

EXPRESSION OF THE RECEPTOR OF ADVANCED GLYCATION
ENDPRODUCTS (RAGE) IN GINGIVAL TISSUES OF SMOKERS AND NON-
SMOKERS WITH GENERALIZED PERIODONTAL DISEASE

By

THOMAS YOUNG HO YOON

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by

Thomas Young Ho Yoon

This document is dedicated to my fiancée and future wife Dr. Judith Lim. Without your love, support and dedication none of this could have ever been possible.

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Abstract of Thesis Presented to the Graduate School
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Thomas Young Ho Yoon

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Chair: Herbert J Towle III
Major Department: Periodontics

Background: The association between smoking and periodontal disease is well established; however the mechanism by which smoking augments the destruction of periodontal tissue is not clear. We hypothesize that there is an increased expression of the receptor of advanced glycation endproducts (RAGE) in gingival tissues of smokers which mediates the tissue toxic effect of advanced glycation endproducts (AGEs).

Material and methods: Gingival biopsies from five smokers and five age and sex matched non-smokers were examined. RNA was extracted from the gingival tissues, reverse transcribed, amplified and run on agarose gel. The bands on the gels were viewed using ethidium bromide and band intensity was analyzed using Scion Image.

Results: For all 10 subjects, a band was observed at the 326 base pairs position, which is the predicted length of the RAGE PCR product. In all pairs, the smoker biopsies expressed a greater level of RAGE as compared to the matched non-smoker tissues.

When viewed as groups, analysis of the band intensity indicated that RAGE mRNA in

smokers was approximately 140 percent of the expression of that measured in nonsmokers (student's t-test, $p = 0.028$).

Conclusions: The presence of increased RAGE expression in human gingival epithelium of smokers with periodontal disease may indicate that AGEs are involved in the periodontal destruction associated with smoking.

CHAPTER 1 INTRODUCTION

There is ample evidence that associate smoking with the destruction of the periodontal apparatus. The exact mechanism by which tobacco products affects the periodontium is not well understood. Numerous reports have documented that smokers have a higher incidence and prevalence of periodontal disease than non-smokers.^{1,2} Smokers have been shown to exhibit different features detrimental to gingival health. It has been reported that smokers have an increased amount of the bacteria *Porphyromonas gingivalis* and *Bacteriodes Forsythus*.³ Other proposed mechanisms for negative periodontal effects of smoking include vascular alteration, altered neutrophil function, decreased IgG production, decreased lymphocyte proliferation, and altered fibroblast attachment and function.⁴

A direct link between tobacco use and the development of advanced glycation products (AGEs) has recently been established.^{5,6} Nor-nicotine, a nicotine metabolite found in high concentrations in the plasma of smokers, was shown to significantly induce the formation of AGEs. AGEs have in turn been shown to induce production of cytokines such as Interleukin 1 (IL-1), IL-6 a, tumor necrosis factor- α (TNF- α) and increase oxidative stress.

Recently we have shown that human gingival cells grown in tissue culture and exposed to 1 μ M nor-nicotine for 72 hours expressed approximately four-fold RAGE compared to non treated cells.⁷ We also hypothesize that AGEs and up regulation of

RAGE may be involved in the pathogenesis of periodontal disease associated with smoking.

The purpose of the present study was to investigate the hypothesis that the gingiva of smokers express elevated levels of RAGE compared to non smokers.

CHAPTER 2 MATERIALS AND METHODS

Gingival biopsy samples were obtained from 22 patients (10 smokers and 12 non smokers) with generalized chronic periodontitis. All subjects were patients of the University of Florida, College of Dentistry, Department of Periodontics. The study was approved by the institutional review board and each participant signed a consent form. Inclusion criteria for patients involved with the study were 1) generalized periodontal disease consisting of loss of attachment $\geq 30\%$ with bleeding upon probing in the remaining dentition 2) no other significant systemic disease 3) current smokers and, 4) those patients who have never smoked or used tobacco products. Smokers were defined as those patients who smoked greater than 100 cigarettes over their lifetime and smoked at the time of the clinical examination.⁸ The exclusionary criteria were 1) lack of periodontal disease 2) previous smokers who had quit 3) significant systemic disease which may have an impact on periodontal status such as diabetes (both type 1 and 2), arthritis (both rheumatoid and osteoarthritis), or any other systemic autoimmune disease, and 4) patients on long term medication.

Periodontal Therapy

All subjects were provided with initial periodontal therapy including oral hygiene instructions, scaling and root planing where necessary and re-evaluation. During the re-evaluation phase, patients were treatment planned for periodontal surgery as indicated for persistent disease. At the time of surgery, gingival tissues were obtained from the

deepest probing depth and immediately “snap frozen” at -70°C until processed for reverse transcriptase polymerase chain reaction (RT-PCR) (Figure 1).

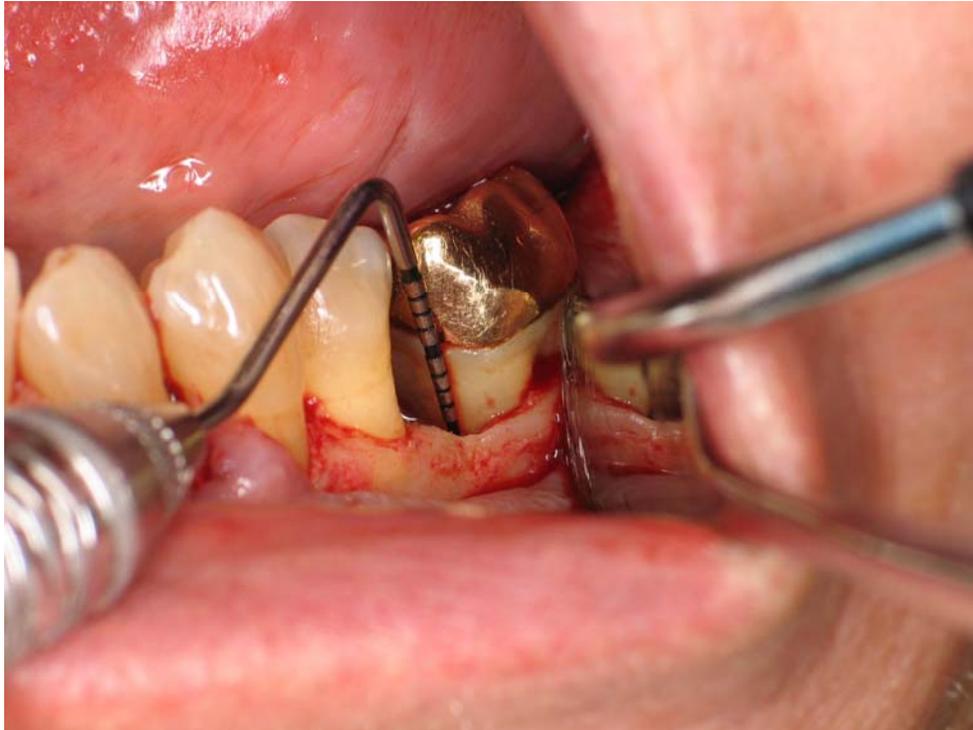


Figure 1. Apically positioned flap surgery depicting a site where the biopsy tissue was harvested

Sample Selection and Matching

One sample was excluded because an informed consent was not obtained. Another sample was excluded because the patient admitted to a previous smoking history after the biopsy was harvested. Five pairs of age and sex matched pairs, which only differed in smoking status, were subsequently chosen for analyses (table 1-1 and 1-2).

Table 1-1. Patient Data Smokers

Pt ID#	Age	Sex	Smoking Status	Type of Procedure	Attachment Loss
1S	60	Male	2ppd x 40yrs (80 pack yrs)	Multiple Extractions	4-6mm
2S	76	Male	2ppd x 55yrs (110 pack yrs)	Multiple Extractions	4-6mm
3S	47	Male	1ppd x 30 yrs (30 pack yrs)	APF With Osseous	4-6mm
4S	57	Female	0.5 ppd x 30yrs (15 pack yrs)	APF With Osseous	4-6mm
5S	62	Female	1ppd x 40yrs (40 pack yrs)	APF With Osseous	4-9mm

Table 1-2. Patient Data Non-Smokers

Pt ID#	Age	Sex	Type of Procedure	Attachement Loss
1S	55	Male	APF With Osseous	4-6mm
2S	77	Male	Single Extraction	3-6mm
3S	43	Male	APF With Osseous	3-6mm
4S	58	Female	APF With Osseous	4-6mm
5S	65	Female	APF With Osseous	5-8mm

RNA Extraction

The presence of mRNA was determined by RT-PCR using a housekeeping gene as a standard. The gingival tissues were thawed and RNA was extracted using a QiaGen RNeasy Mini Kit (QiaGen RNeasy Mini Kit, Qiagen Inc., Netherlands). The DNA was digested by using the QiaGen RNase-Free DNase Kit (QiaGen RNase-Free DNase Kit, Qiagen Inc., Netherlands). Twenty mg of sample was homogenized in 350ul of RLT Buffer with 1% b-Mercapthoethanol. The resulting solution was then centrifuged at 12000g for 3 minutes in order to produce a supernatant. The supernatant was collected

and transferred to a new tube and 350ul of 70% ethanol was added. The resultant sample was placed in an RNeasy mini column and centrifuged at 8000g for 15 seconds. The column was washed with 400ul of RW1 Buffer and also centrifuged at 8000g for 15 second. A solution of 10ul of DNase I and 70ul of RDD buffer was placed in each spin column and incubated at room temperature for 15 min. The spin column was then washed with 350ul of RW1 buffer once and with 500ul of RPE buffer twice. Finally the RNA was eluted by placing 50ul of RNase-Free water directly in the silica-gel membrane and centrifuged for 1 minute at 8000g. The collected RNA was then stored at -70°C .

RNA Quantification

The quantity of the RNA isolated was determined by measuring the absorbance at 260nm. The purity was determined by the 260nm/280nm absorbance ratio. The minimal acceptable ratio considered was >1.75 before the RNA will be used for RT-PCR.

RT-PCR

The RT-PCR was performed using Promega Access RT-PCR System (Promega Access RT-PCR System, Promega Inc., Madison, WI). The RAGE primers used (forward 5'- GACTCTTAGCTGGCACTTGGAT-3' and the reverse 5'- GGACTTCACAGGTCAGGGTTAC-3'), yielded a 326bp product. In a 0.5ml tube 10ul AMV/Tfl 5X Reaction buffer, 1ul 0.2mM dNTP Mix, 50pmol forward primer, 50pmol reverse primer, 2ul 25 mM MgSO₄, 1ul (5u/ml) AMV Reverse Transcriptase, 1ul (5u/ml) Tfl DNA Polymerase, and 1ug of the isolated RNA were combined and brought to a volume of 50ul with RNA-Free distilled water. The first strand of cDNA synthesis was obtained by 1 cycle of 45 min at 48°C , followed by 1 cycle of 2 min at 92°C . The solution was then thermocycled at intervals of 95°C for 12 minutes, then 35 cycles of one minute at 94°C , one minute at 55°C , one minute at 72°C . The products of the RT-PCR are then

separated by agarose gel electrophoresis and viewed with ethidium bromide and UV light. The bands were quantified using densitometry and statistically analyzed utilizing the student's t-test.

CHAPTER 3 RESULTS

The RNA was extracted from the gingival tissues, reverse transcribed, amplified and run on agarose gels as previously described. The bands on the gels were viewed using ethidium bromide and band intensity was analyzed using Scion Image (Scion Image, Scion Corporation, Gaithersburg, MD). For all 10 subjects a band was observed at 326 base pairs, which is the predicted length of the RAGE PCR product. After adjusting for age and gender subjects were matched in pairs (smoker and non-smoker). In all pairs the smoker biopsy expressed a higher level of RAGE compared to the matched non-smoker. When viewed as groups, analysis of the band intensity indicated that RAGE mRNA in smokers was approximately 140 percent of the expression in nonsmokers (student's t-test, $p = 0.028$) (Figure 2). These data suggest that smoking increases RAGE production in the oral cavity.

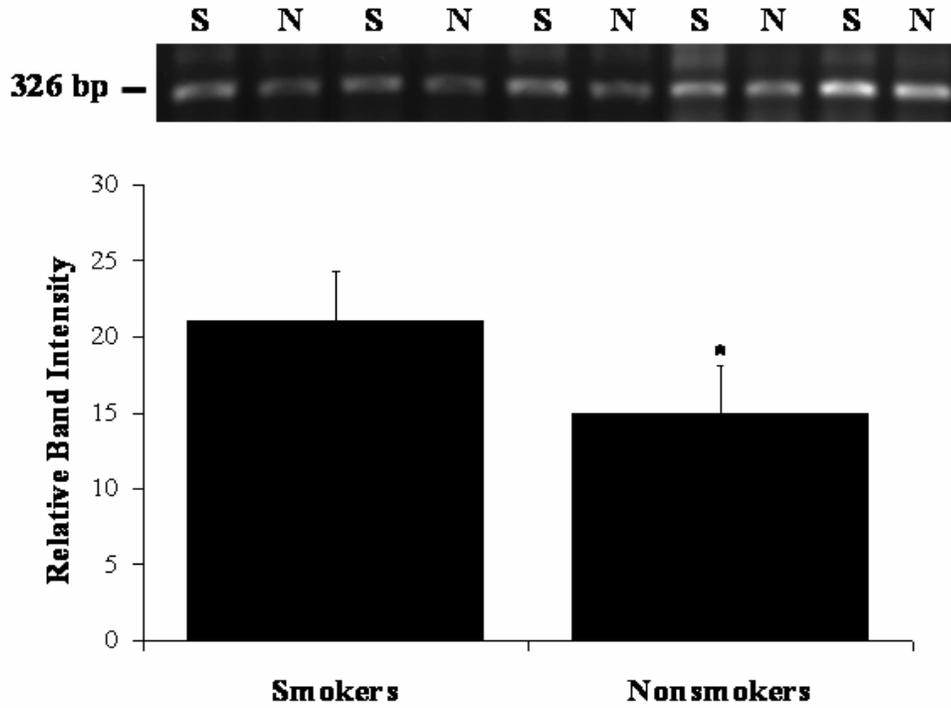


Figure 2. Expression of RAGE mRNA in the gingiva of smokers and nonsmokers. The top image demonstrates the RT-PCR results for RAGE mRNA for smokers and matched nonsmokers (S = smoker, N = nonsmoker). The graph is the summarized RT-PCR band intensity data for all ten subjects. The asterisk indicates $p = 0.028$, student's t-test.

CHAPTER 4 DISCUSSION

In the present study we have demonstrated that after adjusting for age and gender, all five gingival biopsies from smokers expressed higher levels of RAGE compared to the non-smokers. These findings are in accordance with our previous study where we have shown in vitro that gingival fibroblasts preincubated with nor-nicotine, a by product of cigarette smoking, expressed a higher level of RAGE compared with non treated cells. The gingival fibroblasts that were exposed to nor-nicotine were at levels that are similar to physiologic levels in smoker's serum.⁷

Biochemistry of AGE and RAGE

The receptors for AGE (RAGE) are cell surface receptors which are capable of producing a pro-inflammatory response. RAGEs have been shown to be expressed by a variety of cell types including endothelial and smooth muscle cells, lymphocytes, monocytes, and neurons. It has been shown in the literature by Hofmann et al. that once activated by AGE, RAGE can trigger cellular activation with generation of key pro-inflammatory mediators. It has also been shown in the same study that blockade of RAGE can halt delayed-type hypersensitivity and inflammatory states by arresting the activation of central signaling pathways and expression of inflammatory gene mediators.¹⁰ The binding of AGE to RAGE stimulates expression of RAGE itself, and generates oxidative stress, synthesis and secretion of pro-inflammatory cytokines, and chemotaxis.^{9,10}

AGEs are the final end product of non-enzymatic glycation and oxidation of proteins found in plasma and tissues. AGEs are biologically active and may initiate a range of cellular responses including stimulation of monocyte chemotaxis, osteoclast-induced bone resorption, proliferation of vascular endothelial and smooth muscle cells, aggregation of platelets, and stimulation of secretion of inflammatory cytokines, collagenase, and several growth factors.¹¹

AGEs can be either external or internal in origin. Internal sources include certain systemic conditions such as diabetes, Alzheimer's disease, and uraemia.^{12,13,14} External AGEs are produced by the combustion of nicotine in cigarette smoke. Glycation products of cigarette smoke are likely to originate during the curing of tobacco via Maillard-type reactions and have been proposed to confer aroma and flavoring to tobacco products.¹⁵

AGE and RAGE as a Possible Link to Periodontal Disease

Recently it was demonstrated that AGEs can induce apoptosis in fibroblasts. In a study performed by Alikhani et al. the predominant AGE of skin (CML-collagen) induced fibroblast apoptosis in vivo. In vitro experiments demonstrated that CML-collagen induced a time and dose-dependent increase in fibroblast apoptosis when compared to control collagen.¹⁶ It is possible that a similar pattern of fibroblast apoptosis could be found in gingival fibroblast if exposed to AGEs.

AGE deposition and RAGE expression has been associated with other risk factors for periodontal disease.¹⁷ In a study performed by Lalla et al. it was discovered that there is increased formation and deposition of AGEs in the gingiva of diabetic mice when inoculated with either *P. gingivalis* or a placebo control.¹⁸ It was shown however in a later study that periodontal disease in the same murine model was arrested by

administration of soluble RAGE, probably by inhibiting the receptor for interacting with AGE.¹⁹ We have recently examined human diabetic patients with periodontal disease and compared them to age and sex matched controls. Our results showed that the gingiva of diabetic patients with periodontal disease exhibit a higher presence of RAGE when compared to their controls.¹¹ These results suggested that an increased amount of RAGE may contribute to the advanced periodontal destruction commonly found in diabetics.

Conclusion

The exact mechanism of how AGEs activation of its receptor in the gingiva during smoking results in the destruction of the periodontal apparatus is unknown. Smoking promotes the production of AGEs that stimulate increased signaling through their receptors (RAGE) in the gingiva. This may trigger the pro-inflammatory effects of AGEs by stimulating secretion of cytokines that promote destruction of the periodontal apparatus. Since AGEs induce apoptosis in fibroblasts the possibility that apoptosis is involved in the smoking aggravated periodontitis, should be investigated.

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BIOGRAPHICAL SKETCH

Dr. Thomas Young Ho Yoon obtained his BA and DDS from the University of Missouri at Kansas City six year advanced BA/DDS program. After graduation in 2001 he successfully completed a General Practice Residency at Barnes Jewish Hospital in Saint Louis, Missouri. Wanting to further pursue the surgical arts, Dr. Yoon then attended and completed an internship in oral and maxillofacial surgery at the University of Florida. He finished his surgical training with the Department of Periodontology where he served as their chief resident during his final year. Currently, Dr. Yoon practices periodontics in Winter Park, Florida, and maintains a part-time faculty associate position at the University of Florida, Department of Periodontology.