QUANTIFYING ANALYSIS OF ADVANCED GLYOSYLATION END PRODUCTS (AGES) EXPRESSION IN PERIODONTITIS PATIENTS WITH DIABETES TYPE II

Grimm W.-D.1,2, Shchetinin E. V.2, Bobrishev D. V.2, Sirak S. V.2

1Witten/Herdecke University, Witten, Germany
2Stavropol State Medical University, Stavropol, Russian Federation

Diabetes is an established risk factor for periodontitis. We investigated AGES distribution in human gingival tissues and monocyte cytokine expression in diabetic patients with chronic periodontitis (DII/cP). Within indicated surgical procedures gingival biopsies were taken from 10 DII/cP and from 10 healthy controls. Tissue samples were prepared for immunohistochemistry using a monoclonal antibody specific for AGES (6D12, TransGenic Inc.) and IMAGES J was used for quantification. Mean clinical parameters in DII/cP presented typical for severe chronic periodontitis (mt: 10.5; BoP: 84%; PBI: 2.9; cAL: 5.5 mm; rAL: 6.9 mm). In gingival tissue from DII/cP immunohistochemistry showed enhanced accumulation of AGES (17%) compared to controls (13%). This study found that diabetes and periodontitis results in further AGE-accumulation compared to periodontitis alone. These results suggest the possibility that the blockade of RAGE may provide an effective approach in a range of diabetic complications including periodontal disease.

Key words: diabetes, periodontitis, advanced glycosylation end products, immunohistochemistry

Diabetes mellitus is a metabolic disorder resulting in chronic hyperglycemia and hyperlipidemia that ultimately induces diverse multiple systems pathologies. Vascular pathology and resultant oxidative stress increase the risk for atherosclerosis, coronary heart disease, stroke, myocardial infarction, renal disease and periodontitis [2, 17]. An abnormal inflammatory response i.e. a hyper-inflammatory trait has recently been linked to diabetes and is believed to mechanistically contribute to the pathology associated with...
many systemic complications associated with diabetes, including an increased susceptibility to infections, such as periodontal disease. Traditionally, these complications have been attributed to the hyperglycemic state, which over time results in the irreversible covalent modification (glycosylation) of structural proteins and lipids which comprise the extracellular matrix and connective tissues, as well as the vascular tissues. These structural changes result in impaired capillary function, poor blood perfusion of tissues and organs and the release of reactive oxygen species (oxidative stress) triggering a systemic inflammatory process. The activation of inflammation at the systemic level results in the chronic elevation of acute phase reactants such as C-reactive protein (CRP), elevated fibrinogen and lowered albumin – all hallmarks of the acute phase reaction [25].

Multiple studies have demonstrated conclusively the link between diabetes and periodontal disease in human subjects. Consequently, periodontitis has been described as the sixth complication of diabetes mellitus and recent studies show that inflammatory periodontal diseases may increase insulin resistance [20, 26]. Furthermore molecular signatures of inflammation such as activation of NF-kappaB is evident in periodontitis [5].

Prolonged exposure to chronic levels of glucose can lead to the glycosylation of long-lived proteins found in blood and tissues. These glycosylation products, referred to as advanced glycosylation end products or AGEs have been recently implicated as a primary causal factor in the development of complications associated with diabetes [4, 11, 12, 18, 28]. The measure of HbA1c has been a reliable measure of glycemic control over 3-month intervals; however, compared to tissue matrix AGEs with slower turnover rates, the half-life of HbA1c is relatively short. AGE products are a heterogeneous class of molecular structures irreversible in nature, they are characterized by their yellow-brown color and fluorescence, have a propensity to form cross-links, and interact with cellular receptors [3, 27]. Subsequently, there is an increase in AGE deposition in matrix tissues as well as an increase in the number of receptors for AGEs on these tissues and on target cells [1, 2]. These receptors for advanced glycosylation end products (RAGEs) have also been identified and characterized in the literature [16, 31, 33].

The formation of AGEs has been shown to alter basement membrane macromolecules. Results from E. Lalla et al. demonstrate that blockade of receptors for RAGEs results in suppression of both alveolar bone loss and markers of cellular activation/tissue-destructive properties. These data indicate that AGEs stimulate increased production of prostaglandins by monocytes following LPS exposure [22, 23].

Although diabetes is well established as a major risk factor for periodontal diseases the cellular and molecular basis for this association is not clear [38]. High mobility group box 1 (HMGB1) was recently identified as a lethal mediator of severe sepsis and comprises a group of intracellular proteins that function as inflammatory cytokines when released into the extracellular milieu. Y. Morimoto-Yamashita et al. [29] reported that HMGB1 expression in periodontal tissues was elevated in patients with severe periodontitis. In addition, the receptor for RAGE, a receptor for HMGB1, was strongly expressed in gingival tissues obtained from patients with type 2 diabetes and periodontitis compared with systemically healthy patients with chronic periodontitis patients.

In summary, infectious challenge can induce a metabolic diabetic state, which if the infection is ephemeral, is generally considered reversible [21]. The accumulation of final AGEs seems to play an important role in the development of diabetes mellitus (DM)-associated periodontitis; however, some aspects of this issue are still scarcely known, such as the expression of AGEs in DM-associated periodontitis and the clinical factors able to affect their accumulation. This study aimed to clarify these points by evaluating the expression of AGEs in DM-associated periodontitis. Thus, we hypothesize that periodontitis serves as the stimulus of a systemic-based inflammatory response that may represent a previously underestimated metabolic stressor enhancing insulin resistance and impairing insulin secretion.

Material and Methods. Gingival tissue was obtained from 20 patients within extractions and periodontal surgeries.

Prior to biopsy, the periodontal records on six sites were measured. The exam included missing teeth, plaque score, bleeding on probing as percentage of sites, probing depths, and attachment levels. Periodontal measurements were provided at six sites with a periodontal probe by the single calibrated investigator. Periodontal status was expressed in terms of initial (min. 30% of the sites: PD > 4mm), moderate (min. 60% of the sites: PD > 4mm) or severe periodontitis (min. 60% of the sites: PD >5mm).

The biopsy samples were fixed in Bouin’s solution for 24 hours and afterwards decalcified in 10% EDTA, pH 7.4 at 4 °C for 10 days, changing the solutions every 3 days. After rinsing under tap water for 2 hours, the tissue samples were dehydrated and embedded in paraffin wax (Leica Histowax). The embedded specimens were then cut at 5µm thickness and mounted on precharged slides. Sections were dried at 37°C for better adhesion and stained with Hematoxyline & Eosin. The biopsy samples were examined immunohistochemically with a monoclonal antibody specific for AGEs (Fig. 1), 6D12, from the Trans Genic Inc., Japan. The splenic lymphocytes from BALB/c mouse, immunized with AGE-BSA were fused to the myeloma P3U1 cells. The hybrid cells were screened, and the cell line (6D12) with positive reaction AGE-human serum, but negative to BSA was selected through the successive subclonings.
and grown in ascitic fluid of the BALB/c mouse, from which the anti-AGE antibody was purified by Protein G affinity chromatography.

**Fig. 1.** Monoclonal antibody specific for AGEs (6D12). Images were analyzed with respect to sections immunostained with a monoclonal antibody specific for AGEs, 6D12. Sections were used to correct for background and non-specific staining and to set threshold above which staining was considered positive and specific for mouse monoclonal antibody specific for 6D12. Quantification of immunohistochemistry was then performed as follows: multiple sections per condition were imaged under high power (x200) using a Zeiss microscope and attached Sony video camera.

Quantification of immunohistochemistry was performed using three sections. In each section five areas were selected at a 200-fold magnification. The sections were imaged under high power resolution using the Zeiss microscope and attached Sony video camera. Images were analyzed with respect to staining intensity on a computer using IMAGES J. Scion Image is a widely used image processing and analysis program for the PC basing on the NIH Image platform. Thresholding were used to segment an image into objects of interest and background on the bases of gray level. When thresholding was enabled, objects were displayed in black and background was white. Results are presented as the percentage of density differences in comparison with sections derived from non-diabetic patients and stained with monoclonal antibody 6D12.

**Results.** AGEs and their effects on monocytic priming and tissue and cell function help explain the complex relationship between the hyperglycemic state and many of the pathophysiologic changes with diabetes. The presence of infection is usually accompanied by many disturbances in normal metabolic pathways. The host response to the infection is mediated by cytokines, which are considered hormones of the immune system. Cytokines like IL-1β, TNFa and IL-6 during infection and inflammation stimulate hepatic synthesis of acute phase proteins. Interestingly all these cytokines are target genes of the transcription factor NF-kappaB a control regulator of inflammation [32]. These cytokines have been found to be valuable in monitoring infectious or inflammatory processes. Infection has also been identified as an effect modifier in certain disease states such as diabetes (data not shown).

Eleven of the patients were female and 9 were male. Their mean age was 49 years and 51 years, respectively, with a range of 40 to 68 years. The periodontal status of diabetes and control patients is summarized by the mean data of six clinical measurements: probing pocket depth (PPD), clinical attachment level, bleeding on probing (BOP) and plaque scores. All clinical measurements were slightly less severe in the control group versus diabetes group, but the differences were not statistically significant. The results showed, that the mean clinical index as well as the percentage of sites exhibiting BOP in patients with impaired glucose tolerance were not different from the data of the control group. The mean number of sextants exhibiting pocket depth 0 to 3.5 mm, 4.0 to 5.5 mm and 6.0 mm and more did not show differences between the two groups either.

Consistent with earlier observations from our pilot study, gingival tissue from diabetic patients demonstrated enhanced accumulation of AGEs compared with non-diabetic controls, especially within the vascular structures and surrounding epithelial and connective tissues [13]. In contrast accumulation of epithelial/connective tissue and vascular AGE was significantly suppressed in diabetic; indeed these levels were not significantly different than those observed in non-diabetic patients (Fig. 2).

**Fig. 2.** The part A demonstrated the accumulation of vascular AGE in diabetic patients in comparison with the accumulation of vascular AGE in non-diabetic patients (part B).
Quantitative immunohistochemistry demonstrated a 1.3-fold increase in the percentage density in diabetic patients compared to non-diabetic controls (Fig. 3).

Discussion. Even sporadically elevated levels of blood glucose lead to the generation of largely irreversible advanced glycosylation end products, or AGEs. AGEs accumulate in the tissues and plasma of humans in normal aging and do so to a greater degree in those with diabetes. Their earlier and enhanced accumulation in diabetes has been linked to the pathogenesis of vascular and inflammatory cell complications that typify this disorder. The Columbia university research team, Lalla et coworkers, has identified a central cell-surface receptor for these AGEs, termed RAGE, a multiligand member of the immunoglobulin superfamily of cell-surface molecules. RAGE is expressed on a range of cell types, such as endothelial cells, monocytes, smooth muscle cells, and fibroblasts, at low levels in adult healthy animals but at significantly higher levels in settings such as diabetes. Multiple studies have demonstrated that engagement of RAGE by AGEs on cells critically involved in inflammatory responses results in cellular perturbation. For example, in endothelial cells interaction of RAGE with AGEs results in diminished collagen synthesis. Indeed, evidence for perturbed cellular and connective tissue properties has been established in the setting of long-term diabetes [9, 34].

We found that diabetes and periodontitis result in further AGE-accumulation compared to periodontitis alone.

Furthermore our immunohistochemical study just showed a 1.3-fold increase of AGEs due to diabetes in periodontitis patients whereas literature study showed elevated serum antibody AGE elevation in comparison of healthy controls to diabetes patients in a 3- to 10-fold manner [19]. It has been stated that AGEs appear in a lot of conditions as in age in rheumatoid diseases diabetes and periodontitis as a pathologic condition [24]. Both periodontitis and diabetes lead to enhanced AGE-prevalence. A mutual enhancement of pathologic conditions due to AGEs can be assumed.

Glycosylation of long-lived proteins may occur with normal aging; however, the hyperglycemic state accelerates the process, which leads to alteration of tissue and cell function particularly in diabetics. In addition to proteins, lipid moieties are also glycosylated and oxidized [30]. Dyslipidemia involving hypertriglyceridemia and low levels of high density lipoprotein (HDL) is a common finding in diabetics which is thought to be predictive of cardiovascular mortality [36]. Lipoproteins, particularly low density lipoproteins (LDL), are thought to be intimately associated with the development of atherosclerosis [10]. Oxidized forms of these lipids are also believed to be the pathogenic forms of these lipids. Oxidative stresses including products of oxidized arachidonic acid (PGE2 and malonyl dialdehyde or MDA) are potent catalytic inducers of oxidized LDL formation. Infection and poor oxygen perfusion represent the two major causes of oxidative stress. The peroxidation process involving lipoproteins is thought to contribute to atheroma formation by the following interactions: 1) impairment of the LDL receptor recognition of modified LDL and cholesterol transport by HDL, 2) stimulation of platelet aggregation and foam cell formation and 3) formation of immune complexes and reactive oxygen species [6, 34, 37]. The cumulative effects of these mechanisms may subsequently contribute to vascular wall injury and atherogenesis [14].

Gingival tissue from diabetic patients of our study demonstrated enhanced accumulation of advanced glycosylation end products compared with non-diabetic controls, especially within the vascular structures and surrounding epithelial and connective tissues.
factor), and expression of endothelial cell adhesion molecules such as vascular cell adhesion molecule-1. Chang et al. [8] investigated the pattern of palatal wound-healing after graft harvesting under the administration of aminoguanidine, an AGE inhibitor, or N-phenacylthiazolium bromide, a glycated cross-link breaker. AGE deposition, RAGE-positive cells, and inflammation were reduced. RAGE and tumor necrosis factor-alpha were significantly down-regulated. The levels of vascular endothelial growth factor, perilipin, type I collagen, and fibronectin were all increased. In conclusion, anti-AGE agents appeared to facilitate palatal wound-healing by reducing AGE-associated inflammation and promoting the recovery process. Toll-like receptors are the most studied pattern recognition receptors that are present at the cell surface as transmembrane receptors. The toll-like receptor that recognizes lipopolysaccharide on macrophages (toll-like receptor-4) and the RAGES are members of this group.

Conclusions. Binding of pattern recognition receptors activates the nuclear factor-κB signaling pathway that induces immune response genes, especially those for inflammatory cytokines, which are the main initiators of inflammation and the acute-phase response. RAGE has been shown to mediate signal transduction through the generation of reactive oxygen species, which activates transcription factor nuclear factor-κB. These considerations suggest the possibility that the blockade of RAGE may provide an effective approach in a range of diabetic complications including periodontal disease.

Acknowledgements

We thank the medical technologists of the Institute of Pathology of the Ruhr-University Bochum and we thank Susanne Haußmann, medical technologist of the University of Witten/Herdecke, for their support of the study.

References


About authors:

Grimm Wolf-Dieter, PhD, MSc, Professor of Periodontology, Periodontology, Department of Dental Medicine, Faculty of Health, University of Witten/Herdecke, Germany, Private Practice, Praxisteam Hasslinghausen, Mittelstr. 70, 45 549 Sprockhövel, Germany; tel.: +492339911160; fax: +492339911162; e-mail: prof_wolf.grimm@yahoo.de

182
ROLE OF ENDOTHELIAL DYSFUNCTION IN DEVELOPMENT OF NONALCOHOLIC STEATOHEPATITIS

Geyvandova N. I., Nighiyan Z. V., Babasheva G. G.

Stavropol State Medical University, Stavropol, Russian Federation

The aim of the study was to determine the clinical significance of the blood levels of endothelin-1 (E-1) and nitric oxide (NO) as indicators of endothelial function, as well as serum endotoxin (ET) in patients with non-alcoholic fatty liver disease (NAFLD). The body of patients included 142 persons with NAFLD – 90 patients with hepatic steatosis (Group I), 52 patients with non-alcoholic steatohepatitis (NASH) (Group II). All the patients had their plasma content of E-1 determined by ELISA, as well as the level of NO (by colorimetric method). The level of ET in blood serum was determined employing the chromogenic method Hbt LAL. The values for endothelial dysfunction (ED) in the group of the patients with steatosis revealed no difference from the control values. The content of E-1 and NO in the blood of the patients with NASH exceeded those in healthy individuals and in Group I. The ET levels in the blood of the patients in Group II were higher compared to the healthy persons as well the patients with hepatic steatosis. There has been positive correlation identified between the E-1 and HOMA-index, E-1 and ET in case of NASH, yet not in Group I. The dynamics of 1 month into the therapy, which included dietary measures, Metformin and hepatoprotector, revealed a decrease in the blood levels of E-1 and no change in NO. The outcomes demonstrate a role of ED and endotoxinemia in the progress from steatosis to steatohepatitis. It has been shown that, in case of NASH, comprehensive therapy may exert positive effect on endothelial function.

Key words: non-alcoholic fatty liver disease, steatosis, nonalcoholic steatohepatitis, endothelial dysfunction, endotoxinemia

В исследовании определялось клиническое значение содержания в крови эндотелина-1 (Э-1) и оксида азота (НО) как показателей функции эндотелия, а также количества сывороточного эндотоксина (ЭТ) у больных неалкогольной жировой болезнью печени (НАЖБП). Обследовано 142 больных НАЖБП: 90 больных со стеатозом печени – группа I, 52 пациента – с неалкогольным стеатогепатитом (НАСГ) – группа II. У всех пациентов определяли плазменное содержание Э-1 методом ИФА и уровень NO колориметрическим методом. Количество ЭТ в сыворотке крови определялось хромогенным методом Hbt LAL. Показатели эндотелиальной дисфункции (ЭД) в группе больных со стеатозом не отличались от контроля. Содержание Э-1 и NO в крови больных НАСГ превышало показатели у здоровых и в группе I. Уровень ЭТ в крови больных группы II был выше, чем у здоровых и больных стеатозом печени. Выявлена положительная корреляция между Э-1 и HOMA-индексом, Э-1 и ЭТ при НАСГ, но не в группе I. В динамике 1 месяца терапии, включающей диетические мероприятия, метформин и гепатопроте-