Chromogranin A detection in saliva of type 2 diabetes patients

Martine Soell¹,², Ahmed Feki³, Matthias Hannig⁴, Hidehiko Sano⁵, Michel Pinget⁶, Denis Selimović*¹,²,³

¹ Department of Periodontology, Hautepierre Hospitals, University of Strasbourg, France
² INSERM Unit 977, 11 Rue Humann, 67085 Strasbourg Cedex, France
³ Department of Oral Medicine and Oral Surgery, University Hospitals, Strasbourg, France
⁴ Clinic of Operative Dentistry, Periodontology and Preventive Dentistry, University Hospitals, Saarland University, Building 73, D-66421 Homburg/Saar, Germany
⁵ Department of Restorative Dentistry, Division of Oral Health Science, Hokkaido University Graduate School of Dentistry, Kita-13, Nishi-7 Kita-ku, Sapporo, 060-8586 JAPAN
⁶ Pole NUDE, Medecale B, Department of Endocrinology, Diabetes and Metabolic Diseases, University Hospitals, Strasbourg, France

* Corresponding author

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 periods 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 globular 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 splanchic 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 abscess 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 scanscopic 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 posttransduclional modifications like phosphorylations, glycosylations and sulfataions that regulate their biological activity and degradation processes. These modifications depend on the physiological state of the individual organism (2). Diabetes, for example, an metabolo-endocrin 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 arise 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 Hospital 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 14,000 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 Laemml 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 peroxidase 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 peroxidase 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.
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 (Figure 1). 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).

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
We want to thank the Hopital Civil de Strasbourg for supporting us in organizing this study which was partially financed.

**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 applicability of Chromogranin A as a new non-invasive biomarker for type 2 diabetes.

**ACKNOWLEDGEMENTS**

We want to thank the Hopital Civil de Strasbourg for supporting us in organizing this study which was partially financed by the fund: Bourse Jean-Marie Warter, HUS No 3739 dedicated to Dr. Martin Soell and Professor Denis Selimovic for the research project: "Examen de la présence de fragments antimicrobiens dérivés des chromogranines dans le fluide gingival, le sang et la saliva de patients diabétiques avec et sans parodontopathies ". Dr. Soell and Professor Selimovic would like to express their special thanks to Professor Michel Pinget, the President of RESO DIABETE ALSACE and Chef du Pôle NUDE, Medicale B, Service d’Endocrinologie, Diabète et Maladies Métaboliques and Professor William Bacon, Chef de Pôle Odontologie who made this whole study possible.
REFERENCES


(41) Schonwetter B.S., Stolzenberg E.D., Zadoff M.A. Epithelial antibiotics induced at sites of inflammation. Science 1995; 267: 1645


