Experiences with the SELDI-TOF Method for the Detection of the Diabetes Associated Antigen GAD 65 in the Peripheral Blood of Patients with Diabetes and Inflammation

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Abstract

The treatment of diabetes mellitus type 1 is a major challenge in health care. The diabetes associated antigen Glutamic Acid Decarboxylase (GAD 65) has been described as strictly intracellular. However an intracellular antigen would never have access to the immune system. In a previous study with the high sensitive fluorescence correlation spectroscopy (FCS), we detected the GAD 65 antigen in the sera of patients with type 1 diabetes and related autoimmune diseases. FCS is a time consuming measurement procedure and very sensitive to disturbance, which makes unsuitable for a routine screening of the patient sera for GAD 65. Since the measured GAD 65 concentrations were surprisingly high (2.65 microgram/ml) and therefore within the detection range of less sensitive methods, in this study we used the SELDI-TOF (Surface enhanced laser desorption ionisation - time of flight) technique to confirm the FCS results in view of an early diabetes diagnosis. For this purpose we developed an array with the SELDI-TOF method and analysed 4 patient sera (2 diabetes and 2 controls) in a pilot experiment. For one diabetes serum, we found some evidence for the diabetic antigen GAD 65 in the mass spectra in a peak close to albumin with a molecular weight of 65 kDa. The 3 other samples remained negative. Because of the higher concentration of albumin in the sera, a peak evaluation in regard of GAD 65 was difficult and not reliable in this setup. Troubles and experiences with SELDI-TOF for the detection of GAD 65 are discussed.

Key words: Diabetes Mellitus Type 1, autoimmunity, Glutamic Acid Decarboxylase (GAD 65) antigen, Surface enhanced laser desorption ionisation - time of flight (SELDI-TOF)

Introduction

Diabetes mellitus type 1 is an autoimmune disease with various antigen systems involved. It is one of the most debilitating and life threatening autoimmune diseases, where an early diagnosis is desirable and is also the prerequisite for successful prevention and therapy (1-5). Several attempts have been made based on genetic and metabolic analysis (6-10). In patients with critical limb ischemia inflammatory markers like tumor necrosis factor- and monocyte/macrophage product neopterin were described to be elevated in patients with diabetes as compared to non-diabetic (11). Moreover, raised urinary neopterin levels were found in islet cell antibodies (ICA) positive and 10 ICA negative diabetics at onset of disease compared

with age-matched control subjects (12). In longer standing diabetics, neopterin levels were significantly higher in ICA positive than in ICA negative patients.

Glutamic Acid Decarboxylase (GAD 65) is one of the antigens playing a considerable role in diabetes mellitus type 1 and the Stiffman-syndrome (13, 14). However, it is located in the cellular compartment of islet-cells and it has never been shown to exist in the peripheral blood. Research on the circulating GAD 65 antigen has been neglected and its existence in the peripheral blood denied. But such a strictly intra-cytoplasmatic antigen would never have access to the immune system. In a previous publication, we described how we designed the ultrasensitive FCS (fluorescence correlation spectroscopy) technology and found GAD 65 to exist in peripheral blood as

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opposed to the current opinion in the literature (15). The results of the high sensitive FCS method showed, on one hand, that the concentration of GAD 65 in sera was surprisingly high and, on the other hand, the method was very sensitive to influence by other components (other proteins, free dyes etc.) of the human serum.

Therefore, in order to have additional information about the occurrence of GAD 65 in the blood, we used a less complicated and less sensitive method apart from FCS, namely the SELDI-TOF "Surface enhanced laser desorption ionisation - time of flight" -technology. This is well established for the research on biomarkers having the advantage that no additional detection systems such as radioactivity, enzyme- or fluorochrome labelling must be employed (FCS requires labelling with fluorochromes). SELDI-TOF is a method of mass spectroscopy, where specific proteins are identified by characteristic peaks of the mass spectrogram. With the advantages of the SELDI-technique in mind we tried to confirm our previous results and to find further evidence on the existence of GAD 65 in human blood.

Materials and Methods

Specimens

Sera from 4 patients have been investigated. The sera from 2 paediatric patients with early onset of diabetes mellitus type 1 and 2 controls were kindly provided from the serum bank of the Department of Paediatrics of the Medical University of Graz. Controls consisted of persons from normal admissions to the clinic.

Reagents and protein chips

The sera were tested on a PS20 slide (by following a previously defined protocol), provided by Phadia (former Pharmacia), Sweden-diagnostics, Freiburg-Breisgau, Germany. PS20 ProteinChip Arrays are pre-activated with carbonyl dimidazole chemistry that covalently binds to free primary amine groups. The arrays are 8 spot chips with 2 mm diameter spots, spatially compatible with one column of a standard 96-well microplate (9 mm well pitch). The monoclonal GAD 65 antibodies Z1 and Z2 (provided by Phadia) were used and fixed covalently on the PS20 protein chip. Antibody Z1 and antibody Z2 are reacting with different epitopes of the GAD 65 molecule. In a first experiment, sera without dilution were used and in a second experiment, the sera were diluted 1:20. The investigations were made with the CIPHERGEN system (CIPHERGEN Protein Chip Software 3.0) and the calibrations of the spectra were realized in point of view of

the GAD 65. The CIPHERGEN's ProteinChip® System is comprised of a ProteinChip Reader integrated with ProteinChip Software and a personal computer to analyze proteins captured on ProteinChip Arrays.

SELDI-TOF

Mass spectroscopy was carried out according to the SELDI-TOF (Surface enhanced laser desorption ionisation - time of flight) system (16, 17). SELDI-TOF has been proven as suitable tool in the clinical laboratory for the profiling of biomarkers in complex biological specimens such as: serum, plasma, intestinal fluid and urine (18-26). It consists of an aluminium carrier, called the array, a laser unit and a system which measures the time of flight (TOF) of the molecules. The TOF system includes an electric field, induced by the acceleration potential, and a detector. Photomultipliers, avalanche diodes etc. are used as detectors.

Chip array: The different chip array types have either: surfaces that bind many different proteins or surfaces with a specific biomolecular affinity. The chips of the second type are composed of biochemically active surfaces, such as: immobilized antibodies or receptor proteins, and bind only specific molecules. Therefore, they are used to exploit specific molecular recognition mechanisms, enabling the focus on the proteins of interest. After putting the sample on the chip, the weakly bound molecules are washed away. The remaining sample molecules are mixed with small photosensitive molecules. These molecules cause the sample to crystallize, form the matrix as it dries and facilitate the ionization of the proteins.

Laser desorption and ionization: The chip with the samples is put into a vacuum chamber, the flight tube of the mass spectrometer. The laser pulse excites the photosensitive matrix molecules. The energy of the excited molecules is converted into thermal energy which heats up the sample spot. The overheated part of the sample mix explodes into a plume and the molecules are liberated from the array. The protein molecules in the plume collide with the excited matrix molecules, whereby the matrix molecules transfer protons to the proteins and create charged proteins. Due to repeated processes, the proteins can get multiple charges.

Mass spectrum: The counted totals per time interval are displayed in the spectrum. The detection of molecules with the same molecular weight and the same electric charge produce a signal which is called a singleton peak. A peak in the spectrum is then the signal induced by neighbouring singleton peaks. The peak

Figure 1. The spectra of the pure patient sera show very exposed peaks resulting from albumin at 64.697 kDa. The peaks at 50, 75 and 150 kDa result from the antibodies. Because the expected GAD molecule has a molecular weight near albumin (65 kDa), identification in patient sera under these experimental conditions is not possible.

area is proportional to the number of detected molecules. The baseline of in the spectrum is formed by the dark current inside the detector and the detected air molecules.

Results

To realize a diagnostic procedure, every serum was tested in duplicate with different successive dilutions (for example: 1:5, 1:10). We found evidence for GAD 65 in one serum sample in the mass spectrum, which gives us a peak very close to albumin with a molecular weight of 65 kDa. This serum was from a child with an early onset of diabetes mellitus type 1. The concentration of albumin in sera is of course tremendously higher than the concentration of the expected GAD 65. By use of the protein chip PS20 with fixation by covalent bonds of the GAD 65 antibodies, the contamination of the exuberant albumin is less disturbing. Peak evaluation after spiking experiments was done to have comparable results as with FCS (15). The spectra always show the peaks of the antibodies at 50, 75 and 150 kDa.

Evaluation of the spectra resulting from pure sera's on the P20 slides

The spectra of the pure patient sera on the P20 slide display very exposed peaks resulting from albumin (Fig. 1). As reference, albumin was measured showing a peak at 64.697 kDa in the spectrum. Because the expected GAD 65 molecule has a molecular weight of 65 kDa, an interpretation of the spectra of the sera under these experimental conditions is not possible.

Evaluation of the spectra resulting from sera diluted at 1:20

The spectra of the diluted sera (Fig. 2) show a much diminished peak of the reference albumin. For the interpretation of the results, we have calibrated in patient sera the obtained peak intensity at 65 kDa in proportion to the intensity of the albumin peak in the reference. The intensity of the appropriate peak in the spectrum is greater than the peak obtained for albumin by a factor of 8.4 (Table 1).

Discussion

Whereas the current opinion in the literature conforms to the strict intracellular localization of GAD 65, this hypothesis can not explain the immunization and the occurrence of GAD 65 antibodies. Our assumption was that it has to be in the serum of patients and should be found in higher concentrations before the onset of the disease, since GAD 65 has to prime the immune system as an initial step for the following pathology. Biomarkers have been proven to play an important role for the early diagnosis of diseases, such as: cancer, Alzheimer's disease etc. (27-33). Assuming that GAD 65 occurs at the early stage of diabetes mellitus type 1 and remains present during the course of the disease, it would be a suitable candidate as a biomarker.

Therefore, we started our search for the GAD 65-antigen in a soluble form in the peripheral blood. Unknown concentrations let us use the ultrasensitive FCS with the astonishing result of relatively high GAD 65 concentrations (15). FCS is a very sensitive method and is even able to detect single molecules (in "clean systems"), but the technique requires a labelling of the antibodies, which is very difficult to achieve and time consuming. Furthermore, the method is very sensitive to disturbances resulting from other molecules and free dye contamination. In view of a routine diagnosis and starting experiments on a possible biomarker profiling, this time we tried a simpler method where sophisticated labelling techniques can be avoided. SELDI-TOF offered us the simplicity and the already known access to discover biomarkers in the peripheral blood in microgram amounts. This technique has the advantage that no labelling of the antibodies is necessary for the detection of the molecules. Only their molecular weight characterized by the TOF leads to the identification of the expected antigen GAD 65.

By the use of SELDI-TOF and the study of some specimens used in the previous work with FCS we found some evidence for the diabetic antigen GAD 65 in a peak close to albumin. We were successful in fixing GAD 65 in our experiments in a sandwich on the aluminium array. The albumin, produced in the liver, is the most abundant protein in human blood plasma. The reference range for albumin in blood is 35 to 53

Table 1. The intensity of the peak at 65 kDa for specimen 28 is 8.40x the intensity of the corresponding peak for albumin. This serum results from a child with diabetes mellitus type 1 at an early stage.

Specimen	8	25	28	29
GAD/Albumin	0.95	1.70	8.40	2.26
Interpretation	negative	negative	positive	negative

Figure 2. The sera are diluted at 1:20. The spectrum of the diluted serum 28 shows a peak at 65kDa which may results from GAD65. The intensity of the peak is greater, by a factor of 8.4, than the corresponding albumin peak. This serum is from a paediatric patient with diabetes mellitus type 1 at an early stage.

mg/ml. Its concentration is therefore higher, by a factor above 10.000, than the expected value of GAD 65 (2.65 microgram/ml). The molecular weight of albumin is 66.47 kDa and therefore near the molecular weight of Glutamic Acid Decarboxylase (65 kDa). The weights differ only by 2.26%. Because of the tremendously higher concentration of albumin in the sera, a peak evaluation in regard of GAD 65 could not be achieved in a proper manner. Although an array with GAD 65 antibodies was used to pick out the antigen of the sera, it seems that the omnipresent albumin could not be completely washed out, but remained partially on the chip bounded by van der Waals interactions. This leads to a superposition of the GAD 65 peak and the albumin peak at approximately 65 kD, which complicate the proper detection of the antigen. The results of these first trials show that the application of SELDI-TOF for the detection of GAD 65 in human sera, in the manner presented here is not reliable for a routine procedure.

In conclusion, the development of new techniques for the separation of GAD 65 from albumin is necessary for an accurate and reliable detection of the diabetes type 1 antigen. It would be also interesting to find out whether any association might exist between the appearance of GAD 65 in human blood and increased neopterin concentrations which appeared to be a sensitive indicator for the activation of cell-mediated immunity in type 1 diabetes even when ICA were undetectable (12). To obtain further evidence on the existence of GAD 65 in human blood, we used the ELISA (Enzyme-Linked Immunoabsorbent Assay) technique in a further study for the confirmation of the presence of the antigen (manuscript in preparation).

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