Chromogranin A detection in saliva of type 2 diabetes patients

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Abstract

Chromogranin A is present in secretion granules of nerve, endocrine and immune cells and is a precursor of several peptides with antibacterial and antifungal properties at micromolar concentrations.

Our aim in this prospective, double blind study, was to determine the expression of chromogranin A and its peptides at protein level in saliva of type 2 diabetic patients and thereby to obtain a new non-invasive diagnostic means for the future.

Saliva was taken from 30 type 2 diabetic patients and 30 healthy individuals at the same time interval in the morning without any oral stimuli. Circadianic periodics in protein productions have been avoided. The presence of chromogranin A and its derived peptides was determined in whole saliva, after centrifugation at 4°C for 12 min at 14 000 rpm, by SDS-PAGE electrophoresis and Immunoblotting (Western Blot). To ensure same protein concentrations Bradford protein quantification assay has been performed before.

For the first time, we have determined an overexpression of chromogranin A in saliva of diabetic patients in 100% of the individuals.

Chromogranin A, a circulating biomarker for epithelial tumours, is also overexpressed in saliva of type 2 diabetic patients. To confirm our results, more studies with a large amount of patients is necessary.

KEY WORDS: Chromogranin A, diabetes type 2, innate immunity, saliva

Introduction

Since about 20 years ago a large amount of different antimicrobial peptides have been isolated from plants, animals, bacteria, fungi and thereby captured the interest of the scientific community (1). They are present in the hemolymphs of insects and stored in a great amount in the secretion granules of the endocrine and immune system (2). The importance of these molecules is already well established in invertebrates that control the secretion and production by themselves (2). Nevertheless, it has been also shown that vertebrates possess antimicrobial proteins that function as the first border of innate immune defense after infection until the adaptive immune response is established (3). Antimicrobial proteins are thought to be a part of the natural flora and thereby play a role in regulating the balance in the microflora. This model has been established in drosophila in which the antimicrobial peptides have been characterized in the cells representing the endocrine and the immune system. There is a huge number of structural varieties of antimicrobial peptides like different helices, folding and globulary structures maintained by disulfide bridges existing in biological fluids (4). Chromogranin A (CgA), the main member/element of the chromogranins family consisting of water soluble acidic glycoproteins, is a prohormone discovered for the first time in the secretory granules from adrenal medullary chromaffin cells and released into the circulation after splanchnic nerve stimulation together with catecholamines (5). CgA, also present in a lot of endocrine and neuroendocrine cells (6), nerve cells (7) and immune cells (8) is overexpressed in many cancers (9) and neurodegenerative diseases like Alzheimer's disease (10) or Parkinson's disease (11), for example. CgA is routinely used as a diagnostic marker: ELISA tests to determine CgA levels are undertaken to diagnose predictively these cancers and neurodegenerative diseases (9,10). Many natural peptides with antibacterial and antifungal properties are obtained after the posttranslational proteolytic processing of CgA (8,12,13). Among these peptides, some are processed from the N-terminal fragment (vasostatin-I and vasostatin -II) of the CgA. These peptides are active against Gram-positive and Gram-negative bacteria and can also prevent fungal growth (8,14), but they are not toxic for eukaryotic cells. The liberation of intact CgA and derived antibacterial peptides from polynuclear neutrophiles indicates their role in inflammatory processes (14) as well as their role in the communication between the neuroendocrine system and the immune system (8). CgA was also recovered in biological fluids implicated in defence mechanisms like abcess fluids or saliva (7,15,16). The secretion granules from the chromaffine like cells of the medullo-infrarenal system store a complex mixture of the molecules which are today known as catecholamines, and additionally a large number of precursor proteins of the antimicrobial peptides like chromogranins, secretogranins and proencephalin A. They are active against bacteria and fungi at micromolecular concentrations in a synergistic pattern. Their mechanism of action is based on lytic effects and investigations by confocal laser scan microscopy (CLSM) showed that after labelling the peptides with rhodamine the peptides penetrate into the fungi and lead to cell arrest and cell death. This mechanism of action has no effect on mammalian cells and hereby indicates a genetically determined specificity to differentiate target cells by receptor interaction (17). Chromogranins underly posttransductional modifications like phosphorylations, glycosylations and sulfatations that modulate their biological activity and degradation processes. These modifications depend on the physiological state of the individual organism (2). Diabetes, for example, an metabolo-endocrinic disease is a disorder in which the physiological state is changing according to the pathobiochemical disbalances as well as in the course of time and the duration of disease. It is also a disease which is more frequent and constantly increasing in our modern industrialized society. Meanwhile diabetes mellitus became a global health problem leading to several medical complications. A good example for such an development is France and the USA. There are more than 20 million people living with diabetes mellitus in the United States of America only. The French National Assurance Administration did an estimation about the development of diabetes cases based on the data from 2000 to 2005 and came to the conclusion that in the course of 10 years the number of diagnosed diabetes cases will double and that today already a constant augmentation of diabetes patients is approximately 6%. For 2016 an estimated number of 2.8 million patients who suffer from diabetes in France will cause immense costs on the health assurance system (18,19). Diabetes, nevertheless, is also an important risk factor for many other systemic and inflammatory diseases like heart disease, pulmonary diseases and periodontitis that is now the most frequent infectious disease on our planet. There is a need to understand the pathological mechanisms that hide behind these sometimes very complex clinical pictures and to develop preventive and curative therapy options for the future (20).

Hereby the control of the metabolism of diabetes patients is one of the goals in preventive diabetes therapy (21). Another goal is to discover the pathological mechanisms that rise after non physiological regulation of the metabolism caused by a lack of glycaemic control along the preventive recall therapy (22). The correlation between antimicrobial proteins, innate immune responses and diabetes is not well understood and the state of art literature does not reflect the importance on this highly interesting topic, except typical diabetic infectious diseases like the diabetic foot (23). For this topic constellation only 3 publications could be matched in PubMed. The aim of this work was to evaluate the presence of CgA in saliva of diabetes type 2 patients suggesting a correlation between diabetes type 2 and concentrations of CgA in saliva. Further, CgA should be tested for being appropriate as a non-invasive biomarker for diabetes.

Materials and Methods

Patients

A total of 60 patients was included in this study. 30 patients with type 2 diabetes mellitus and 30 healthy individuals were recruited for the control group. Diagnosis of the patients underly following criteria: Inclusion criteria were defined as follows:

-No (other) systemic diseases
-No administration of antidepressiva within the past six months.
-No history of cancer
-No administration of antibiotics in the last 6 months
-No renal failure or diseases
-No oral chronic infectious diseases like periodontitis, for example

To avoid interference with circadianperiodic effects on gene expression of CgA, all patients visited the ambulance of the Pôle NUDE Service d'Endocrinologie, Diabète et Maladies Métaboliques, Hôpitaux Universitaires de Strasbourg between 7 a.m. and 11.00 a.m. for the routine recall recruitment of blood samples and general examinations. The study has been approved by the local committee of the University of Strasbourg and Hopital Civil on the basis of the World Medical Association's declaration of Helsinki as a statement of ethical principles for medical research involving human subjects, including research on identifiable human material and data (Helsinki 1964-Seoul 2008).

Collection of the saliva samples and further processing

Saliva samples were taken from 30 patients each with or without diabetes type 2. All samples were taken without oral salivary gland stimulation. The patients were instructed to salivate directly into Falcon tubes (Falcon 50 ml, Germany) including 500µl RL (24,25,26) buffer with protease inhibitors (Complete cocktail tablets, Roche, Germany) for preserving proteins from degradation processes. Samples were directly transferred into ice containers at least 4 °C. Then all samples were aliquoted to 1.5 ml Eppendorf tubes. The samples were then frozen at -80 Celsius for future use. Before performing the Bradford assay, the Eppendorf tubes were centrifuged at 4 °C for 12min at 14000 rpm. After that the supernatant has been taken for calculating whole protein concentration by Bradford assay. The presence of chromogranin A and derived peptides was determined in whole saliva by Bradford assay, SDS-PAGE electrophoresis and immunoblotting (Western Blot) of chromogranin A.

Electrophoresis

After Bradford assay the calculated amount of the protein (25 µg) per well from whole saliva solution samples was transferred into further Eppendorf tubes (1,5ml) and equilibrated in 2x Laemmli solution. The solutions were then denatured at 95 °C for 5min prior to loading on a 4-12% SDS-polyacrylamide gel (NuPAGE, Invitrogen, USA). Electrophoresis was performed in MES SDS running buffer (Invitrogen, USA) at 120V for about 3 hours.

Immunoblot Analysis

Proteins were electrotransferred (Semi-dry, Biorad, USA) to polyvinyl difluoride membrane (Amersham Biosciences, Uppsala, Sweden) and immunodetected with specific mouse monoclonal antibody raised against Chromogranin A (1:1000) (sc-53013, Santa Cruz Biotechnology, Santa Cruz, USA) after blocking the blots with 3% blocking buffer (0,1% Tris buffer saline-Tween) overnight at 4 °C. An anti-goat antibody conjugated to horseradish peroxydase was used as a secondary antibody (1:10 000 dilution, Santa Cruz Biotechnology, Santa Cruz,, USA). To detect beta-actin, the blots were incubated with mouse monoclonal anti-beta-actin antibody (Santa Cruz Biotechnology, Santa Cruz, USA) overnight at 4 °C in blocking buffer (0,1% Tris buffer saline-Tween). An anti-mouse antibody conjugated to horseradish peroxydase was used as a secondary antibody (1:10 000 dilution, Santa Cruz Biotechnology, Santa Cruz, USA). The fluorescence immunodetection was performed with the SuperSignal West Extended Duration Substrate Kit (Pierce, PERBIO Science Brebières, France) according to manufacturer's instructions.

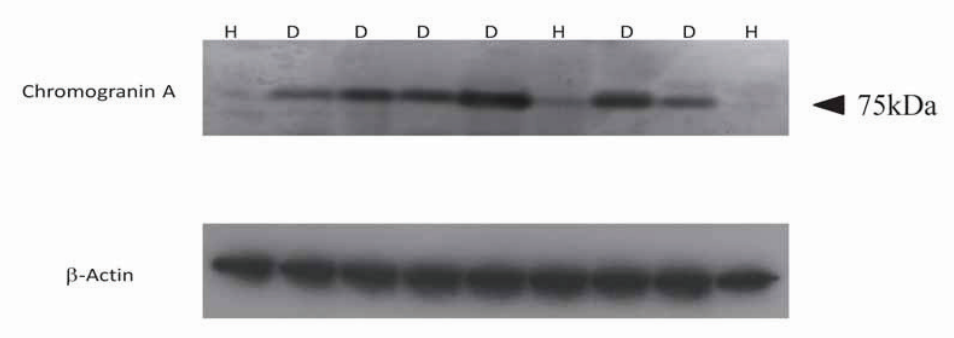

FIGURE 1. Western Blot analysis reveals overexpression of Chromogranin A in the saliva of diabetes patients type 2 (D) in comparison to healthy controls (H). Beta-actin gene expression has been used as control for equilibrating protein concentrations by Bradford assay

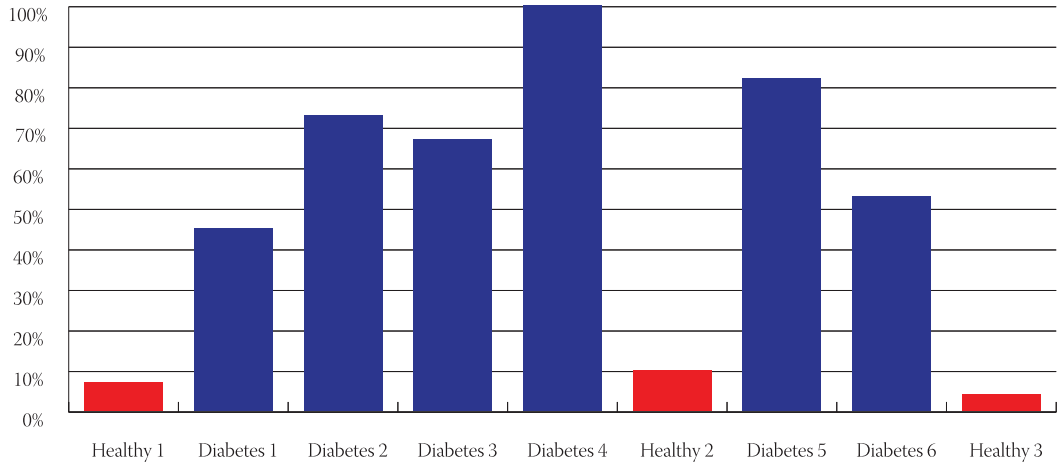

FIGURE 2. Representative gene expression profile (band intensity) of different type 2 diabetes patients (blue) in comparison to healthy subjects (red)

Results

Chromogranin A Western Blot analysis show a strongly higher significance of gene expression in the saliva of all type 2 diabetes patients than compared to all controls (Figure1). Although there are strong differences in protein expression among the diabetes patients, a 4times fold gene expression in comparison to control never falls below the value (Figure 2). The mean values compared to controls result even in a more clear signifancy, 8% for the control group to 76% for type 2 diabetes patients (Figure 3).

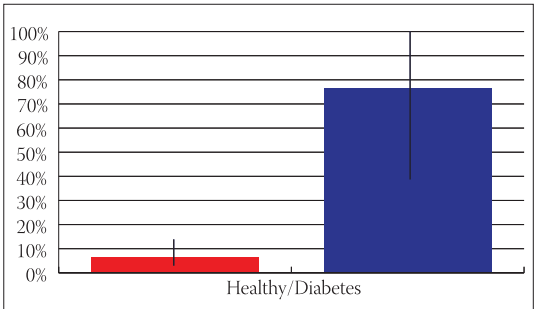

FIGURE 3. Mean value of the gene expression (protein concentration) between 30 healthy subjects and 30 patients with type 2 diabetes

Discussion

In our study we demonstrated, for the first time, the overexpression of the antimicrobial precursor protein and biomarker for cancer Chromogranin A in saliva of patients with type 2 diabetes. This observation leads us to different questions concerning the biological and pathobiological sense of such an overexpression in the oral cavity. Human saliva implicates a variety of totally different aspects of biological functions that mirror its characteristics as a barrier between the inner system of an organism and the outer world. The role of saliva in the health of the patient is up until our time not well investigated, especially concerning systemic diseases. The

detection of salivary gland hypofunction and the psychological states of an individual are well documented in the state of art literature (27). In this context there are also some reports concerning CgA expression in saliva, stress factors and dry mouth syndrome (28-30). These studies show that the psychological state of a patient has impact on the production of specific proteins and thereby mirrors a psychological state or pathological condition although there are also contradictory results (31,32). Thus, such diagnostic means performed in the saliva could prevent maybe some complications caused by such conditions (33). It has to be emphasized that the psychological aspect that has to be proved further in double-blind large clinical studies, did not play any role in our investigation as the expression of CgA in the control subjects clearly show. But what about infectious diseases in the oral cavity? What influence do they have on Chromogranin A expression and where are the effector cells and proteins located exactly if only partially in the salivary glands where chromogranin A has been already detected (34)? There is a scientific discussion about the interrelationship between diabetes type 2 and periodontitis, for example (20). Our group is working on innate immune responses and oral health, and we know that some pathogens from different strains are able to induce antimicrobial peptides and proteins in totally different types of tissue and cells (data not shown). Some of these imply a long history of evolutionary processes by finally developing a strategy using angiogenetic and innate immune responses for their own essential purposes (35). The mucosal epithelium has immense importance in host defence and immune surveillance. This specialized interaction results in either passive coexistence between microbe and host, as in the case of commensal microbes, or a breach of the mucosal barrier and subsequent cell injury, as in the case of microbial pathogens (36). Accordingly, the oral epithelium is able to secrete a variety of defence effector molecules including pro-inflammatory cytokines (37,38) and antimicrobial proteins (39,40) to counteract any invading pathogens. Immune responsiveness to many microbial pathogens depends on a family of pattern recognition receptors known as Toll-like receptors (TLRs), which are the major innate recognition system for microbial invaders in vertebrates (41). In this infection and inflammatory cascades we can build up a correlation between interleukin- 1 beta (IL-1 β)(42-45) and TNF-alpha (TNF-α) (46-48) that are overexpressed in sera of diabetes type 2 patients and the possible influence of these cytokines on the cells in the salivary glands. Challenging primary cells with different cytokines like IL-1 β, TNF-α, IL-6 and so on or Lipopolysaccharides (LPS) and other bacterial toxins, we can see a strong induction of antimicrobial proteins compared to control cells (data not shown). Therefore, the role of the microvascular networks, especially in the oral cavity, might play a key role in the phylogenetically ancient innate immune defense system. In this context, it is important to take into account that microvascularisation in type 2 diabetes is harmed depending on severity and duration of the diagnosed diabetic pathological picture. It is challenging for the future to shed light on the mechanism of action concerning our observations in saliva of diabetes patients.

Conclusion

Chromogranin A, a circulating biomarker for epithelial tumours, is also overexpressed in saliva of type 2 diabetic patients. Further randomized double-blind studies with larger amount of patients are needed to confirm our results and to prove the applicableness of Chromogranin A as a new non-invasive biomarker for type 2 diabetes.

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