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White Blood Cell Signaling and Defense Mechanisms in Patients with Diabetes Mellitus Type 2 and Periodontitis

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Abstract

White blood cell membrane and surface structures are affected by the metabolic disorders and complications found in diabetes mellitus. Therefore, cellular activation, signal propagation, intracellular signaling as well as bactericidal effector functions are altered.

Most likely diabetic symptoms can be corrected by the systemic intervention and treatment of the patients (Anti-diabetic Therapy/ADT, i.e. anti-diabetic medication, diet and dietetic supervision, physiotherapy and physical exercises). We hypothesize that simultaneously blood cell functions will improve.

Gum diseases like periodontitis have long been associated with and termed complications of uncontrolled diabetes mellitus. Vice versa, after diabetic conditions are corrected, periodontitis treatment will be proven effective, when oral hygiene regimen, full mouth decontamination (FD, i.e. the oral use of topical antiseptics prior and after professional mechanical tooth cleaning, tooth as well as root surface planing, polishing as well as gum and soft tissue decontamination in combination with systemic antibiotics) are performed. To reinforce gum healing, reinfection prevention (RP) as well as supportive periodontal therapy (SPT) will be administered by dental professionals on an individual basis and a detailed schedule.

If periodontal pockets critical for participant's self care are not eliminated by FD including RP and SPT, and niches > 5mm after 6 month persist, patients are informed and offered surgical intervention as indicated for gum disease elimination.

Key words:

Diabetes Mellitus Type 2; Chronic periodontitis; Human polymorphonuclear neutrophils; Cellular signalling

Scientific background

Diabetes mellitus is a heterogeneous group of disorders with the common characteristic of altered carbohydrate metabolism. In this proposed study research will focus on individuals suffering from Diabetes Mellitus type II (DM). The increase in obesity, which is considered a major risk factor for diabetes mellitus type II, and the high rates of impaired fasting glucose and glucose tolerance found in NHANES III, indicate that diabetes mellitus type II continues to be an expanding health problem in modern societies [1,2].

Chronic periodontitis results from an extension of the inflammatory process initiated by bacteria in the gingiva to the supporting periodontal tissues. It is becoming increasingly apparent that the relationship between diabetes and periodontal disease is reciprocal. Periodontitis, like other infections, has a significant impact on diabetic control. Equally, diabetes is a significant risk factor for the development of periodontal disease and can aggravate the severity of periodontal infections [3,4].

Factors that increase the severity of inflammatory diseases, such as altered PMN function, have been associated with pathogenesis of diabetes [5-7]. Recent data suggest that PMN become pre-activated during diabetes and release mediators that promote the tissue damage associated with periodontitis.

Diagnosing diabetes mellitus and the respective criteria have undergone significant changes in the last decades [8,9]. Likewise, the diagnosis of periodontitis has been reclassified. Clinical attachment level (CAL) is frequently used as gold standard for monitoring periodontitis progression or treatment outcome [10-12].

Employing up to date standards of diagnosing these 2 separate diseases, several general trends became apparent: Poorly or uncontrolled diabetes mellitus was associated with increased susceptibility to oral infections, in particular periodontitis [4,13]. The incidence of periodontitis was found increased among diabetic patients, with an onset in childhood and puberty and culminating during the aging of the patient population [14-19]. Severity of periodontitis and frequency was increased in diabetic patients that showed also systemic complications [20,21]. An increased susceptibility did not correlate with increased levels of dental plaque and calculus [19-22]. Already these early investigations corroborate the hypothesis that there was a pathogenesis promoting association between these two different diseases, especially in un- or poorly controlled diabetic patients.

More recent work strongly discussed this bilateral association of diabetes and periodontitis [23].

Hence, further research is mandatory to elucidate the role of periodontitis as comorbidity of diabetes and *vice versa*.

Among phagocytic cells polymorphonuclear neutrophils (PMN) form the first line of defense against invading organisms and particles. Specific receptors recognize chemotactic factors e.g. formyl-Met-Leu-Phe (fMLF), released either by the foreign organisms or during the opsonization process, leading to movement (chemotaxis) of the phagocyte up the chemotactic agent's concentration gradient to its origin, where the invading entity is reached. Foreign organisms become opsonized, so that they present immunoglobulins or complement fragments on their surface, or are complexed, as antigens with the appropriate immunoglobulin, to form immune complexes (HIC). When in contact with the opsonized particle or HIC, the phagocyte shifts from migratory to phagocytic (effector) processes, recognizing the invader. The liganded receptors then initiate a series of signals, resulting in phagocytosis of the invading entity and release of the phagocyte granules' contents (lytic enzymes, defensins, cationic proteins, etc.) as well as oxidative products, predominantly into the phagocytic vacuoles [24]. Initial, rapid fMLF- and Fcγ-receptor mediated activation processes by PMN in suspension, observed in real time, in purified intact, naïve cells (neither permeabilized, fixed, transformed or transfected) and under controlled conditions had been defined [25,26]. Marked stimulus-dependent differences between these mechanisms had been noted in, providing insights into the actual biochemical events that regulate stimulus-induced effector functions [27].

Hypotheses

PMN membrane and surface structures are affected by the metabolic disorders and complications found in diabetes mellitus type II [28-30]. Therefore, receptor binding, signal propagation, intracellular signaling as well as bactericidal effector functions are reduced.

When diabetic symptoms are corrected by the systemic intervention and treatment of the patients, PMN functions will then normalize and reach the functionality comparable to the those cells derived from control individuals.

Periodontitis known as the 6th complication of diabetes mellitus [4] worsens, when diabetes is uncontrolled. Vice versa after diabetic conditions are corrected, periodontal treatment will be proven effective, when oral hygiene regiment, full mouth decontamination and if needed surgical intervention are performed for pocket elimination and gain of clinical attachment.

Specific Aims

To investigate if cytosolic $Ca2^{+-}$ ($\Delta[Ca2^{+}]i$) and pH (ΔpHi) signaling responses and bactericidal effector functions of PMN dependent upon the status of diabetic control and are reduced or increased when compared to age and gender matched controls.

To determine the biochemical basis for diabetic PMN alteration of motility as well as bactericidal functions: production of superoxide and release of elastase, respectively

To characterize the molecular basis of the observed alterations in the regulation of cytosolic calcium ($\Delta[Ca2^{+}]i$) and pH (ΔpHi) exhibited by diabetic PMN

To investigate if the pre-activated state and altered bactericidal functionality of diabetic PMN are reversed when the patients' glycemic control is normalized, blood glucose levels as well as periodontal disease are corrected

To evaluate, if systemic and periodontal intervention can lead to clinical attachment gain in patients with diabetes mellitus

Methods and Study Design

The primary study purpose is basic science. It had been submitted to clinicaltrials.gov and can be identified via NCT01848379. The study allocation is randomized (single blinded outcome assessor). The endpoint classification is an efficacy study with an intervention model by parallel assignment.

Inclusion criteria

Recently diagnosed patients suffering from type II diabetes, with/without periodontitis will be included. The diabetic condition should be severe, indicated by a glycated hemoglobin (HbA1c) ≥ 8.5% [31,32].

On a daily base, age, gender and race matched control individuals will be invited for participation. The population age may vary between 18–70 years. Patients and controls should have at least 12 natural teeth without subgingival fillings, crowns or caries. Diabetes and periodontitis patients should exhibit a periodontal screening index (PSI) above 3 at base line. Participants should be otherwise healthy. (at SY1; for this and the following abbreviations cf. Study Design).

Control individuals should either be free of diabetes, periodontitis or both diseases, respectively. For all interested participants gingival inflammation during the study period phase II and III is reduced by periodontal prophylaxis and professional administered or for home use instructed reinfection prevention (cf. study design, PRO).

Exclusion criteria

Patients will be excluded if they have clinical features known to influence the natural history of periodontal disease or are unable to comply with the study. The exclusion criteria are:

Pregnancy

Smoking

Low body mass index (BMI <18.5 kg/m^2)

Severe cardiovascular disease including coronary artery disease, cerebral vascular disease, peripheral vascular disease, valvular heart disease, and congestive heart failure

Other major illnesses including cancer, liver disease, pulmonary disease, chronic infectious disease other than periodontitis (HIV, hepatitis, etc.), rheumatological disease, hematological disease, or any condition requiring hospitalization or chronic medical therapy other than diabetes

Major psychiatric illness requiring treatment, or that might interfere with the ability to understand or cooperate with the protocol

Ongoing alcohol or drug abuse; all forms of medication or illegal substance abuse

Systemic enteral or parenteral medication, in part daily vitamin or anti-oxidative supplementation and certain calcium channel blockers (i.e. Nifedipine); but anti diabetic drugs or insulin substitution

Allergies to antibiotics or adjuvant medication/antiseptics as well as dental materials in use (including gloves) in particular those against topical antiseptic solutions i.e. chlorhexidine/N',N''-hexane-1,6-diylbis [N-(4-chlorophenyl) (imidodicarbonimidic diamide)] or povidone iodine/2-Pyrrolidinone, 1-ethenyl-, homopolymer, compound with iodine

Severe dental disease defined as severe dental caries, and/or severe pulpal disease requiring surgical correction, or any other mucosal or dental condition not readily treated, or requiring extensive dental, oral surgical or prosthetic treatment, or any other oral treatment which could affect the outcome of periodontal therapy or diseases or syndromes that require systemic medication

Systemic, topical or inhaled steroid treatment for more than 30 consecutive days within 6 weeks of baseline

Any periodontal treatment within 6 months prior to baseline

For controls: a periodontal screening index (PSI) >1

In addition, dental diseases requiring treatment or concurrent medication may confound the ability to test the hypothesis that treatment of diabetes and/or periodontal disease prevents "pre-activation" of polymorphonuclear leukocytes. Therefore, these patients will be excluded

Severe dental diseases defined as profound dental caries, and/or severe pulpal disease requiring surgical correction, or any other mucosal or dental condition not readily treated, or requiring extensive dental, oral surgical or prosthetic treatment, or any other oral treatment which could affect the outcome of periodontal therapy or diseases or syndromes that require systemic medication

Systemic, topical or inhaled steroid treatment for more than 30 consecutive days within 6 weeks of baseline.

Any periodontal treatment within 6 months prior to baseline

For controls: PSI >1

Allergies (cf. above)

Dental and Periodontal Examinations (PAR1-4)

If a participating individual meets all inclusion criteria, he/she will undergo a baseline dental examination (PAR1) to categorize the presence and extent of periodontal disease. The examination will also include new radiographs (panoramic), unless recent films (within 6 months) are available. The oral examination will be performed by the study periodontist employing a Florida probe. At this time the examiner will include a detailed survey of face, lymph nodes, lips buccal mucosa, floor of the mouth, tongue, hard and soft palates, gingiva, edentulous ridges, and teeth. Then the following features will be quantified employing an electro-mechanical probe.

Clinical attachment level: Clinically and quantitatively, loss of attachment is defined as the distance in mm from the cemento-enamel junction (CEJ) to the base of the pocket. The measurement recorded is the distance from the CEJ to the gingival margin (GM), resulting in a positive or negative value depending on whether the GM is coronal (+) or apical (-) to the CEJ. Clinical attachment level is calculated according to the formula: (CAL=pocket depth–[GM–CEJ]) [33]. For assessment of errors associated with measurement of pocket depth and clinical attachment level, trained and calibrated examiners will follow the protocol established by Grossi et al. [34].

Probing depth: Probing depth also called Periodontal Probing Depth (PPD) is defined as the distance in millimeters from the gingival margin to the base of the sulcus or periodontal pocket. It is measured at six sites per tooth (distobuccal, midbuccal, mesiobuccal, distolingual, midlingual, and mesiolingual) of all teeth present.

All clinical examination will be performed by one trained dental examiner using the pressure calibrated Florida probe, but the two oral hygiene indices (cf. PBI and PLI).

Bleeding on probing: Bleeding on probing will be determined by recording the presence or absence of bleeding following probing to determine pocket depth. This parameter will be expressed as % bleeding sites out of all examined sites in the dentition and will be documented with the Florida probe software.

Modified papillary bleeding index: PBI will be determined on each inter-proximal papilla except those mesial of the medial incisors by a modified procedure based on the studies Saxer et al, [35]. A score of 0 to 4 will be given instantaneously after papilla probing with 0: no bleeding; 1: light, spotted bleeding; 2: moderate bleeding, several spots or a distinct line; 3: bleeding likely to fill the inter-proximal space and 4: almost spontaneous profound bleeding.

Modified dental plaque index: Plaque will be assessed on 4 surfaces per tooth: lingual, buccal, inter-proximal (mesial and distal) [33,36].

Decayed and missing teeth: Decayed and missing teeth will also be recorded. The enrolled individuals will have the following clinical periodontal inclusion criteria consistent with the 1999 American Academy of Periodontology classification of chronic generalized, severe periodontitis [37]: Presence of at least 12 natural teeth and involvement of at least 6 teeth with pocket depth ≥ 6 mm and loss of attachment ≥ 5 mm in three aspects of each involved tooth. These criteria will be applied after accounting for necessary tooth extraction. Patients who require urgent dental procedures will not be considered eligible.

Description of periodontal intervention

The goal of periodontal therapy is achievement of a clinically defined state of periodontal health. Beginning after the baseline exam (PSI/PAR1) participants will receive instructions and an oral hygiene regimen as well as professional tooth cleaning (PRO) performed by one trained instructor. The underlying intention is to reduce gingival inflammation. Therefore, a full examination (PAR2) will evaluated the periodontal conditions. If necessary, in addition to dental hygiene, participants will receive staged periodontal therapy:

Non-Surgical therapy scaling and root planing (FMDAB)

Based on clinical exam PAR2, patients will receive complete subgingival debridement with scaling and root planing in combination with local antiseptics under local anesthesia using hand and ultrasonic instruments by on experienced operator. All 4 quadrants will be completed within 2 days of the week of clinical appointment PRO and the patients will receive systemic antibiotic therapy (metronidazole and amoxicillin) during this time. A large body of evidence supports this approach for management of severe periodontal disease in the diabetic population [38]. This therapy will consist of scaling and root planing of all four quadrants of the dentition in combination with local antiseptics as well as systemic antibiotic therapy [39,40].

(Ciprofloxacin will substitute for amoxicillin in the case of penicillin allergy.) The results of scaling and root planing will be evaluated after 3 months (PAR3). If and when individual teeth do not respond to this intervention, more comprehensive periodontal surgery will be performed. After FMD AB intervention, the patient will be seen for maintenance therapy (PRO). After approximately 3 months, the results of therapy will be evaluated again (PAR3). Further scaling and root planing alone, or in combination with antiseptics or antibiotics will be performed if necessary (PRO). The goal of this comprehensive therapy is a state of periodontal health characterized by the absence of BOP and PPD <5 mm.

Periodontal surgical therapy (SUR)

Patients with an inadequate response documented at PAR3 (i.e. BOP positive >20% [41-43] in combination with PPD >6 mm [44] and FK grades II or III as well as progression of clinical attachment loss [42]) will be informed of treatment needs and that additional SUR could correct clinical niches. To date the clinical literature suggests that numerous patients will in fact require surgery [45] and that a surgical approach has a good chance of achieving minimal probing depths [46-48]. The periodontist will use his/her best judgment and select from one or all of the following procedures: flaps with or without osseous surgery, root resective or access flap therapy.

At 52 weeks all participating patients will be re-evaluated (PAR4). Again, any sites with continued involvement (persistence of pockets >6 mm [44] and failure to stabilize progression of clinical attachment loss) will receive re-treatment. Fortunately, prior clinical experience and the published literature suggest that refractory cases are relatively rare (2-3%). The re-treatment and/or periodontal maintenance therapy will consist of a site-specific subgingival debridement with scaling and root planning under local anesthesia using hand and ultrasonic instruments and locally delivered antiseptics. Additionally patients' compliance and oral hygiene will continuously be evaluated to ensure stability of their periodontal condition.

Description of diabetes intervention (SY1-4)

The collaborating departments have developed strategies to effectively manage diabetic patients. To date dentists/periodontists and physicians are instantaneously able to share medical background information of the participating individuals. The investigators will screen patient history (after contacting the referring physicians) and obtain the reports of participants' diabetic status, along with most recent HbA1C level, and other measures of diabetes management or preventive interventions (c.f. below section: Parameters). Under some circumstances it might become necessary to have acute supportive interventions for patients with un-controlled HbA1C levels by their physician. Therefore, patients suffering from diabetes mellitus type II are hospitalized in the initial phase of the study. As we progressively move patients toward better control, the HbA1C levels will be reduced, making the need of instantaneous intervention of their physicians less frequent.

In this study we will compare relevant parameters (e.g., $\Delta[Ca^{2+}]i$, ΔpHi, chemotaxis, and bactericidal functions, e.g. extracellular release and total production values of ROS/superoxide, degranulation of human neutrophil elastase by phagocytic leukocytes) of these patients vs. controls before these treatments and after successful medical/dental intervention. When possible, every effort will be made to examine the cells from these patients over a wide range of time points to determine if the pre-activated state is prevented/reversed, and whether these changes in leukocytes precede, coincide or lag behind alterations in blood glucose, HbA1c, Glcmdp or oral pathology.

Demographic data and human subjects

Demographic as well as other data will include genetic markers of both diabetes and periodontal disease risk, differences in behavioral and attitudinal factors with relevance to both diabetes and periodontal disease risk, such as self-care behaviors (both oral and general health), other health related behaviors such as alcohol consumption and use of tobacco products (recognized as a risk factor for periodontitis as well as diabetes), food selection and nutritional intake, variations in access to and use of professional and dental care services, and related socioeconomic variables (e.g. education, income). Blood to be used in these studies will be obtained from healthy (non-diabetic and non-periodontitis) individuals and patients with diabetes mellitus and/or periodontitis. The same investigator will clinically examine all patients and healthy control individuals. A written consent form provides for refusal to participate. The Study population will predominantly recruit local residents as patients and controls. Thus the cohorts will consist out of Caucasian participants enriched with a few individuals with a Mediterranean descent. Since patients and controls are aimed to be age and gender matched, special care will be given to find representative healthy individuals.

Inclusion of women: Every effort will be made to ensure that women will be adequately represented. Women will be included in the proposed research program and will represent 50% of the individuals recruited.

Data and wafety: The clinical and analytical data will be stored anonymously in separate, encrypted databases. The Department of Medical Statistics will support the blinded design in terms of the assessment, the analysis of clinical as well as laboratory parameters. As mentioned, Dr. Rolf H Boedeker of the Department of Medical statistics at the Justus Liebig University will serve as the outside monitor and consultant for these studies.

Interview

After providing consent, individuals will be interviewed by study personnel to collect detailed information about demographics, years of education, employment history, and physical activity. A complete medical and dental history will also be obtained, including detailed information about medication use, history of alcohol consumption (current and lifetime drinks per day), smoking history (current packs of cigarettes per day, life smoking in pack-years, and time since last cigarette smoked), reproductive history, weight history and use of vitamins, food supplements and herbal and other non-traditional medication use. Detailed information will be specifically collected about oral hygiene and periodontal status.

Data management and sample size calculations

Data Management e.g., case report forms and computer entry, will be managed and linked through the electronic medical record a specialized, periodontal data handling system the Florida Probe software. All computer data will be secured and accessible to only the appropriate investigators and for statistical evaluation.

Sample size

The sample size calculation was performed based on precursor studies, on data from collaborating scientists [49-53] as well as from our laboratory [25,26,54-56].

Based on these preliminary data, it is estimated that all hospitalized diabetic patients will exhibit poorly controlled diabetes initially. Furthermore it can be anticipated that approx. 50% of those with poorly controlled diabetes will become well-controlled diabetics following medical intervention. Assuming an initial prevalence of poorly controlled diabetics of 100%, a 50% reduction in poorly controlled diabetics following therapy, significance level of 0.05 for a 2-tailed test, a sample size of 15 would be necessary. Individuals added to account for attrition resulting in a total of 17 individuals in each group (i.e. diabetes patients with periodontitis, periodontitis patients and healthy controls).

Statistical analysis

For conducting these analyses, a one-way analysis of variance model will be assumed with a significance level of α=0.05 and three levels to include: 1) diabetes with poor control 2) periodontitis patients and 3) orally and systemically healthy individuals. Analyses differentiate the levels of maximal changes in cytosolic Ca^{2+} concentration and pH, the expression of ROS/superoxide/peroxide, degranulation (elastase release, EA), PMN chemotaxis, glucose levels at the time of venipuncture as well as glycated hemoglobin. Additional analyses will be conducted utilizing 2-sample t-tests (2-tailed testing, significance level of α=0.05) to insure statistical detection of any biologically significant differences between diabetics with poor glycemic control and respective controls.

Adjustment for age, gender and smoking status will be considered. These associations will also be tested individually within each group.

Outcome Measure	Parameter Name	Unit	Time Course	Abbreviations
Primary	CAL	mm	BL, 6 and 12 M	PAR1 to PAR4 (A)
Secondary	PPD	mm	BL, 6 and 12 M	
	BOP	mm	BL, 6 and 12 M	
	GR	mm	BL, 6 and 12 M	
	FK	Grad I, II & III	BL, 6 and 12 M	
	BMI	kg/m^2	-3W, BL, 6 and 12M	SY1 to SY5 (C)
	HbA1C	mmol/mol	-3W, BL, 6 and 12M	
	DD	month	-3W or BL	
Other	$[Ca^{2+}]i$	µM	-3W, BL, +2W, 6 and 12M	SY1 to SY5 (B)
	pHi		-3W, BL, +2W, 6 and 12M	
	ROS	rel. FU	-3W, BL, +2W, 6 and 12M	
	EA	rel. FU	-3W, BL, +2W, 6 and 12M	

BL: Base Line Visit; W: Week; M: Month

Table 1: Parameters [8,9]

To have an accurate estimate of the medical history, current status and diabetic conditions of all participating individuals' parameters from whole venous blood will be assessed. These parameters, to be monitored prior and after medical and/or periodontal treatment, will be measured in the Department of Clinical Chemistry at the Universtätsklinikum Gießen & Marburg GmbH.

Change of the initial (-3 weeks) Gingival Crevicular Fluid (GCF) flow will be assessed at baseline. GCF: a quantitative and qualitative measure of the serum like exudate in the gingival crevice will be performed.

Global luminol dependent chemiluminescence of stimulated neutrophils (CLt) at (-3 weeks) as a measure of total ROS will be performed ex vivo with a kinetic chemiluminescence assay after receptor activation of neutrophils. Additionally the extracellular luminol dependent chemiluminescence of Stimulated Neutrophils (-3 weeks) will be monitored.

The change of cellular immune responses will be analyzed via the determination of leukocyte subsets, i.e. T-lymphocytes from the peripheral venous blood samples.

Parameter set (B) will be assessed depending on stimuli (chemoattractants and/or FcγR agonists), kinetically in our laboratory on a Hitachi F-7000 spectrofluorimeter as previously described [25,26].

Hygiene indices (D) will be assessed prior to information and/or (re-) instruction (MOT) by only one examiner and recorded in pre-printed spread sheets:

PBI: modified Papilla Bleeding Index

PLI: modified Plaque Index

Digital panoramic radiographs will be taken, if not already in file within the dental school and or from a referring colleague and not outdated /older than 6 month and/or assessed at Baseline (BL) as well as one year after FMD therapy.

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