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Expression of the receptor of advanced glycation end products in gingival tissues of type 2 diabetes patients with chronic periodontal disease: a study utilizing immunohistochemistry and RT-PCR

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Abstract

Objectives: Relationship between diabetes and periodontal disease is well established. It has been shown that advanced glycation end-products (AGEs) might exert noxious effects on gingival tissues through its receptor. Evidence for the role of receptors of AGE (RAGE) in periodontal disease was verified in a murine model for diabetes. However, the presence of RAGE in human gingival tissues has not been demonstrated previously. In this study we demonstrate the presence of RAGE in human periodontium in patients with chronic periodontitis with and without type 2 diabetes.

Material and Methods: Gingival biopsies from eight patients with both type 2 diabetes and chronic periodontitis and 14 healthy control subjects with chronic periodontitis were immunohistochemically stained for RAGE. Five samples from the study groups and four controls were subjected to reverse transcriptase coupled to polymerase chain reaction (RT-PCR) for quantitative determination of mRNA for RAGE.

Results: On immunohistochemistry, positive staining for RAGE was seen in the endothelium and the basal and spinous layer of the inflamed gingival epithelium in both type 2 diabetes and non-diabetes tissue with no statistically significant difference between both groups. RT-PCR, however, showed a 50% increase in mRNA for RAGE in the gingiva of diabetic patients when compared with controls ($p < 0.05$).

Conclusions: Although there was no change in the staining intensity for RAGE between both groups, the increase in the mRNA for RAGE in the type 2 diabetes gingival epithelium may indicate a possible involvement of this receptor in the periodontal destruction in type 2 diabetes.

Key words: gingiva; receptor of advanced glycation end products; type 2 diabetes

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There is ample evidence in the periodontal literature associating diabetes and periodontal disease; however, the exact biological mechanism responsible for this link is unclear (Taylor et al. 1996). Advanced glycation end products (AGEs), the final products of non-enzymatic glycation and oxidation of proteins, are found in the plasma and accumulate in the tissues during both aging and at an accelerated rate in diabetes (Stern et al. 2002). The formation

of AGEs is irreversible and the degree of modification correlates with the life span of the modified protein. AGEs are biologically active and may initiate a range of cellular responses including stimulation of monocyte chemotaxis, osteoclast-induced bone resorption, proliferation of vascular smooth muscle cells, aggregation of platelets, and stimulation of secretion of inflammatory cytokines, collagenase, and several growth factors. The biological effect of AGEs is mediated, at least partly, by the receptor of advanced glycation end-products (RAGE) (Li & Schmidt 1997). An integral membrane protein, RAGE, forms a central part of the cell surface binding site for AGEs (Hofmann et al. 1999). RAGE is a multiligand, signal transduction receptor belonging to the immunoglobulin superfamily and it is expressed by a variety of cell types including endothelial cells, smooth muscle cells, lymphocytes, monocytes, and neurons. Binding of ligands to RAGE stimulates expression of RAGE itself, and generates oxidative stress, synthesis and secretion of pro-inflammatory cytokines, and chemotaxis (Brett et al. 1993, Hofmann et al. 1999, Tanaka et al. 2000). RAGE was shown to be induced by AGEs and TNF-α (Tanaka et al. 2000). Thus, activation of RAGE propagates a chronic inflammatory disease state, which may further support the generation of AGEs.

Using immunohistochemistry, RAGE has been demonstrated in vivo in the vasculature including endothelial cells, smooth muscle cells, and mononuclear cells, as well as in other cells including a variety of epithelial cells (Brett et al. 1993). AGEs and RAGE are often concentrated in joints of subjects with rheumatoid arthritis and amplify the immune/inflammatory response in this disease process (Hofmann et al. 2002). It was demonstrated that human synovial fibroblasts constitutively expressed RAGE mainly in synovial intima (Basta et al. 2002). These studies have suggested that AGEs may form in inflammatory foci, as a result of oxidation or activated myeloperoxidase pathway (Basta et al. 2002). AGEs through RAGE may prime pro-inflammatory mechanisms in endothelial cells thereby amplifying inflammatory effects in atherogenesis and chronic inflammatory disorders (Hofmann et al. 2002). Furthermore, RAGE induces the expression of various cytokines and growth factors to mediate the trans-differentiation of epithelial cells to form myofibroblasts and the loss of epithelial E cadherin staining (Oldfield et al. 2001). The link between AGEs and the chronic process of inflammation has also been mentioned in relation to periodontal disease in diabetic patients and animal models for periodontal disease; however, to our knowledge, the presence of these receptors in the human gingiva has not been demonstrated.

The purpose of this study was to demonstrate the presence of RAGE in human gingival tissues in type 2 diabetics and non-diabetic subjects with chronic periodontal disease

Material and Methods

Thirty patients participated initially in the study. Fifteen patients with type 2 diabetes mellitus and 15 age- and sex-matched non-diabetic patient controls attending the Departments of Periodontics at the University of Florida, College of Dentistry.

The study was approved by the institutional review board and each participant signed a consent form.

All periodontal evaluation was performed by one periodontist according to a uniform classification (Armitage 1999). Inclusion criteria for the study were (1) well-controlled type 2 diabetes, defined by fasting blood glucose levels of <126 mg/dl as reported by the patient (2) generalized periodontal disease consisting of loss of attachment $\geqslant 30\%$ with bleeding upon probing in the remaining dentition, and (3) no other significant systemic disease. The exclusionary criteria were (1) uncontrolled type 2 diabetes, (2) lack of periodontal disease, and (3) significant systemic disease. The protocol included a complete medical history, baseline periodontal recordings and full-mouth radiographs. All subjects were then treated with initial preparatory care consisting of instruction in proper plaque control, root planning and scaling with anesthesia where indicated, and dental prophylaxis. Following a 6-week period, they were re-evaluated; the study group and only 26 patients that required periodontal surgery with active periodontal inflammation were retained for the study. At the time of surgery, tissues harvested from the circumferential tooth area were retrieved and either immediately placed in 10% neutral buffered formalin for immunohistochemistry or snap frozen and kept in -70°C until processed for reverse transcriptase coupled to polymerase chain reaction (RT-PCR).

Immunohistochemistry

Paraffin-embedded blocks of gingival biopsies were sectioned at 5 μm, blocked with acetone for 5 min, and then incubated with a 1:400 dilution of antibody to RAGE (goat anti-human R&D, Flandres, NJ, USA). A horseradish peroxidase anti-goat system (R&D) was used according to the procedure recommended by the manufacturer. RAGE had been previously shown to be stained immunohistochemically in formalin-fixed, paraffin-embedded tissues. For negative controls, gingival sections were incubated with goat anti-human IgG1 as primary antibody. For positive control human connective tissue with demonstratable small blood vessels was used. Endothelial cells were found to stain positively for RAGE. Staining intensity was graded as strong (+++), moderate (++), or weak (+) according to concentration and distribution of the receptor in the sectioned tissues (percentage of positively stained cells) (Markopoulos et al. 2000).

RT-PCR: Out of the 26 patient samples, we had enough tissue in only five samples from type 2 diabetes patients and four samples from controls for both immunocytochemistry and RT-PCR studies.

RNA extraction

RNA was extracted from the cultured cells or from tissue using QiaGen RNeasy Mini Kit (QiaGen Inc., Valencia, CA, USA) and the DNA was digested using the QiaGen RNase-Free DNase Kit (QiaGen). Twenty milligrams of sample was homogenized in 350 μl of RLT buffer with 1% b-mercapthoethanol, then centrifuged at $12{,}000 \times g$ for 3 min. The supernatant was transferred to a new tube and 350 μl of 70% ethanol was added. The sample was placed in a RNeasy mini column and centrifuged at $8000 \times g$ for 15 s. The column was washed with 400 μl of RW1 buffer and also centrifuged at $8000 \times g$ for 15 s. A solution of 10 μl of DNase I and 70 μl of RDD buffer was placed in each spin column and incubated at room temperature for 15 min. The spin column was then washed with

350 μl of RW1 buffer once and with 500 μl of RPE buffer twice. Finally the RNA was eluted by placing 50 μl of RNase-free water directly in the silica-gel membrane and centrifuged for 1 min at 8000 × *g*. The collected RNA was stored at –70°C.

RNA quantification

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

RT-PCR

Semi-quantitative RT-PCR was performed using Promega Access RT-PCR System (Promega US, Madison, WI, USA). The RAGE primers used (forward 5′-GACTCTTAGCTGGCACTTGGAT-3′ and the reverse 5′-GGACTTCA-CAGGTCAGGGTTAC-3′) yielded a 326 bp product. In a 0.5 ml tube, 10 μl AMV/Tfl 5 × reaction buffer, 1 μl 0.2 mM dNTP mix, 50 pmol forward primer, 50 pmol reverse primer, 2 μl 25 mM $MgSO_4$, 1 μl (5 U/ml) AMV reverse transcriptase, 1 μl (5 U/ml) Tfl DNA polymerase, and 1 μg of the isolated RNA were combined and brought to a volume of 50 μl with RNA-free distilled water. The first strand of cDNA synthesis was obtained by one cycle of 45 min at 48°C, followed by one cycle of 2 min at 92°C. The solution was then sequentially thermocycled at 95°C for 12 min, then 35 cycles for 1 min at 94°C, 1 min at 55°C and 1 min at 72°C. The products of the RT-PCR were then separated by agarose gel electrophoresis and viewed with ethidium bromide and UV light. The bands were quantified using densitometry and statistically analyzed.

Results

Immunohistochemistry

Out of 26 subjects undergoing periodontal surgery, only 22 samples (eight diabetes and 14 non-diabetes) were found to demonstrate adequate amounts of epithelium and connective tissue bands and were included in the study for evaluation and analysis. The negative controls did not stain. The positive control section from human connective tissue specimen showed positive staining of endothelial cells lining the small blood vessels (Fig. 1A). All studied sections stained positive for RAGE. Seven out of eight sections from diabetic patients were strongly positive (III). One out of eight specimens from diabetic patients was moderately stained (II). Eight out of 14 from non-diabetic patients were strongly positive (III), and six out of 14 were moderately positive (II). The staining was positive for cell membranes for both the epithelial and endothelial cells. In the epithelium, the basal and spinous cell layers were significantly stained (Fig. 1B). Data was analyzed via analysis for linear trend in proportions for diabetes and staining intensity, and their interaction with statistical significance at the 5% level. The epidemiology information (EPI INFO) software program was used to determine statistical significance. Because of the immunohistochemistry study failed to show a statistical difference between the staining intensity between diabetes and control groups ($p = 0.15$), the sections were subjected to a RT-PCR quantitative analysis of mRNA for RAGE.

RAGE mRNA expression in periodontal tissues of diabetic patients

RT-PCR was used to evaluate the expression of RAGE in gingival tissue collected from five diabetic and four non-diabetic patients. The RT-PCR product was electrophoresed on agarose gels and viewed with ethidium bromide. The bands were photographed (Fig. 1C) and band density was measured using the image analysis software Scion Image (Scion Inc., Gaithersburg, MD, USA). Fig. 1C demonstrates that samples from diabetic patients had a 50% higher levels of RAGE mRNA expression than those from non-diabetic patients ($p < 0.05$).

Discussion

In the present study by using immunohistochemistry, we have demonstrated the expression of RAGE on the gingival tissues of patients with chronic periodontitis with or without type 2 diabetes. RAGE was present on the small blood vessels in the lamina propria, but more significantly on the basal membrane layer of the epithelium and stratum spinosum. The staining was membranous, the type 2 diabetes samples stained more intensely than those from healthy subjects, although statistical significance could not be found. This finding was further confirmed by the significant increase in mRNA for RAGE in the diabetic gingiva as demonstrated by the RT-PCR.

The relationship between glucose levels and periodontal disease is well documented (Katz et al. 2000). Grossi & Genco (1998) proposed a model for the bi-directional relationship between periodontal disease and diabetes mellitus. In their hypothesis, periodontal infection produces whole-body insulin resistance resulting in hyperglycemia (Grossi & Genco 1998). It has been hypothesized that accumulation of AGEs and enhanced expression of RAGE may contribute to the exaggerated tissue injury (Zhou et al. 2003). As a result of long-term hyperglycemia there is formation of AGE products that leads to the accumulation of AGEs in the vessel wall. This has been implicated in the pathogenesis of diabetic complications (Brownlee et al. 1988). In diabetic tissues, expression of the receptor for AGEs (RAGE) is enhanced in a manner overlapping with that of its ligands (Brownlee et al. 1988). An important role for RAGE in excessive cellular activation, and enhanced pro-inflammatory pathways in diabetic tissues was evident by the findings that blockade of RAGE suppressed vascular hyperpermeability in diabetic rats and atherosclerotic lesion development in diabetic apoE-null mice (Zhou et al. 2003). In parallel with those observations, indices of endothelial cell activation and macrophage migration/function were suppressed in the presence of RAGE blockade (Vlassara et al. 1994). Recent observations that AGEs and RAGE together with some inflammatory markers form in many states, such as atherosclerotic lesions, renal failure, and aging, have suggested that the biology of RAGE and its ligands extends beyond diabetes (Vlassara et al. 1994). RAGE also binds S100/calgranulins, members of a family of pro-inflammatory cytokines (Hofmann et al. 1999). S100/calgranulins binding may lead to ligation of RAGE-bearing cells in the injured/inflamed milieu, thereby providing a mechanism for sustaining cellular perturbation and tissue injury (Hoffmann et al. 1999). The result is generation of AGEs, leading to functional changes of many blood proteins. As a result, AGE proteins amplify the magnitude of

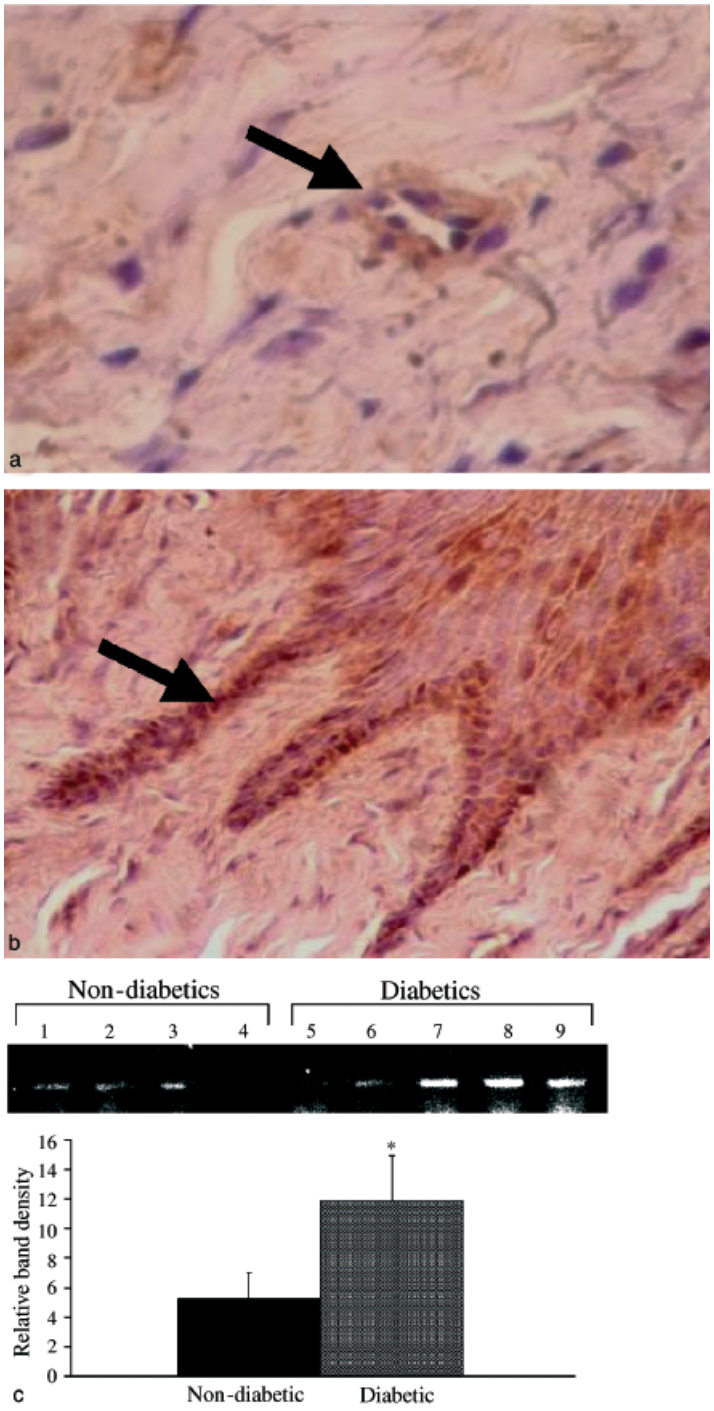

Fig. 1. (a) Positive control section. Human connective tissue subjected for receptor of advanced glycation end products (RAGE) staining. Note positive staining of endothelial cells on a small blood vessel (arrow). Final magnification × 125. (b) Gingival tissue from patient with chronic periodontitis and type 2 diabetes, positive staining for RAGE. Note staining of the epithelial basal and spinous layers as well as endothelial cells of small blood vessels in the superficial lamina propria (arrow). Final magnification × 125. (c) RAGE mRNA in diabetic and non-diabetic gingival tissue expressed as relative band density. Total RNA was isolated from gingival tissue from four non-diabetic and five diabetic periodontal patients. Reverse transcriptase coupled to polymerase chain reaction was performed on the RNA using the primers for RAGE (forward 5′-GACTCTTAGCTGGCACTTGGAT-3′ and the reverse 5′-GGACTTCACAGGTCAGGGTTAC-3′). These primers yield a product of 326 bp. The products were electrophoresed on agarose gels and viewed with ethidium bromide (top of figure). The gel bands were then quantified by densitometry (bottom graph). Asterisk indicates $p < 0.05$, Student's *t*-test when compared with non-diabetic patients.

macrophage cytokine response, which further may amplify the periodontal disease. In this model, a self-feeding perpetuation system of catabolic response and tissue destruction is created, resulting in more severe periodontal disease and increased difficulty in controlling blood glucose levels (Grossi & Genco 1998).

Schmidt et al. (1996) hypothesized that AGEs present in diabetic gingiva may be associated with a state of enhanced oxidant stress, which is a mechanism for accelerated tissue injury.

Using a diabetes mice model Lalla et al. (1998) showed increased AGE deposition and expression of vascular and monocytes RAGE were demonstrated in diabetic gingiva compared with non-diabetic controls. Furthermore, using the same mice model these authors succeeded in arresting periodontal disease in the diabetic mice by administration of soluble RAGE, probably by blocking the receptor from interacting with AGEs (Lalla et al. 2000).

Holla et al. (2001) suggest that the development of chronic periodontitis could be influenced by polymorphism of the RAGE gene independently of diabetes.

In the present study, we have demonstrated the expression of RAGE in the gingival tissues of both diabetic and non-diabetic patients with periodontal disease. The target cells for RAGE expression were found to be epithelial and endothelial cells. RAGE had been identified previously on different epithelial cells including in kidney sections of diabetic patients and healthy respiratory and skin tissues (Brett et al. 1993). The biological role of RAGE in the epithelial cells in healthy and disease state has not been investigated yet, although some studies suggest a role for this receptor in the process of aging of cells (Stern et al. 2002).

RAGE has some biological characteristics that suggest it may play a pivotal role in the pathogenesis of periodontal disease. It is a tissue receptor, associated with pro-inflammatory response and is expressed differentially in an increased amount with aging (Schmidt et al. 1995). As an important co-factor, increased expression of receptors for adhesion molecules was found on endothelial cells of diabetic patients (Schmidt et al. 1995) and these molecules were also associated with severity of periodontal disease (Joe et al. 2001).

We hypothesize that the effect of AGEs, mediated through their receptors, involves not only alteration of the vasculature bed (Hori et al. 1996) but possibly by a direct effect on the epithelial cells.

This study is the first to demonstrate the expression of RAGE on epithelial cells of human gingiva with periodontal disease. The increased mRNA for RAGE might suggest a possible overproduction at the protein level, however, this cannot be decided by our immunohistochemical staining.

In our immunohistochemistry, we did not find statistically significant difference between the type 2 diabetes and non-diabetes groups. This could be partially attributable to the insensitivity of the staining technique in immunohistochemistry or because of biological causes that deplete the amount of RAGE in the gingiva of type 2 diabetes patients.

Our patient data did not include potentially important information such as smoking habits, diet, BMI, and did not include actual measurement of fasting serum glucose or glycosylated hemoglobin; however, this preliminary study provides further evidence to the link between diabetes type 2 and periodontal disease. Further studies comparing and analyzing the expression of RAGE in normal healthy periodontium and gingiva of diabetic patients are warranted.

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