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Short communication

Differential expression of alpha II spectrin in monocytes of tuberculosis patients ☆

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ABSTRACT

Monocytes play a crucial role in immune response to tuberculosis. The present study focuses on identifying differences in the monocyte proteome profile of tuberculosis patients, household contacts and healthy controls. Differential protein expression was studied by two-dimensional (2D) gel electrophoresis. One of the spots consistently showed either lower intensity or was absent in patients and was identified as alpha II-spectrin. The decreased expression of αII-spectrin was further validated by quantitative PCR (qPCR) and western blot analysis. This study suggests the possible role of decreased levels of αII-spectrin in the pathology of tuberculosis.

1. Introduction

Tuberculosis continues to be the leading cause of morbidity and mortality worldwide [1]. Despite several studies the immune mechanisms involved in the disease protection have not yet been fully understood. Granuloma, which is the hallmark of tuberculosis consists of tubercle bacilli infected macrophages which are surrounded by mononuclear cells like fibroblasts, leukocytes and monocytes. The role that the newly recruited monocytes play inside the granulomas is still unknown. It has been observed that monocytes isolated from healthy controls undergo apoptosis when exposed to *Mycobacterium tuberculosis* (MTB) in vitro whereas those from patients underwent both necrosis as well as apoptosis [2]. It was also observed that such an alteration in the fate of monocyte was reversible following drug treatment to eliminate the bacteria [3]. Recent studies have shown that monocytes from healthy individuals when infected with MTB in vitro have altered ability to differentiate into macrophages; this was attributed to apoptotic events taking place during differentiation in vitro [4]. It would therefore be interesting to study the proteome profile of monocytes in tuberculosis patients in comparison to their healthy household contacts (HHC).

Of late proteomics has been used extensively to identify differences which help in the identification of markers associated with the disease. Several studies have been carried out for identifying protein signatures in different disease conditions [5–7]. Studies have also been carried out to determine the differences in proteome during monocyte to macrophage differentiation [8,9]. Although monocytes from tuberculosis patients and controls have been studied as mentioned above, to the knowledge of authors, no proteomic study of such monocytes appears to have been done. In this study an attempt has been made to detect any differences in the adherent cell population derived from the peripheral blood mononuclear cells (PBMCs) of patients, HHC and healthy controls. The proteomic study was further confirmed by using qPCR and western blot analysis.

2. Material and methods

2.1. Isolation of peripheral blood monocytes

PBMCs were isolated from peripheral blood of five patients as well as their HHC and healthy controls. The patients were identified on the basis of positive sputum acid fast bacilli (AFB) staining and chest X-ray reports. The HHC and healthy controls included in the study are asymptomatic. All patients were males while HHC and controls included both males (two) and females (three) each for proteomic study and western blot analysis. The age of the individuals included in the study ranged between 25 and 40 years of age.

All procedures used in the study were approved by Institutional Ethics Committee for Human Research of the Faculty of Science, M. S.

Abbreviations: MTB, *Mycobacterium tuberculosis*; HHC, household contacts; PBMCs, peripheral blood mononuclear cells; AFB, acid fast bacilli; EDTA, ethylene diamine tetraacetic acid; PBS, phosphate buffered saline; 2-D, 2-dimensional gel electrophoresis; IPG, immobilized pH gradient strips; qPCR, quantitative PCR; HRP, horseradish peroxidase.

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University of Baroda. Written informed consent was obtained from all subjects.

Isolation of monocytes was carried out within 5 h of collection as described previously [10].10 mL of blood was collected from each individual in ethylene diamine tetraacetic acid (EDTA) coated vacutainer tubes and monocytes were isolated by density gradient centrifugation on Ficoll-hypaque followed by adherence for 2 h in T25 tissue culture flasks in medium containing RPMI-1640. The non-adhered mononuclear cells were separated from the adhered monocyte preparation by washing vigorously with phosphate buffered saline (PBS). The remaining adherent cells were considered as the monocyte preparation, used in subsequent processing for analysis by 2-dimensional electrophoresis (2-DE).

2.2. Protein sample preparation from monocytes

Cell lysis of the monocytes was performed using 1 mL of lysis buffer (5 M Urea, 0.25% 3-[(3-cholamidoproplyl) dimethylammonio]-1-propanesulfonate (CHAPS), 0.25% Tween 20, 100 mM dithiothreitol (DTT), 10% isopropanol, 5% glycerol, protease inhibitor, 100 mM sodium orthovanadate). This was followed by protein precipitation of monocyte lysate using Methanol Chloroform method [11]. Protein precipitates were then resuspended in 2D sample/rehydration buffer (Bio-Rad) and the protein was estimated using Bradford assay.

2.3. Two-dimensional electrophoresis

Following protein estimation, an aliquot equivalent to 25 μg total protein was used for rehydrating the immobilized pH gradient (IPG) strips (Bio-Rad, USA) pH 3–6 overnight in rehydration buffer. First dimension isoelectrophoresis (IEF) was performed in three steps: at 250 V for 20 min, at 4000 V for 2 h, 40,000 Vh for 4 h. Prior to the second dimension, the IPG strips were equilibrated with Equilibration buffer I (Bio-Rad) for 15 min. This was followed by equilibration for 15 min with Equilibration buffer II (Bio-Rad). After equilibration and alkylation, the proteins separated by IEF were further separated on a 10% polyacrylamide gel. The gels were subsequently treated with a fixative solution followed by staining using a silver staining kit (Fermentas, USA).

2.4. Spot identification

Spot selected for analysis was excised from silver stained gels and sent to Vimta Labs, Hyderabad, India for identification. Briefly, the spots were subjected to in-gel trypsin digestion and peptides generated were subjected to MALDI/TOF analysis. Following the acquisition of peptide mass fingerprint, they were compared with SwissProt data base and the protein was identified.

2.5. Total RNA isolation and reverse transcription

To confirm the results obtained from 2D and spot identification, qPCR was done using monocytes of patients ($n = 10$) and controls ($n = 10$). Both the groups included five males and five females each. Total RNA was isolated from monocytes using commercially available kit (Bangalore Genei, India). The quality of the RNA samples was analyzed by inspecting the integrity of 28S and 18S bands on agarose gel electrophoresis. cDNA was reverse transcribed from 5 μg of each total RNA sample using RT-PCR kit (Thermo Fisher Scientific, Surrey, UK). The quality of cDNA preparation was further confirmed by beta actin amplification.

2.6. Quantitative PCR

Quantitative PCR (qPCR) was done on a Real-Time thermal cycler (Bio-Rad) using specific primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and αII-spectrin. The primers used for αII-spectrin were: SPEC FP 5′-AGC AAG CAC CAG AAG CAC CAG G-3′ and SPEC RP 5′-TCA GCT AAG GCA GCC AGG CG-3′, and for GAPDH were GAPDH FP 5′-TGA GCA CCA GGT GGT CTC C-3′ and GAPDH RP-5′-TAG CCA AAT TCG TTG TCA TAC CAG-3′. The qPCR was done according to the following amplification conditions: initial denaturation at 94 °C for 30 s, followed by 45 cycles of denaturation at 94 °C for 10 s, annealing and extension for 30 s at 60 °C and finally the fluorescence was recorded. The amplification was done in triplicate for each sample and cycle threshold (Ct) was used for gene expression analysis. The expression level of αII-spectrin in each sample was normalized to GAPDH expression of the same sample with the help of CFX manager software (Bio-Rad) for gene expression analysis. The product specificity was confirmed by single peak in melt curve analysis. To check the genomic DNA contamination in sample, the negative controls were set with the total RNA without reverse transcription which did not give any recordable fluorescence (data not provided). Statistical analysis was performed using GraphPad Prism software (GraphPad, San Diego, CA, USA). The Mann–Whitney U-test was applied for group differences ($p < 0.05$).

2.7. Western blotting

The protein isolated from monocytes was separated by SDS-PAGE and transferred to nitrocellulose membranes. After blocking for 1 h in PBS containing 1% gelatine the membrane was incubated with mouse anti-αII-spectrin monoclonal antibody (1:1000, BD Bioscience) overnight at 4 °C. The membrane was then washed thrice with PBS containing 1% Tween-20 followed by 1 h incubation with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody. Subsequently, the membrane was washed thrice with PBS containing 1% Tween-20 and immunoreactive bands were detected by using TMB/H_2O_2 (Bangalore, Genei).

3. Results

3.1. Differential protein expression in tuberculosis patients

We hypothesized that monocytes from tuberculosis patients, their HHC and healthy controls would have distinct proteome profiles. We therefore analyzed the total protein content of monocytes obtained from five individuals of each group. Since our focus was to identify a few proteins that are either positively or negatively correlated with disease state, we analyzed a small amount of the protein extract on a 7 cm IPG strip followed by analysis of 7 × 8 cm SDS-PAGE gels. Using PD Quest software, approximately 450–650 spots could be detected in these gels as can be seen from the figure (Fig. 1) which has representative 2D gel of patients (Fig. 1a), HHC (Fig. 1b), and healthy controls (Fig. 1c), following 2D separation of 25 μg of protein sample, and silver staining. It is apparent that this is a very small fraction of the protein content of monocyte when compared to that reported by others [12]. Since the focus of the present investigation was to detect differences between the proteome of monocytes obtained from tuberculosis patients, their HHC and healthy controls, loading of a greater amount of protein on the strips used would make resolution of individual spots more difficult. As can be seen from the figure (Fig. 1-d,e and f), which shows the magnified images of selected area of the above mentioned 2D gels respectively, only a small number of proteins are visibly up or down-regulated under these conditions.

3.2. Protein identification

Following MALDI/TOF analysis, one of the spots (spot 1) was identified as αII-spectrin which was found to be absent from monocytes of all patients, and not that of any of the HHC or healthy controls. Protein identification following acquisition of peptide mass fingerprint was based on 14 mass values being matched out of a total of 30 mass values searched, and covering 7% of the sequence. Also, the identified protein had a Mowse score of 55, where a Mowse score of 53 at $p < 0.05$ was