperglycemic conditions (Figures 4-9, 4-10, 4-11). What is not known from our experiments is whether or not increased production of RAGE also results in increased production of sRAGE. It can be considered unlikely however, as even if sRAGE expression was increased, it is not produced in sufficient quantities to block the pro-inflammatory response, as demonstrated by the increase in pro-inflammatory cytokines seen with CML stimulation when no sRAGE was added. (Figures 4-1, 4-3, 4-4, 4-6, 4-7, 4-8).

Soluble RAGE has been shown to be produced by the body in response to infection. It was recently demonstrated to be present in amniotic fluid when microorganisms are present (90). Its use as a method for blocking local effects of RAGE ligation may be limited, as it has been shown by other groups that as well as blocking the effect of RAGE, it may have pro-inflammatory effects. Pullerits et al. showed that ligation of sRAGE results in release of pro-inflammatory cytokines IL-6, TNF-α, and MIP-2 by monocytes and macrophages (91). In their study, they looked at high mobility group box chromosomal protein 1 (HMGB-1), a potent pro-inflammatory cytokine that is a RAGE ligand. HMGB-1-induced arthritis in mice was suppressed with administration of sRAGE, but in a separate experiment they showed a marked increase in the number of polymorphonuclear and mononuclear cells in the peritoneal cavity of mice administered with intra-arterial sRAGE. They hypothesized that, while blocking the local effects of inflammation, sRAGE deviates inflammation into the peritoneal cavity. Furthermore, apart from sRAGE’s ability to induce cytokine release, they showed that sRAGE induces neutrophil chemotaxis in vitro (91). So although several groups have found that sRAGE blocks the effects of RAGE, administration of sRAGE as a therapeutic approach to block local pro-inflammatory effects may have systemic consequences. It must be elucidated through further research if the benefits of blockade of RAGE outweigh these concerns.
An unexpected finding of our research was that stimulation with TLR2 ligand (LPS from *P. gingivalis*) resulted in the release of anti-inflammatory cytokines IL-4 and IL-10 (Figures 4-2 and 4-5). This may happen to help maintain homeostasis. Since TLR2 is only activated by small components of bacteria, the anti-inflammatory effects may serve to ensure that body tissues do not respond simply to fragments of dead bacteria. Other TLR’s and heterodimers of TLR’s produce pro-inflammatory effects, so it can be hypothesized that the overall immune response becomes pro-inflammatory when multiple bacterial components are encountered. Our lab has also shown that in diseased periodontal tissue, TLR2 expression is down-regulated and TLR4 and RAGE expression is up-regulated, further demonstrating the pro-inflammatory nature of periodontal disease.

In conclusion, exposure of gingival epithelial cells to conditions simulating uncontrolled diabetes results in an increase in pro-inflammatory cytokine production, a decrease in anti-inflammatory cytokine production, as well as alterations of innate immune receptor expression which leads to a hyper-inflammatory state. Further work is needed to understand the mechanism through which glucose promotes these effects.


BIOGRAPHICAL SKETCH

Dr. Jamie Amir completed his dental training at Guys’, King’s and St. Thomas’ School of Medicine and Dentistry in 2003. He was a Fellow at the Center for Implant Dentistry at the University of Florida College of Dentistry from 2003 to 2005, then undertook the Internationally Educated Dentists program at the University of Florida to receive licensure to practice dentistry in the state of Florida. He completed his post-doctoral residency in Periodontics at the University of Florida in the summer of 2009, after which he joined an existing private practice in Melbourne, Florida.