INNATE IMMUNE RECEPTOR EXPRESSION IN PERIODONTALLY DISEASED AND HEALTHY TISSUES

By

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To my wife, who has tirelessly supported my goals throughout this journey.

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The innate immune system, of which toll-like receptors (TLRs) and receptor for advanced glycated end products (RAGE) are an integral part, has been implicated in the pathogenesis of periodontal disease. Recent work has shown expression of RAGE to be up regulated in gingival tissues from patients suffering from chronic periodontitis and diabetes, although the reason for the increased expression has not been elucidated. However, RAGE has been implicated in a number of other pro-inflammatory processes and has been implicated in the disease progression of coronary artery disease, hypertension, arthritis and Alzheimer's disease. Additionally, toll-like receptors two (TLR-2) and four (TLR-4) are up regulated in chronic periodontitis as a result of bacterial infiltration by *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis*. It was the purpose of this investigation to determine whether there was an association among the up-regulation of RAGE, TLR-2, TLR-4 in patients diagnosed with chronic periodontitis.

To determine the possible associations, gingival tissue samples were collected from patients during periodontal surgeries, after which, RNA isolation and real time polymerase chain reaction (qPCR) were used to quantify expression of RAGE, TLR-2 and TLR-4. Our results demonstrate that RAGE and TLR-4 expression were up regulated in chronic periodontitis

patients, whereas TLR-2 expression was down regulated when compared to expression in healthy gingival tissues, with a correlation coefficient of r=0.9964, indicating that in a given participant when RAGE expression was increased, so was TLR-4 expression while TLR-2 expression was decreased.

CHAPTER 1
INTRODUCTION

Periodontal disease is one of the most prevalent diseases in the United States, with nearly one quarter of the population being affected with some form of the disease. World estimates, according to the World Health Organization put more severe forms of the disease within the range of ten to 15% worldwide.[1] While periodontal disease itself is not necessarily life-threatening, there is an increasing body of evidence supporting the notion that periodontal disease may significantly contribute to systemic diseases that carry a higher morbidity and mortality, such as diabetes, cardiovascular disease and cerebrovascular disease.[2, 3] Although specific links have not yet been elucidated, many of these diseases share the common link of chronic inflammation, increased levels of inflammatory products and markers, as well as higher levels of Advanced Glycation Endproducts, or AGEs.[4]

The immunological response begins with the innate immune system, which is highly conserved throughout nature. The innate response is targeted specifically toward common molecular identifiers of pathogens, known as pathogen associated molecular proteins, or PAMPs. PAMPs sequences identified include lipopolysaccharides (LPS), peptidoglycans, bacterial DNA, as well as lipoproteins.[5] On the front line of the innate immune response are immunologic receptors initially identified in mice in 1985 – Toll-like receptors, or TLRs. TLRs recognize these conserved patterns and trigger a cascade within the innate immune response.[5]

Many organisms known to contribute to periodontal disease also express many of these PAMPS. For instance, the LPS from the bacteria *A. actinomycetemcomitans and P. gingivalis* bind to TLR-2 and TLR-4 respectively.[5] Several studies have indicated an increase in the expression of these innate immune receptors under inflammatory conditions.

AGEs form via a non-enzymatic reaction from reducing sugars, such as glucose with amino groups in proteins, lipids and nucleic acids, by way of a series of reactions, Schiff bases and Amadori products are produced.[4] They can be formed exogenously, such as in tobacco and cooked foods, or endogenously due to spontaneous reactions – especially in those persons with high circulating blood glucose levels, such as diabetics.[6] AGEs are insoluble and are capable of permanently modifying proteins after which they bind to the endogenous receptors for known as RAGE resulting in a pro-inflammatory response.

The inflammatory nature of periodontitis has been well established. Periodontitis has been associated directly with pro-inflammatory mediators and cytokines, such as interleukin 1 (IL-1), interleukin 6 (IL-6) and tumor necrosis factor α (TNF-α), mainly through the induction of the innate immune response using TLR ligation.[7] In addition, studies indicate a relationship between RAGE signaling and those induced by TLR ligation. Therefore, determining a relationship between chronic bacterial infection, such as in chronic periodontitis, and an association with an increase in the expression of RAGE, TLR-2, and TLR-4 where a clear relationship between periodontal disease and these receptors has not been established is of interest.

In order to elucidate a possible relationship among RAGE expression and TLR-2 and TLR-4 expression, periodontally healthy and patients diagnosed with chronic periodontitis were recruited to donate gingival tissues from surgical procedures. Here, the level of gene expression for RAGE, TLR-2 and TLR-4 was analyzed using real-time polymerase chain reaction (qPCR). In addition, histological sections were used to determine the cell types harvested in these tissues as well as the expression pattern of RAGE in these tissues.

These data demonstrate that periodontally diseased tissue expressed a higher amount of RAGE and TLR-4 and a lower amount of TLR-2 when compared to periodontally healthy

tissues. We were able to determine that epithelial, endothelial, fibroblast, etc. cells were included in the cell types analyzed for gene expression. In addition, mononuclear cells were found to be present in the diseased tissues as well. Here, RAGE protein expression was also qualitatively up regulated on most cells types, not just the immune infiltrate. These data indicate that increases in gene expression was not solely due to increases in immune cell infiltrate

Hypothesis: The state of inflammation or periodontal health status has an effect on the expression level of innate immune receptors.

CHAPTER 2
BACKGROUND

The Periodontium in Health

The periodontium, or the supporting structures surrounding teeth, consists of several tissues. These tissues serve not only to support the teeth, but, to provide protection against bacterial infiltration and disease. These tissues consist of bone, cementum, connective tissue, and epithelium.[7] In health, these separate tissues comprise a very strong support structure for the teeth that provide adequate protection against bacterial plaque. The normal, healthy structure of the healthy periodontium has been studied extensively. Dimensionally, the epithelium attaches to the tooth anywhere from 0.67-1mm apical to the cementoenamel junction (CEJ) and is approximately 1mm in dimension. The connective tissue, typically comprised of collagen, ground substance and fibroblasts, attaches to the tooth from that point on and comprises an additional millimeter.[8] The connective tissue attachment approximates the bone and from the bone to the apex of the tooth root, the periodontal ligament (PDL) and bone are continuous. (Figure 2-1) There is minor variation among people, as well as tooth type with respect to the above dimensions. The sum of these measurements is termed the "biologic width".[8] In health, these measurements do not stray far from one millimeter each for the sulcus, epithelial attachment and connective tissue attachment. The epithelium serves to provide protection from bacterial invasion into the PDL and bone, as well as mechanical protection. In disease, through repeated insult, the epithelial attachment may become weakened and eventually detached.[8] The lack of attachment of the epithelial barrier allows a bacterial plaque biofilm to form subgingivally, making removal of the plaque very difficult for the patient. With time, this plaque is able to harden into calculus – a mineralized form of the bacterial plaque that serves as a

constant irritant to the connective tissue attachment. Given more time, plaque accumulates further apically and the disease progresses.[9]

The main function of bone in the peridontium is to support teeth in the process of mastication. Forces in the human masticatory system sometimes reach 600-750N in Americans and Europeans. The separate tissues in the system, such as the PDL, tend to distribute the load through a slightly movable system. While the PDL does provide some of the support, the majority of forces are borne by the mandible and the maxilla. The main functional capacity of the PDL is to stabilize the tooth within its bony socket, as well as to provide somatosensory information and nutrition.[10] The destruction of these tissues results in loss of attachment, increased probing depth and ultimately, loss of the tooth due to lack of proper bony support.

Periodontal Tissue Destruction

Diagnosis of periodontal disease is currently accomplished according to collection of clinical data – namely probing depth, recession, bleeding upon probing, furcation involvement and mobility of teeth. Probing depth gives us an indication as to the current status of the PDL attachment around a tooth by determining the depth, to the nearest millimeter, to which the probe will reach. Typically, this is in the two to three millimeter range in health and does not extend beyond the epithelial attachment. In disease, the periodontal probe passes the epithelium and extends into connective tissue.[11] Recession indicates tissue loss from the cemento-enamel junction (CEJ) to the gingival margin. Bleeding upon probing indicates the possibility that inflammation is present at a particular site. The presence of bleeding does not necessarily indicate disease, however.[12] A tooth can be described as furcation involved when there exists enough bone loss as to expose the area between roots to probing. Mobility indicates the lateral movement of a tooth. Lack of mobility is typically considered a sign of better prognosis and health.[16] The sum of probing depth and recession, from the level of the CEJ is termed

attachment loss. The higher the degree of attachment loss, the more support has been lost from the tooth.

Periodontal disease is an inflammatory disease classified according to the type of tissue involved, the location, duration and severity of the disease. The current classification system, established in 1999, is widely used to diagnose periodontal disease.[13] In addition to non-microbially induced forms of the disease, such as viral infections, fungal infections, etc. the system includes bacterial origins (plaque-induced) of the disease. For periodontal diseases of bacterial origin, diagnoses range from chronic to aggressive, localized to generalized, and slight to severe, dependent upon the amount of attachment loss that has occurred.[13]

Tissue destruction (or attachment loss) in periodontitis is the result of several factors. Many bacterial species have been implicated in the process of periodontal destruction. In 1998, Haffajee and Socransky identified several bacterial species associated with periodontal diseases. In addition to identifying some of the present bacteria via DNA checkerboard hybridization, they attempted to associate certain species with more severe forms of the disease. Several groups were described, with the red and orange complexes being associated more closely with disease than the other groups.[14] Two species of bacteria that have been readily identified with periodontal disease, both chronic and aggressive, respectively, are *Porphorymonas gingivalis* and *Aggregatibacter Actinomycetemcomitans*. Each of these species is associated with the "Red" complex and "Green" complexes, respectively.[14]

The immune response mounted by the body to the bacterial infiltration releases a host of inflammatory mediators that are able to destroy the periodontal tissues. Soon after the junctional epithelium has been violated, access to the PDL and underlying connective tissues is possible.[15] Bacterial toxins, such as lipopolysaccharides, induce the production of inflammatory mediators,

such as proteases, cytokines and prostaglandins to assist in resistance of the bacterial invasion. This response, intended to neutralize bacteria, also has the untoward effect of destroying the periodontal tissues along with the bacteria. The resultant tissue destruction is the basis for the loss of tissue seen in periodontitis.[17]

Extension of the inflammation is made possible by the proliferous blood supply within the periodontium. Vessels extending into the connective tissue, periosteum and bone allow migration of bacteria, bacterial toxins, and pro-inflammatory mediators through out the tissues. The resultant immune response via pro-inflammatory cytokines, such as IL-1, TNF-α and matrix metalloproteinases (MMPs) leads to the resorption of bone and other connective tissues.[17] This inflammatory process is capable of destroying supporting periodontal tissues over a long period of time, as with chronic periodontitis, or within a short time frame, as with aggressive periodontitis.

Risk Factors for Periodontal Disease

As with any disease, the risk for developing periodontal disease can be modified by outside, controllable factors. These modifiable traits are often called risk factors.[18] Within a population of patients, use of specific tools to identify which patients are at risk for a certain disease is often of great value in prevention or modification of the severity of a disease. Several factors have been associated with increased risk of developing periodontal disease, including socioeconomic status and age. High on the list, however, are tobacco smoking, as well as diabetes.[19] The negative effects of smoking on oral health have been well-documented. Tobacco smoke, in addition to being an exogenous source of AGEs, retards healing, vascularization, and can impair collagen growth.[20] The effects of diabetes have also been well-documented, with

impaired healing and host immune response specifically contributing to the morbidity of periodontal diseases.[20]

Treatment for Periodontal Disease

Although both bacteria and inflammatory processes contribute to the initiation and progression of periodontal diseases, the majority of treatments to date are aimed at the removal of bacterial complexes. Indeed, the first line in treatment for periodontal disease is non-surgical treatment, which typically takes the form of scaling and root planing. Scaling is defined as removal of supra and subgingival bacterial deposits (plaque and calculus). By root planing, the outermost layer of cementum from a tooth root is removed and "planed" to an even consistency, thereby providing for smooth, deposit-free root surfaces. The removal of bacterial deposits, combined with root surface modification, allows reattachment of periodontal tissues, typically in the form of a long junctional epithelium (LJE).[21] Outcome measures for periodontal health include reduction of probing depths, gain of attachment levels, and evidence of bleeding upon probing.

After traditional, non-surgical periodontal treatment is performed and the results do not attain health, surgical treatment of periodontal disease becomes the next line of treatment. With surgical treatment, the gingival tissues are reflected, oftentimes removing a small (1-3mm) collar of diseased tissues, granulomatous or tissues that are undergoing an intense inflammatory response and replace lost bone and other connective tissues are removed and discarded, and scaling and root planing is performed with better visualization of bacterial deposits.[22] The enhanced visibility of involved teeth allows for more complete removal of deposits and recontouring of tooth roots. Typically, the remaining bone around involved teeth is recontoured in order to achieve "positive architecture" of the supporting bone.[22] The goal of these treatments

results in the removal of bacteria, allowing for decreased insults to the immune system. This, in turn, leads to a decreased activation of the immune response and the resultant tissue destruction.

Toll-Like Receptors and Periodontal Disease

Periodontitis is characterized by an active increase in the immune system, both innate, as well as adapted, as a result of bacterial insult. The "early" periodontal lesion can be histologically typified by the acute invasion of lymphocytes, which includes monocytes, B and T cells. Later in this progression, the "established" lesion consists of an intense neutrophil invasion. The advanced lesion, however, is the first stage of the lesion that can be described as having resultant bone loss and supporting tissue destruction. Throughout these stages of the periodontal lesion, a common thread is the involvement of the innate immune response. Integral in this response are toll-like receptors (TLRs).

Toll-like receptors are intracellular and extracellular proteins that form the first response to bacterial and viral infiltration. They often initiate a response based upon bacterial or viral products that are present, such as lipopolysaccharides, peptidoglycan, flagellin, foreign DNA and RNA, as well as bacterial byproducts. TLRs are homologous to the IL-1 strucuture, but, contain a number of leucine-rich repeats in their structure. The leucine-rich repeat areas correspond to certain PAMPs and TLRs are capable of dimerizing to recognize different bacterial products.[5]

Specifically, it has been shown that both TLR-2 and TLR-4 are up regulated in the presence of bacterial insult within the periodontium, although which cell types are involved is unclear.[24, 25] Lipopolysaccarides and other components of bacterial products function as PAMPs, trigger a signaling cascade from TLRs on specific cells within the innate immune system and architecture of the periodontium, including monocytes, neutrophils, dendritic cells, epithelial and endothelial cells. Triggering of TLR-2 and TLR-4, among other toll-like receptors, result in the upregulation of many soluble mediators such as cytokines, chemokines and growth factors.[26]

These soluble mediators initiate multiple cell signaling pathways result in the activation of T cells and B cells, the hallmark of the established periodontal lesion and part of the adaptive immune system. Chronic stimulation of toll-like receptors, as is found in plaque-induced periodontitis, releases a continuous stream of pro-inflammatory mediators,[5] one of the mechanisms responsible for the tissue destruction seen in periodontitis.

Advanced Glycation Endproducts and Periodontal Disease

AGEs form via a non-enzymatic reaction from reducing sugars, such as glucose with amino groups in proteins, lipids and nucleic acids. By way of a series of reactions, Schiff bases and Amadori products are produced, forming AGEs.[4] This process has been described as early as the 1900's and is commonly referred to as the Maillard reaction. They can be formed exogenously, such as in tobacco and cooked foods, or endogenously due to spontaneous reactions – especially in those persons with high circulating blood glucose levels, such as diabetics.[6] AGEs are insoluble and are capable of permanently modifying proteins, and bind to an endogenous receptor known as RAGE.

The receptor for AGE is a cell surface receptor that is capable of inducing a proinflammatory response. RAGE expression occurs within a wide variety of cell types, including epithelial, endothelial, smooth muscle, lymphocytes, monocytes, as well as neurons.[4] Activation of RAGE triggers cellular activation of proinflammatory mediators, such as interleukin 1 (IL-1), IL-6 and tumor necrosis factor alpha (TNF-α). Once bound, RAGE stimulates further expression of RAGE via a positive feedback mechanism and generates oxidative stress, synthesis of proinflammatory cytokines, as well as chemotaxis.

RAGE also has the unique capability of binding many types of ligands. Some of the ligands, in addition to AGEs, that have been shown to bind RAGE are S100/calgranulins, High

Mobility Group Box-1 (HMGB1) and amyloid beta peptide (Abeta).[38] S100 has been shown to act as a pro-inflammatory cytokine, triggering a cascade of release of IL-1, IL-6, and TNF-α, as well.[31] MHGB1 is a proinflammatory protein that has been implicated in a growing number of transformational processes, including breast cancer and adenocarcinoma.[32]

AGEs have been implicated in the pathogenesis of tissue destruction as a result of diseases such as diabetes and cardiovascular disease, as well as the natural aging process. Macular degeneration, atherosclerosis, diabetic neuropathy, as well as periodontitis all have a link to increased expression of RAGE, as well as higher levels of circulating AGEs.[28] Signaling pathways pursuant to RAGE ligation results in higher levels of inflammatory mediators, as well, such as C-reactive protein, and an increased degree of overall systemic inflammation.[29]

RAGE expression has been previously been associated with risk factors for periodontal disease, such as diabetes.[6] Lalla et al. also reported increased formation and deposition of AGEs in the gingival of diabetic mice inoculated with *P. gingivalis*. Additionally, a study using the same murine model found that periodontal disease was arrested by administration of soluble RAGE (sRAGE), most likely through the inhibition of the RAGE-AGE interaction.[6] Finally, Katz et al. has shown human subjects with diabetes and periodontal disease to have a higher expression of RAGE in gingival tissues compared to controls. These evidence together point to the possible relationship of RAGE expression and periodontal disease status. [33]

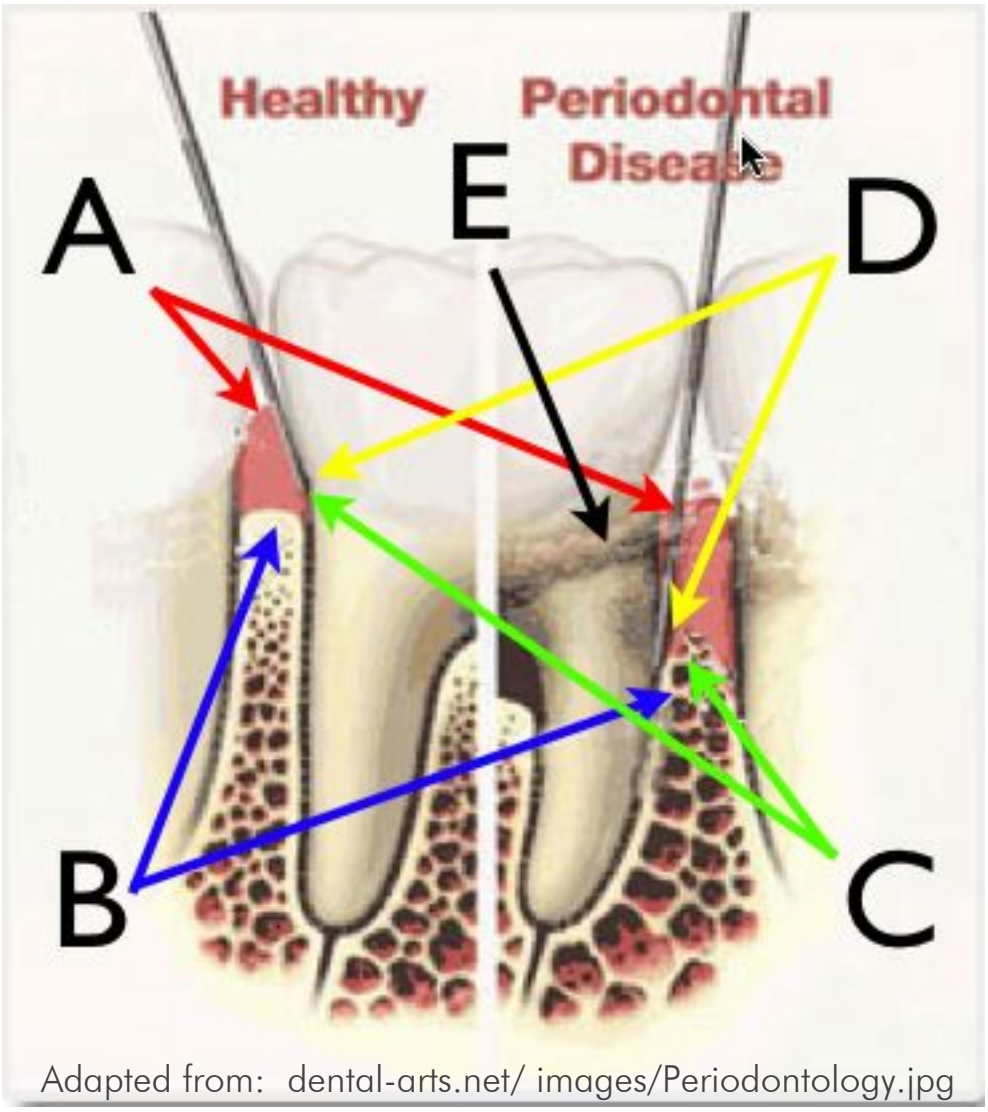

Figure 2-1. Periodontal Health. Diagram of a tooth in health (left) and disease (right) (A) Note the level of the free gingival margin. Apical migration of the free gingival margin is termed recession. (B) Note the height of the alveolar process. Bone levels are reduced in disease. (C) Note periodontal probe extending deep within the pocket on the diseased site. In disease the periodontal probe extends beyond the epithelial attachment and into the connective tissue. (D) Note junctional epithelium near CEJ in health and long junctional epithelium in disease. (E) Note the presence of plaque and calculus and its access to periodontal disease.

CHAPTER 3
MATERIALS AND METHODS

A prospective observational study was conducted to determine if periodontally healthy and diseased tissues had a difference in expression of RAGE, TLR-2 and/or TLR-4. Quantification of gene expression for these three genes was completed utilizing real-time polymerase chain reaction (qPCR). In order to determine which cell types were contributing to the expression patterns observed, gingival samples were also analyzed using hematoxylin and eosin immunohistochemistry on parrafin embedded sections.

Participant Population

Fifty patients were recruited from the University of Florida College of Dentistry, Department of Periodontology. All patients consented to the study, following Institutional Review Board approval. Inclusion criteria were as follows: diagnosis including chronic periodontitis or health, an age range between 18 and 70 years of age, necessity for surgical treatment (periodontal surgery, dental implant surgery, etc.). Patients were excluded if they had a history of severe or chronic systemic disease, were pregnant or lactating or were taking medications known to affect the gingiva.

Surgical Procedure

Tissue harvesting was completed in conjunction with multiple types of oral surgical procedures performed by different surgeons. The majority of diseased specimens were removed as waste tissue during surgery to treat moderate to advanced chronic periodontitis. The healthy specimens were removed as waste tissue during procedures not related to periodontitis, such as dental implant placement, uncovery, clinical crown lengthening, or distal wedge surgeries. Patients were seen for regular follow-up seven to fourteen days post surgery.

Tissue Preparation and Storage

Following each surgical procedure, equal portions of the sampled tissue were placed in Trizol, RNA later, transfer Media and/or formalin. After which, the specimens were stored at -80°C or room temperature (RT) until RNA harvesting or histology respectively could be performed. Formalin-fixed tissues were sent for embedment in paraffin, sectioning and slide preparation.

RNA Harvesting

Samples frozen in Trizol were allowed to thaw under a fume hood, after which 200 □L 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 4°C at 12,000g. 500 □L of isopropynol was then added to the resulting supernatant in a new tube and left to incubate at -80°C overnight. After which, samples were centrifuged at 4°C for 10 minutes at 12,000g. The supernatant was then decanted and 1000ul of 75% EtOH was added to each tube, vortexed for 15 seconds, and centrifuged at 4°C for 5 minutes at 7500g. The supernatant was again decanted and allowed the tubes were allowed to air dry for 15 minutes. Finally, 20 □L of RNase/DNase free H_20 was added to each tube, which were then incubated at 65°C for 1 hour. Resultant RNA was stored at -20°C until Reverse Transcription (RT) could be performed.

Reverse Transcription (RT)

cDNA was reverse transcribed using normalized RNA and a standard reverse transcription master mix containing 5X buffer (Invitrogen), 1mM DTTs (Invitrogen), 2.5mM dNTPs (Invitrogen), Superscript III reverse transcriptase (Invitrogen), oligonucleotides (Invitrogen), and RNase, DNase free water in a final volume of 25□L using the thermocycler conditions: 40°C for

40 minutes, 70° for 15 minutes , hold at 4°C. All cDNA was stored at 4°C until qPCR could be performed.

Real-time Polymerase Chain Reaction (qPCR)

10ul of cDNA were placed into individual wells of a 96-well polypropylene RNase/DNase-free plate (USA Scientific) along with a standard qPCR master mix including 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). Standard curves that were generated from serial dilutions of each gene were used to measure mRNA transcript copy number. Each gene was detected in independent qPCR 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] X [GAPDH corrective ratio]. The GAPDH corrective ratio was calculated as [lowest GAPDH copy number within sample set]/[GAPDH copy number for cell of interest].

Hematoxylin and Eosin Staining

Tissue specimens in formalin were sent for paraffin embedment, sectioning into 5μm slices and placement on slides. Upon receipt, the slides were then soaked in successive baths for five minutes at a time: xylene, xylene, 100% ethanol, 95% ethanol, 70% ethanol, deionized water. Following this, the sections were soaked for five minutes in Harris Hematoxylin, rinsed in deionized water. The slides were then soaked in bluing solution for one minute, (1.5mL NH4OH in 98.5mL of 70% ethanol), followed by rinsing in deionized water. The slides were soaked in

an Eosin bath for five minutes, after which were rehydrated in ethanol and xylene baths: 70% ethanol, 95% ethanol, 100% ethanol and xylene for five minutes each. Cover slips were then placed on the slides using Permount and dried under the fume hood for one hour. Sections were visualized using a Zeiss microscope and fields captured using Image Pro-Software (BioRad).

Immunohistology

Tissue specimens in formalin were sent for paraffin embedment, sectioning into 5μm slices and placement on slides. Upon receipt, the slides were soaked in successive baths for five minutes at a time: xylene, xylene, 100% ethanol, 95% ethanol, 70% ethanol, deionized water PBS. The slides were quenched by incubating in 0.5% H2O2 for 30 minutes, followed by rinsing for five minutes in PBS. Slides were probed with goat anti-hRAGE (1:500) and allowed to incubate in a humidified chamber overnight at 4°C. After a rinse in PBS for five minutes, slides were probed with anti-goat HRP (1:1000) and allowed to incubated in a humidifier for one hour at room temperature. The slides were then developed with 3'3'-daminobenzidine (DAB) substrate in a humidified chamber for 45 minutes. Slides were counterstained by washing in water for five minutes, dipping three times in hemotoxylin, followed by two successive five minute baths in distilled water. Slides were then rehydrated in ethanol and xylene baths: 70% ethanol, 95% ethanol, 100% ethanol and xylene for five minutes each. Cover slips were then placed on the slides using Permount and dried under the fume hood for one hour. Sections were visualized using a Zeiss microscope and fields captured using Image Pro-Software (BioRad).

CHAPTER 4
RESULTS

Currently, a total of 47 subjects have participated in this study. Thirty nine of which donated tissue samples consisting of epithelium and connective tissue (Fig. 2-2, 2-3). Thirty four (19 diseased, 15 healthy) of these samples were analyzed via PCR for gene expression of RAGE, TLR-2, as well as TLR-4. RAGE was chosen, as it is a marker allowing for measurement of exposure to exogenous and endogenous AGEs. TLR-2 and TLR-4 were chosen, as they represent innate immune response to lipopolysaccharide of particular bacterial species involved in periodontitis – *P. gingivalis and A. actinomycetemcomitans* respectively.

Patients that participated had a diagnosis chronic periodontitis (moderate or severe; localized or generalized) necessitating surgical intervention, or were considered periodontally healthy, undergoing an alternate procedure related to dental implantology or removal of healthy tissues for clinical crown lengthening or tissue wedge removal. Patients were considered having periodontitis if they met the disease classifications set forth by the American Academy of Periodontology. (13)

The demographics of the participant population are summarized in Table 1. Briefly, among the 19 subjects studied in the diseased population, there were 7 smokers and 12 non-smokers. In addition, 3 of these periodontally diseased participants were diabetic and 1 of these participants was a diabetic smoker. Males comprised 6 of the subjects, with 13 being females. With respect to the healthy population's 15 subjects, 2 were smokers, with no diabetics present in the periodontally healthy group. Five males and 10 females made up the healthy population.

Expression of Receptor for AGE in Periodontal Tissues

RAGE expression occurs in many different cell types, including epithelial, endothelial, as well as gingival fibroblasts. (4) Expression is a result of insult by endogenous, as well as

exogenous sources. The binding of RAGE to its ligand, AGE, triggers an immune response that involves release of pro-inflammatory mediators and cytokine release resulting in tissue damage. (28)

It has been hypothesized that diseased periodontal tissues express RAGE to a higher degree than periodontal tissues that are not diseased. To determine the validity of this hypothesis, qPCR was performed on 19 diseased and 15 healthy tissue samples harvested during periodontal surgeries. RAGE-specific primers were used to determine expression and GAPDH-specific primers were used as a normalization control. After the data was normalized for cDNA content, it was determined that there was a significantly higher (*p value* $\leq$0 .0004, Mann-Whitney) expression of RAGE in periodontally diseased tissues versus normal, healthy tissue samples (Fig. 4-1, 4-2). These analysis included tissues from diabetic participants and well as those from smokers. Both of these conditions have been shown to increase the expression of RAGE, therefore, we repeated the analysis in the absence of these participant samples. Here the difference in RAGE expression remained significant. (*p value* $\leq$0.0004, Mann-Whitney Test) (Fig. 4-4)

Expression of Toll-Like Receptor 2 in Periodontal Tissues

Toll-like receptors, upon binding to ligands, signal the transcription of many pro-inflammatory cytokines. Again, TLRS are expressed in many of the cells associated with the periodontium and periodontal lesions. TLRs have also been described to be up regulated in response to bacterial stimuli as seen in periodontal disease. In addition the LPS of periodontal pathogen *P. gingivalis* has been demonstrated to induce an immune response through ligation of TLR-2. Therefore, in order to elucidate as to whether periodontal disease classification correlated positively with TLR-2 expression qPCR was performed on tissue samples utilizing TLR-2-specific primers, normalized to GAPDH. After normalization, it was determined that

diseased tissues expressed TLR-2 to a lesser degree than healthy periodontal tissues (*p value* $\leq$0.0007, Mann Whitney Test) (Fig 4-1, 4-3). Again, after adjusting the analysis without smokers and diabetic patients the decreased expression of TLR-2 remained significant (*p value* $\leq$0.0007, Mann Whitney Test) (4-4).

Expression of Toll-Like Receptor 4 in Periodontal Tissues

Induction of expression of TLR-4 occurs in a similar manner to that of TLR-2, being expressed by multiple cell types within the periodontium, such as epithelial cells, fibroblasts, cementum, osteoblasts and osteoclasts. (5) In addition, the LPS of multiple periodontal pathogens such as *A. actinomycetemcomitans* and *Fusobacterium nucleatum* ligate TLR-4 inducing a pro-inflammatory response. (5)

Similar to TLR-2 expression experiments, our intent was to establish the relationship between periodontal health status and TLR-4 expression in periodontal tissues. Just as with here we used TLR-4 specific primers and qPCR with periodontally healthy and diseased tissues again normalizing to GAPDH. In these experiments, TLR-4 was shown to be up regulated in periodontally diseased tissues (*p value* $\leq$0.0001 via the Mann Whitney Test.) (Fig 4-1, 4-3) Adjustment to the data analysis by removing those with a smoking and/or diabetes status did not alter the significance of the up regulation of TLR-4 observed in periodontally diseased tissues (4-4).

Comparison of Expression Patterns for RAGE, TLR-2 and TLR-4

While the above experiments demonstrated that both RAGE and TLR-4 were up regulated in periodontally diseased tissues, it was not clear whether those patients with high levels of RAGE also had high levels of TLR-4. At the same time the inverse relationship of TLR-2 and TLR-4 expression by a given participant was not addressed. To answer these questions, we performed regression analysis to determine if there was a correlation between the expression of

RAGE, TLR-4 and TLR-2 within a given individual. Here we determined if RAGE expression was high in diseased tissues, TLR-2 expression was consistently low in the same individual (r=-0.7201) (Fig 4-5). Additionally, if RAGE expression was high, along with decreased TLR-2 expression TLR-4 expression was also relatively high (r=0.9964) (Fig 4-5). Therefore we can state that both increased RAGE and TLR-4 expression directly correlate with periodontal disease status. The opposite held true for TLR-2 expression, with high expression levels having an inverse relationship with periodontal disease status (Fig 4-5).

Analysis of Cell Types of the Periodontal Tissue Expressing RAGE

Utilizing histological analysis, we were able to determine that epithelial, endothelial, and fibroblasts, among other cells were included in the cell types analyzed for gene expression. In addition, mononuclear cells were found to be present in the diseased tissues as well. Hemotoxylin and Eosin stained sections (figures 4-6, 4-7) were prepared in order to determine typical cellular contents of samples taken. These cell types were identified in healthy, as well as diseased tissues.

Additionally, RAGE expression was qualitatively determined by utilization of immunohistochemistry. Analysis of expression in healthy and diseased tissues revealed relatively little expression in the healthy segment of tissues. (Figure 4-8) The diseased tissues, however, demonstrated strong expression in areas of many cell types, including epithelial, endothelial, fibroblast and macrophage cells. (Figure 4-9) RAGE protein expression was also qualitatively up regulated on most cells types, not just the immune infiltrate.

Table 4-1. Demographic data of participant population. Table indicates sex, smoking status, diabetes status and number of participants that were diabetics in addition to smokers.

	Healthy (15)	Diseased (19)
Males/Females	5/10	6/13
Smokers/Non-Smokers	2/13	7/12
Diabetics/Non-Diabetics	0/15	3/16
Smoker+Diabetic	0	1
Age Range	18-57	25-70

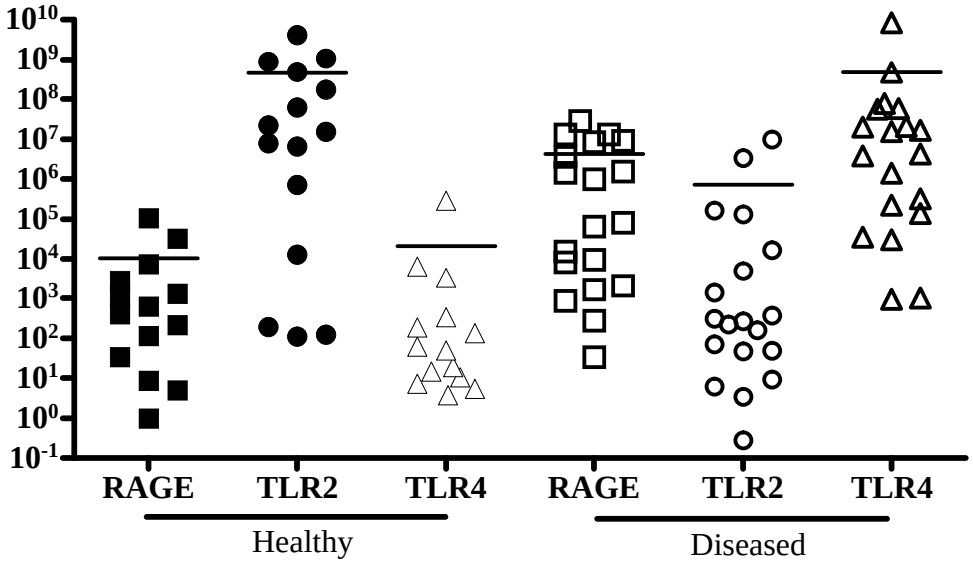

Figure 4-1. Quantitative gene expression in periodontal tissues. Periodontal tissues from 19 diseased (black) and 15 healthy (white) participants were subjected to qPCR. Gene expression for RAGE (squares), TLR-2 (circles) and TLR-4 (triangles) was normalized to GAPDH and copy number quantitated using amplicon specific standard curves. *p value < 0.0004 RAGE healthy vs. diseased, # p value < 0.0007 TLR-2 healthy vs. diseased, ^p value <0.0001 TLR-4 healthy vs. diseased.

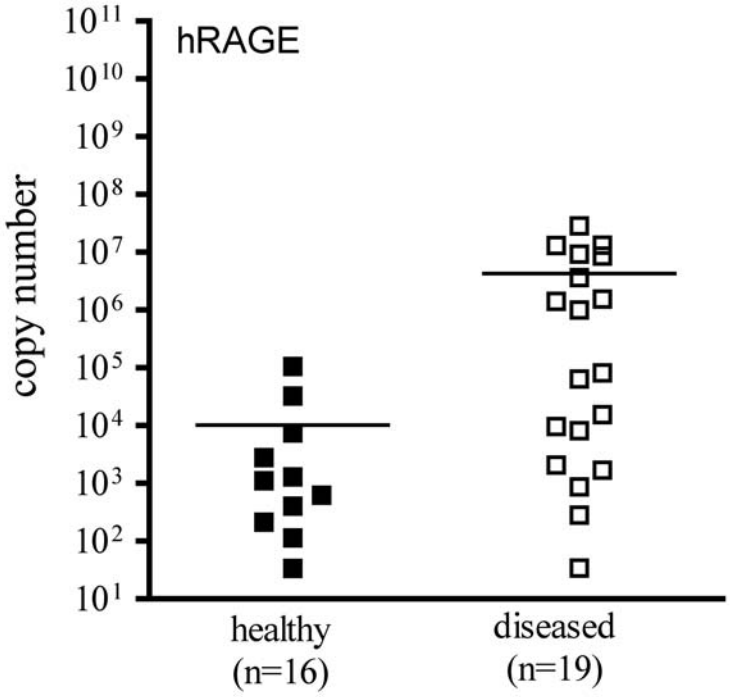

Figure 4-2. RAGE expression in healthy and diseased subjects. Differences between expression in healthy and disease subjects were significant ($p<.05$)

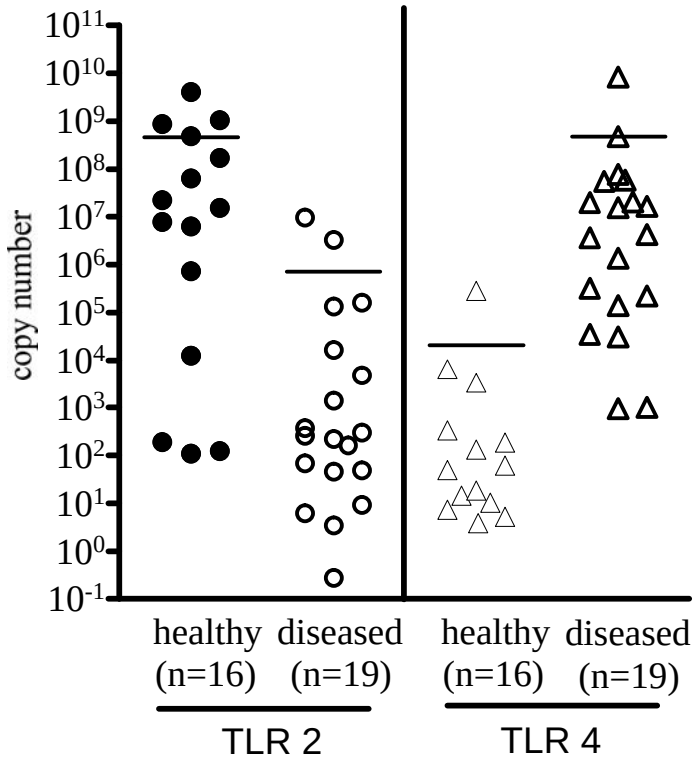

Figure 4-3. TLR-2 and TLR-4 expression comparison for healthy and diseased subjects. Differences between TLR-2 expression in healthy and diseased subjects was significant ($p<.05$). Differences between TLR-4 expression in healthy and diseased subjects was significant ($p<.05$).

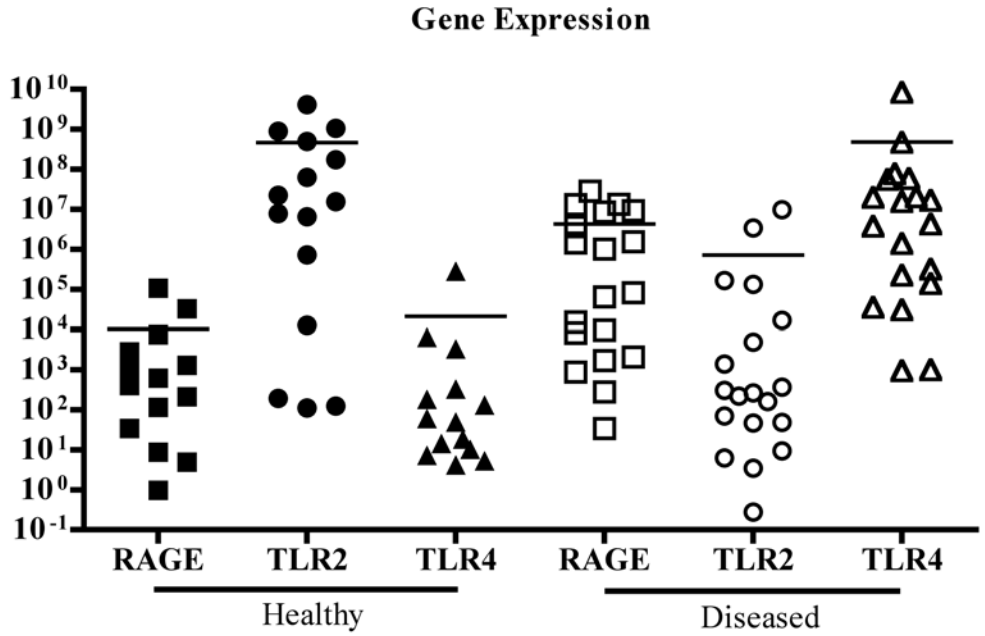

Figure 4-4. Gene expression in all subjects, excluding smokers and diabetics. Exclusion of smokers and diabetics yielded similar results, with differences among RAGE, TLR-2 and TLR-4 staying significant ($p<.05$) after exclusion.

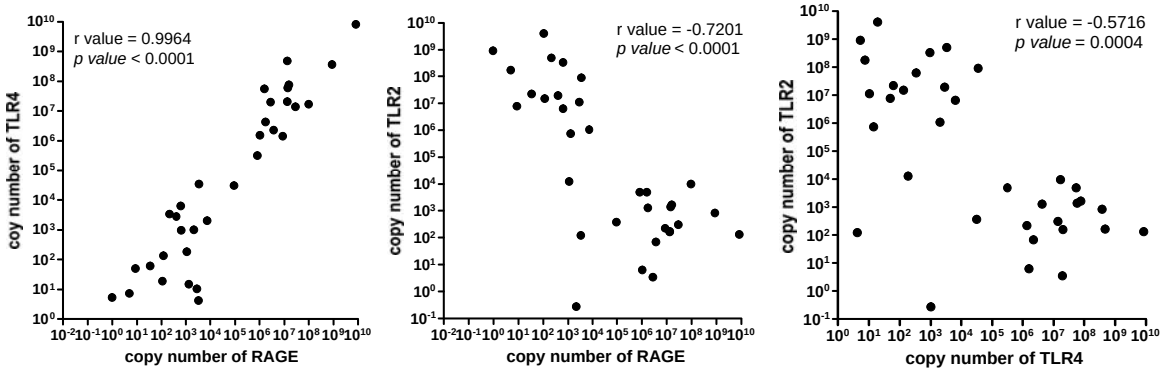

Figure 4-5. Correlation Data among RAGE, TLR-2 and TLR-4. RAGE-TLR-4 correlation r=0.9964, p<.0001. RAGE-TLR-2 correlation r=-0.7201, p<.0001. TLR-2-TLR-4 correlation r=-0.5716, p=.0004.

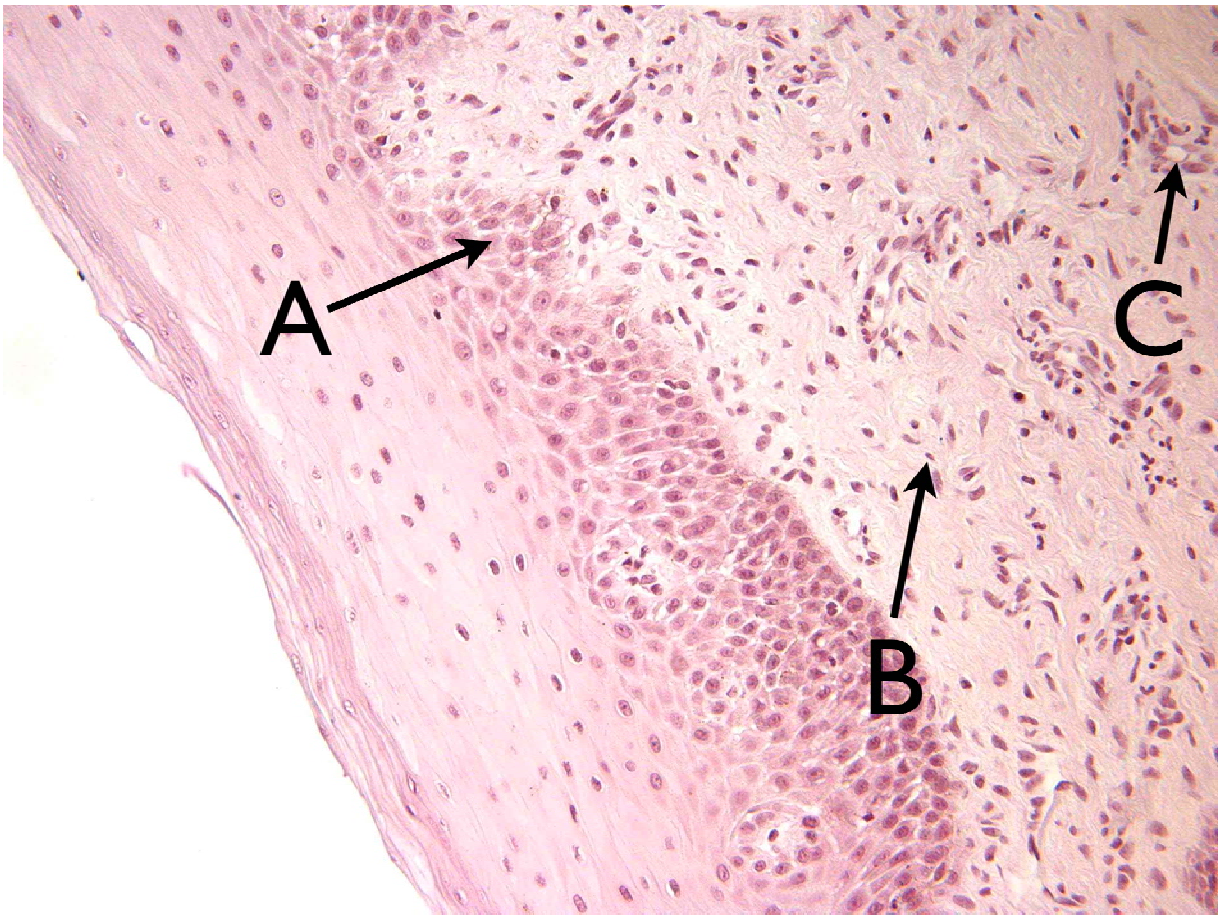

Figure 4-6. Histological examination of healthy periodontal tissue. Representative section of healthy tissue which was sectioned and stained with hemotoxylin and eosin for histological examination of cell types present in sample (n=5). Note the presence of epithelial cells (A), fibroblasts (B) endothelial cells (C) In addition, note the lack of inflammatory infiltrate and hyper-vascularity.

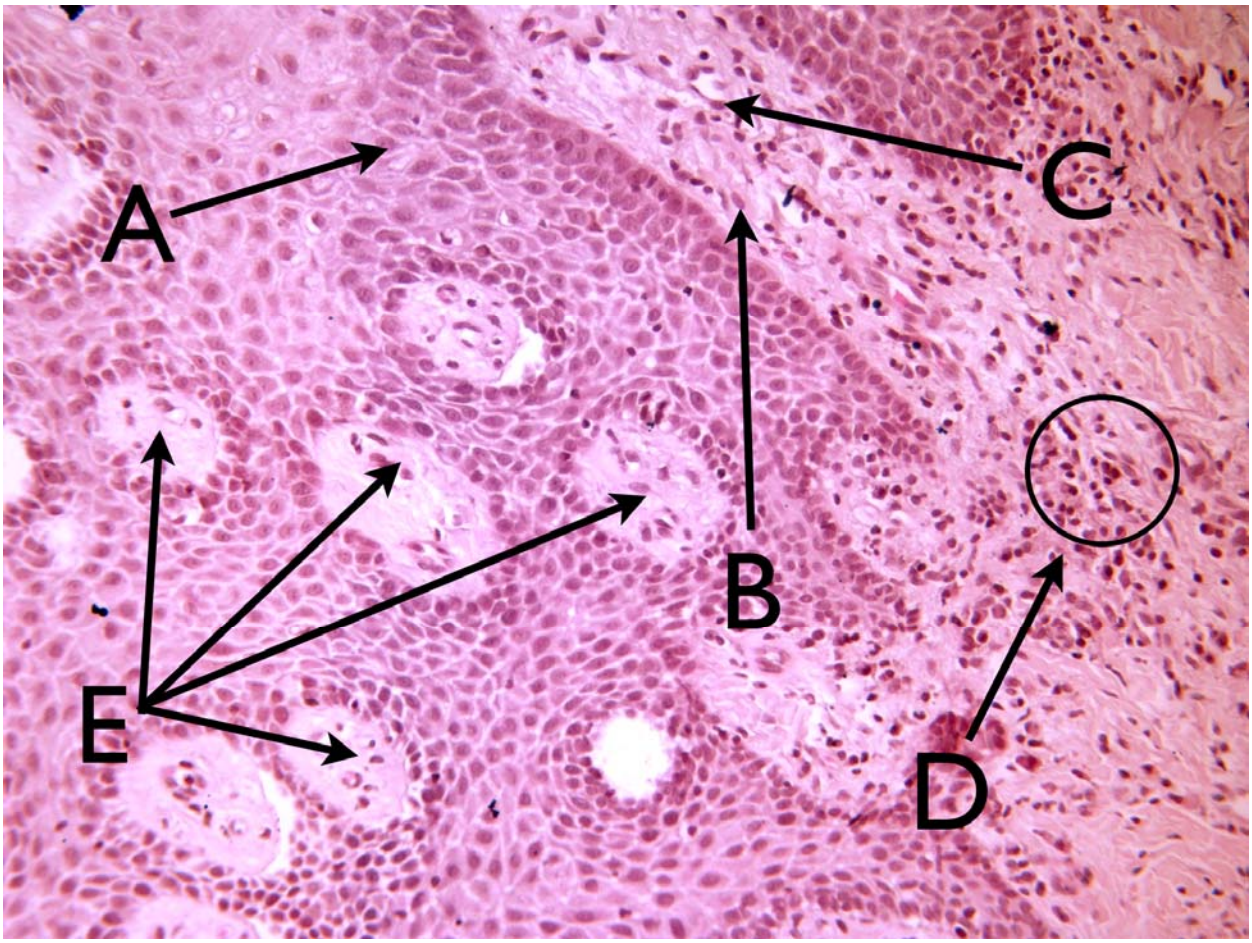

Figure 4-7. Histological examination of healthy periodontal tissue.. Representative section of diseased tissue which was sectioned and stained with hemotoxylin and eosin for histological examination of cell types present in sample (n=5). Note the presence of epithelial cells (A), fibroblasts (B), endothelial cells (C) macrophages (dark-staining cells). In addition, note the inflammatory infiltrate (D and Circle) invading connective tissue as well as the high degree of vascularity.(E).

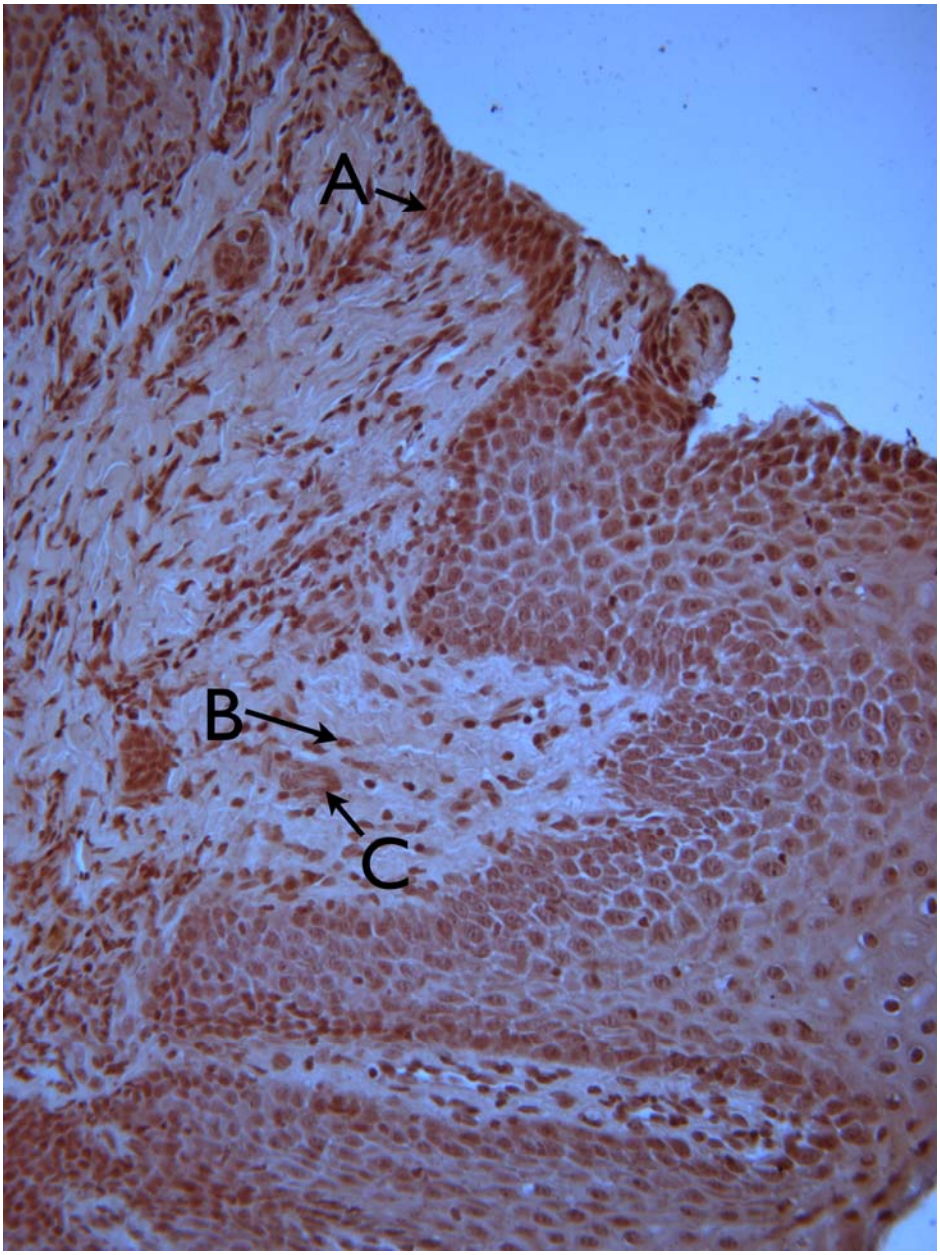

Figure 4-8. Immunohistology staining for RAGE in healthy periodontal tissues. Darker brown staining areas indicate RAGE expression. Expression is seen in epithelial cells (A), fibroblasts (B) and endothelial cells (C). Note distinct lack of heavy macrophage infiltration, as well as normal vascularity.

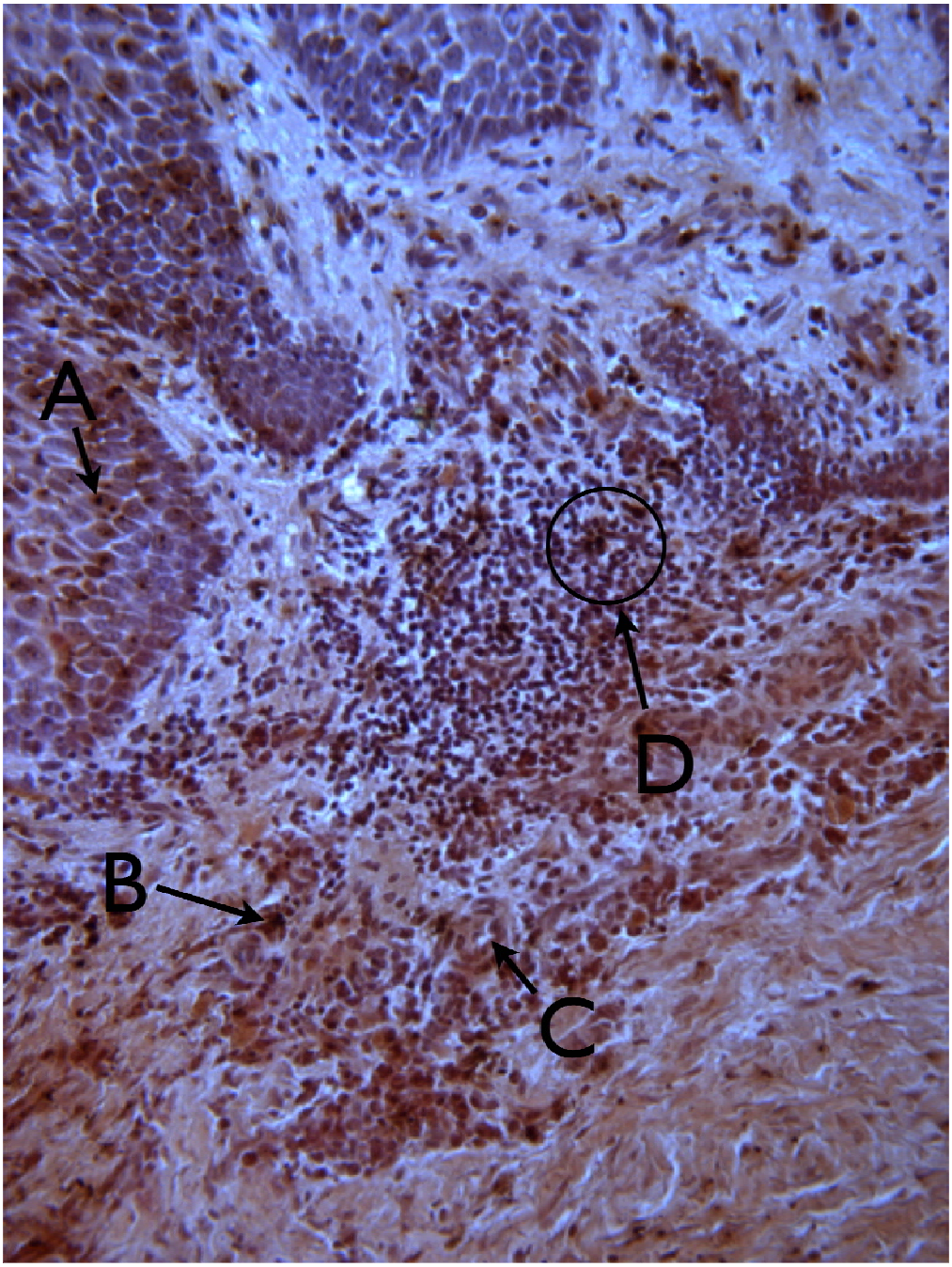

Figure 4-9. Immunohistology staining for RAGE in diseased periodontal tissues. Darker brown staining areas indicate RAGE expression. Expression is seen in epithelial cells (A), fibroblasts (B) endothelial cells (C) and macrophages (D and circle).

CHAPTER 5
DISCUSSION

Periodontal disease, as well as many other degenerative diseases, is characterized by tissue damage as a result of an inflammatory response. The goal of periodontal therapy is reduction of the inflammatory response by removal of offending pathogens and their products, which results in improved tissue health and increased support for the affected dentition.[40]

It was the intention of this investigation to quantify the expression of innate immune receptors commonly found to be up regulated as a result of periodontal disease, as well as expression of RAGE, which has been shown to contribute to a host of inflammatory-based diseases.[4] Utilizing qPCR, expression of innate immune receptors TLR-2 and TLR-4 and RAGE were quantified in peridontally diseased and periodontally healthy tissues. It was demonstrated that both tissue types expressed varying amounts of all three receptors studied (Fig. 4-1, 4-4). However, there were significant differences between expression for diseased and healthy tissues (Fig. 4-1, 4-4).

RAGE, which is capable of binding several different ligands, (AGEs, S100, HMGB1) was shown to be up regulated in periodontally diseased versus healthy tissues (Fig. 4-1, 4-2, 4-4). While it has been demonstrated that RAGE expression is increased in diseased gingival samples in the past, those studies involved patients with diabetes and/or smoking.[33] To our knowledge, this is the first demonstration of increased RAGE expression without confounding variables already shown to increase RAGE expression (Fig. 4-4).

Interestingly, TLR-2 did not follow the expression pattern that has been traditionally elucidated[5] (Fig. 4-3). In contrast to the expected increased expression in diseased tissues, a down-regulation was demonstrated in this population of patients (Fig. 4-3). Reasons for this difference are unknown at this time. However, recent work in our lab (Amir et al.) has shown an

anti-inflammatory response by gingival epithelial cells (GEC) when stimulated with LPS from *P. gingivalis*. This response resulted in the release of anti-inflammatory cytokines IL-4 and IL-10. This type of response does not lead to the clearance of pathogen and therefore may be the result of a bodily response to maintain homeostatic integrity in the face of dead bacterial components some of which maybe from commensal organisms consistently stimulating the epithelial component of the innate immune system. Therefore, in the face of an intense bacterial infection such as periodontal disease the immune system down-regulate those receptors, which, upon ligation result in anti-inflammatory responses, allowing for a proper immune response to occur. It would be interesting to determine if there is a relationship with state of disease progression and TLR-2 expression. One would hypothesize that once the bacterial insult has been controlled by treatment and the pro-inflammatory response is no longer needed, that TLR-2 expression levels may rise to again maintain homeostasis between commensal organisms and the innate immune response.

Unlike TLR-2 expression, , there was a significant and definitive increase in TLR-4 expression in diseased tissues as compared to periodontally healthy tissues (Fig. 4-1, 4-3, 4-4). The up regulation of TLR-4 in diseased periodontal tissues seems to be the prototypical response of the innate immune system[25] Here, TLR-4 ligation by PAMPs, which are highly conserved structures in many pathogenic bacterial species results in a robust pro-inflammatory response in order to immediately destroy and/or control the offending agent.. It has been hypothesized by many that TLR-4 and RAGE interact to augment and/or enhance inflammatory responses under certain conditions. Indeed recent research, has demonstrated a correlation between TLR-4 ligation and RAGE ligand interactions. Here, low density lipoprotein (LDL) AGEs were used to stimulate human coronary artery endothelial cells, as well as human and mouse macrophages.[41]

When TLR-4 loss of function was induced or TLR-4 binding was blocked by a competitive inhibitor, in mouse macrophages, cytokine synthesis was markedly reduced.[41] These results indicated that there may be an activation of TLR-4 signaling pathways by stimulation with AGE. In addition, HMGB1 a RAGE ligand and pro-inflammatory cytokine. implicated in cancers, mostly due to its pro-angiogenic factor, seems to potentiates the pro-inflammatory response through activation of NFkB, using a similar signaling pathway as TLR-2 and TLR-4, again resulting in a positive feedback mechanism that signals for other inflammatory mediators.[42] These data taken together coupled with the data presented here indicate there may be implications for RAGE and TLR interactions beyond the cardiovascular system and cancer, such as the dentogingival complex.

We were able to determine that epithelial, endothelial, fibroblast, etc. cells were included in the cell types analyzed for gene expression. Hematoxylin and Eosin stained sections were analyzed for the above cell types. It was determined that representative sections of healthy and diseased tissues contained the above cell types, regardless of disease status. However, there were heavy immune cell infiltrates present within the diseased tissues. (Figures 4-6, 4-7)

Immunohistology was also completed for patient samples. Healthy and diseased tissues were shown to express RAGE, although, diseased samples demonstrated a much higher intensity in certain locales. RAGE protein expression was also qualitatively up regulated on most cells types, not just the immune infiltrate. There appeared to be expression from all identifiable tissue types, however, the expression was not homogeneous per tissue type. The healthy tissues showed a more homogeneous expression profile, indicating normal, homeostatic expression, without distinct expression in any one area or tissue.

Correlation analysis was also completed with the data. It was confirmed that patients who exhibited an increased expression of RAGE also had increased expression of TLR-4 and a decreased expression of TLR-2.

What remains to be seen, however, is the interaction among AGE levels, the innate and adapted immune responses and chronic disease states. With increasing elucidation of the mechanisms at a cellular level by which the interactions occur, the question continues to remain as to whether there are implications for the body as a whole, and to what extent. AGE levels have documented effects on disease process such as cardiovascular disease, sequalae from diabetes and periodontal disease. The growing perception seems to point toward an interrelationship among all of these diseases and that there is an oral-systemic connection. In essence, that the presence of periodontal disease may have a causal effect for chronic inflammatory disease due to its contribution to the regulation of TLR and RAGE expression. At this point, the evidence is strong for association, but, not causality.

As expected, the receptor for AGE seems to be a likely participant in this relationship. RAGE has the capability to bind to several ligands (AGE, Calgranulin/S100, HMGB1), which is unique in terms of immune receptors. Additionally, RAGE is expressed in a variety of tissues, and systems, including endothelial, fibroblasts, macrophages, etc. Due to the ability for RAGE to bind to many ligands and its ubiquitous nature throughout tissues, coupled with the potential of the innate immune response, RAGE up regulation may hold the key to understanding many of the mechanisms chronic inflammatory diseases.

While quite a bit is known about RAGE expression and its association with inflammatory diseases, such as periodontal disease as described here, further research is certainly needed to elucidate the mechanisms of how it contributes to disease initiation and progression.

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BIOGRAPHICAL SKETCH

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