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Identification of a 48 kDa form of unconventional myosin 1c in blood serum of patients with autoimmune diseases

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ABSTRACT

We searched for protein markers present in blood serum of multiple sclerosis (MS), rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) patients in comparison to healthy human individuals. We used precipitation/extraction methods and MALDI TOF/TOF mass spectrometry, and identified a protein with Mr ~46 kDa as a fragment of human unconventional myosin IC isoform b (Myo1C). Western blotting with specific anti-human Myo1C antibodies confirmed the identity. Screening of blood serum samples from different autoimmune patients for the presence of Myo1c revealed its high level in MS and RA patients, relatively low level in SLE patients, and undetected in healthy donors. These data are suggesting that the level of p46 Myo1C in blood serum is a potential marker for testing of autoimmune diseases.

1. Introduction

Blood serum has been extensively explored as a source of markers, as it may contain not only blood proteins *per se*, but also proteins originating from all tissues of the body [1,2]. It is estimated that up to 10,000 different proteins (and/or its fragments) may be present in the blood serum, and most of them are in very low concentrations [3]. Selection of a protein preparation, and especially enrichment procedures, may aid in successful search for markers [2,3].

Autoimmune diseases are characterized by auto destructive processes. Initiation of these processes may differ for different autoimmune diseases, but they may also include similarities related to execution mechanisms of cells and tissues degradation. Therefore, it is possible that different autoimmune diseases may share even presentation of markers. Multiple sclerosis (MS) is an inflammatory disease in which the myelin sheaths around axons of brain and spinal cord are damaged, thus, leading to demyelination and scarring [4]. This results in a wide range of symptoms. Mechanisms of MS have autoimmune character, due to cellular and humoral immune reactions towards brain tissue self-antigens, mainly the myelin basic protein (MBP) [5]. The key feature of the MS patients is the formation of inflammatory sites in brain tissue, which may lead to presence of tissue proteins leaking into the blood [4,5]. RA and SLA also characterized by inflammation and tissue demerged which can elevate of amount of cellular proteins in blood serum [6,7]. For purification and concentration of proteins for MALDI TOF/TOF a 2,2,2-trichloroacetic acid (TCA)-induced protein precipitation is frequently used [8,9], although in our hands TCA-extracted proteins remain out of the study.

We attempted to identify the proteins in TCA-extracted fraction of blood serum of MS, RA and SLA patients versus normal human donors and to estimate their potential diagnostic value.

Here, we report identification in human blood serum of unconventional myosin Ic (Myo1C) that is ubiquitously expressed in vertebrates. We propose that this protein could serve as a potential marker for MS, RA and SLE autoimmune disorders.

Abbreviations: MS, multiple sclerosis; PA, rheumatoid arthritis; SLE, systemic lupus erythematosis; NHD, normal healthy donors

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2. Materials and methods

2.1. Human blood serum samples

Serum was obtained from the peripheral blood of 28 MS patients (diagnosed according to the McDonald diagnostic criteria for MS), 12 SLE patients (diagnosed according to ACR criteria for SLE), 12 RA patients (diagnosed according to ACR/EULAR Rheumatoid Arthritis Classification Criteria) and of 12 healthy volunteers. The samples were collected under the approval of Bio-Ethics Review Board of the Danylo Halytsky Lviv National Medical University in accordance with the regulations of the Ministry of Health of Ukraine. A documented consent was obtained from all patients included in the study, and the form of the informed consent was approved by the Bio-Ethics Review Board of the Danylo Halytsky Lviv National Medical University.

2.2. Preparation of TCA-soluble proteins

According to our protocol, 1 ml of blood serum was diluted in 2-fold with phosphate buffer saline (PBS), and then 100% TCA was added to 10% of final concentration. After 30-min incubation at −20 °C, the solution was centrifuged for 15 min at 10,000 g. The supernatant containing TCA-soluble compounds was isolated and mixed with acetone in 1:6 ratio, followed by incubation at −20 °C for 18 h. The precipitate was pelleted by centrifugation for 10 min at 10,000 g. The pellet was diluted in distilled water and protein concentration was measured at 280 nm using a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies, USA). The solution was stored at −20 °C until use.

2.3. SDS-electrophoresis and Western-blot analysis

SDS-electrophoresis in 12% PAG [10] of blood serum TCA-extracted proteins was followed by gel staining with Coomassie Brilliant Blue G. For Western blot analysis, proteins were transferred from the gel onto a nitrocellulose membrane. The membranes were blocked (1 h at 20 °C) with 5% non-fat milk in the PBS buffer containing 0.05% Tween-20. The blots were washed with PBS-Tween-20, three times for 5 min each, and then probed with the specific Abs. To identify 46 kDa polypeptide in a pool of TCA-extracted proteins of blood serum, polyclonal anti-MYO1C (N-terminal region) rabbit antibody (AVIVA SYSTEM BIOLOGY, product number ARP56292) in 1:1,000 dilution was incubated overnight at 4 °C. Corresponding secondary antibodies were used for ECL detection. Generated images were scanned, digitalized and images were quantified using ImageJ software.

2.4. MALDI-TOF mass spectrometry

Individual electrophoretic protein bands were excised from the gel and subjected to the in-gel trypsin digestion as was described earlier [11]. To identify the proteins, their tryptic digests were subjected to peptide mass fingerprinting (PMF) followed by post-source decoy (PSD) fragmentation. MALDI TOF/TOF mass spectrometry was performed on an Ultraflex instrument (Bruker, Germany). Samples were mixed with alpha-cyano-4-hydroxy-cinnamic acid, and loaded on a metal target plate. Mass spectrum was collected in the positive mode. i.e. for MH+ ions. Internal calibration was performed with tryptic peptides generated upon auto-digestion of trypsin added to samples (842.51, 1045.56 and 2211.10 Da). Collected mass spectra were used for searches with Profound tool of NCBInr database. Search criteria were as follow: no limitations of species and pI, mass selection as for 10–100 kDa, MH+, complete alkylation of Cys, partial oxidation of Met, one missed cleavage by trypsin, and tolerance of 0.5 Da. Selected main peaks were subjected to PSD by LIFT technology developed by Bruker and available for Ultraflex instruments. Fragmentation spectra were collected, and delta masses of observed fragments were used for calculation of the sequence.

2.5. Statistical analysis

Significance of the difference in a typical experiment was assessed by Student's *t*-test. The level of significance was set at 0.05. Three levels of significance were used $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

3. Results and discussion

3.1. TCA-extracted blood serum proteins and their characterization

For isolation of the TCA-extracted proteins from blood serum, a procedure including treatment of blood serum by 10% trichloracetic acid, pelleting of the insoluble debris by centrifugation and following precipitation of the soluble compounds with acetone in 1:6 ratio were used. Fig. 1 shows a typical SDS-PAG electrophoretic profile of the acetone precipitated proteins. We observed that the TCA-soluble fractions contained proteins with the molecular mass of 66 kDa (Fig. 1, lanes 1–7) and 46 kDa (Fig. 1, lanes 5, 6).

3.2. Identification of MyoC1-like protein in a pool of TCA-extracted blood serum proteins

We focused further on identification of detected proteins, which are shown in Fig. 1. To identify the polypeptides with molecular mass of 66 kDa and 48 kDa, MALDI TOF/TOF mass spectrometry analysis based on peptide mass fingerprinting was used.

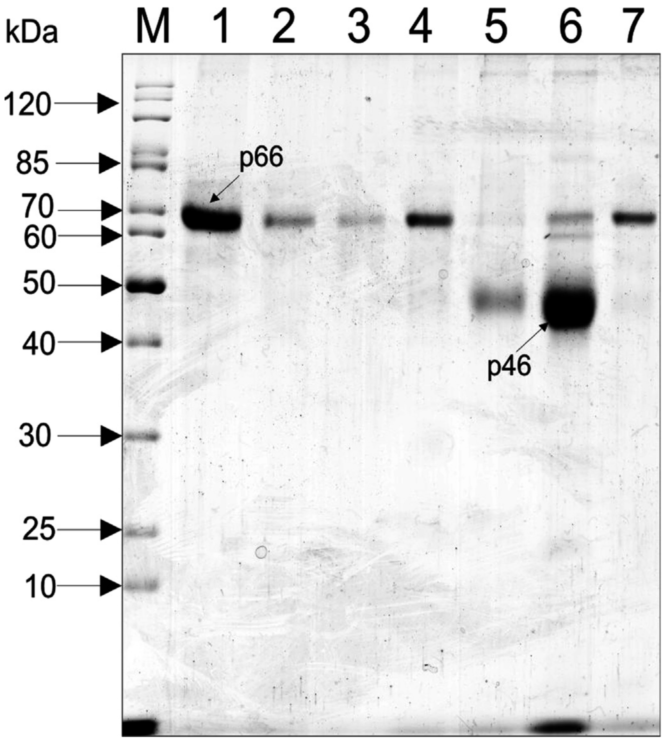

Fig. 1. A typical SDS-electrophoresis in 12% PAG of TCA-extracted proteins isolated from blood serum of multiple sclerosis patients. M, molecular masses protein standards. Lanes 1–7, the samples of TCA-extracted proteins. Arrows point at the polypeptides which were subjected to MALDI-TOF mass spectrometry.