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Two Dimensional Protein Map Standardization of Human Bone Marrow Stromal Cells

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Abstract

Human bone marrow stromal cells (BMSCs) have been hailed as a promising source for cell therapy and tissue regeneration due to its multipotent nature. Bone marrow stromal cells differentiate into various tissues at the cues from its surrounding. Proteomics is a powerful tool to elucidate cellular mechanisms. The present study established a reproducible protocol to display the complete qualitative 2-D protein map of BMSCs. Results showed the standard optimization of the 2-D protein mapping at pH 3-10 with 60 µg protein loading in freshly isolated mononuclear cells, cultured BMSCs (P0) and BMSCs (P2). This protocol could be further used, along with identification of differentially expressed protein by mass spectrometry, for thorough understanding of the molecular events involved, which would enable a better control of the various differentiation processes of BMSCs.

Keywords: Proteomics; 2-D gel electrophoresis; Bone marrow stromal cell (BMSC)

Abbreviations: BMSCs: Bone Marrow Stromal Cells; 2-D: Two Dimensional; IEF: Isoelectric Focusing; SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis; PBS: Phosphate Buffered Saline; MSCs: Mesenchymal Stem Cells

Introduction

In proteomic approach, the technique mostly used with sufficient resolving power is 2-D gel electrophoresis, which provides a platform for the simultaneous separation of proteins in a complex mixture by isoelectric focusing (IEF), and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [1,2]. It also offers better visualization of the whole proteome and hence, the ease of subsequent comparison and characterization [3,4]. Genomic and proteomic research now complement each other, and are partners for comprehensive large-scale studies that will contribute to the better understanding of disease and drug action. Proteomics also provides a systematic approach for the quantitative and qualitative mapping of the whole proteome [5,6].

Cell-based therapies are used for the treatment of various medical conditions. Bone marrow stromal cells (BMSCs), also known as mesenchymal stem or mesenchymal progenitor cells, are the major component of the bone marrow microenvironment. They play an important role in promoting growth and development of hematopoietic cells. Proteome profiling of bone marrow stromal cells may reveal important insights into the mechanisms of normal and dysregulated hematopoiesis [7].

Over the past several years, analytical methods based on 2-D gel electrophoresis coupled with mass spectrometry have been applied to explore the proteome of BMSCs [8-10]. Despite all these efforts, there are multiple challenges for the protein resolution and identification. At the same time, investigators may use various strategies to circumvent some of these issues; for instance, the use of organic solvents, detergents and non-specific peptidases can improve protein solubility.

Therefore, in this study, it was thought worthwhile to establish a reproducible protocol to display the complete qualitative 2-D protein mapping of the bone marrow stromal cells. This protocol could be used later, along with identification of differentially expressed protein by mass spectrometry for thorough understanding of the molecular events involved, which would enable a better control of the various differentiation processes of BMSCs.

Materials and Methods

The study procedure complied with the institutional guidelines on use of human subjects in research. Consent was obtained from three patients with prolapsed spinal disc by Tissue Engineering Centre, Universiti Kebangsaan Malaysia Medical Centre, Universiti Kebangsaan, Malaysia, as main principal investigator. The patients underwent posterior instrumentation in the Ampang Puteri Specialist Hospital. All three patients were males, aged 27, 39 and 40 years, respectively. Bone marrow aspirate was aspirated from the iliac crest using a 50 ml heparinized syringe (Greiner, Austria), with an 18G (Terumo Corporation, Japan) needle, as part of the surgical procedure for bone grafting. An additional 5 ml of bone marrow aspirate was obtained and dispensed immediately into EDTA vacutainers (Greiner Bio-One, Austria) for the study.

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Mononuclear cell isolation

Mononuclear cells were isolated from the aspirated bone marrow *via* gradient centrifugation over a Ficoll-Paque Plus layer (GE Healthcare, Sweden) at 400×g for 30 minutes, followed by washing twice with phosphate buffered saline (PBS) (Gibco, USA), pH 7.4. A fraction of the freshly isolated mononuclear cells were subjected to protein extraction, as detailed below. The remaining cells were resuspended in culture medium (DMEM, Gibco, USA)+15% fetal bovine serum (Invitrogn, USA), and plated into a 9.6 cm^2 cell culture plate (Nunc Bio-One, USA). Only the adherent cells were subsequently maintained in culture.

Cell culture

All cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 (Jouan, Duguay Trouin, SH). Fresh medium was added on the third day. Medium was changed only after substantial cell attachment twice a week. Upon confluence (Figure 1), cells were detached by the addition of 0.05% trypsin-EDTA (Gibco, USA) solution, and counted with a haemacytometer (Weber Scientific International Ltd. Middlesex, England) by trypan blue dye (Gibco, USA) exclusion method. Cells were then resuspended in culture medium and plated into a new culture plate, at a standard density of 5,000 cells/cm^2. Cells were counted with every passage until passage 2 (P2).

Protein extraction

For each sample, 200,000 cells were pelleted for protein extraction, respectively. Samples were washed twice in PBS (Gibco, USA), and were subsequently lysed at 4°C with lysis buffer (10 mM Tris-HCL, Ph 7.5, 1 M $MgCl_2$, 1 M EGTA, 0.1 mM phenylmethyl-sulfonyl fluoride, 5 mM β-mercaptoethanol, 0.5% 3-(3-cholamidopropyl) dimethylammonio-1-propanesulfonate, 10% glycerol), for 30 minutes to release intracellular proteins. Lysed samples were then centrifuged at 13,000 rpm at 4°C for 30 minutes, to pellet the cell debris. Crude cellular proteins were then transferred to a cryotube (Becton Dickinson, USA), snap-frozen and kept in liquid nitrogen. This approximately yielded 100 µg crude cellular proteins. Protein concentration was determined by using Pierce kit [11]. The proteins were then separated, in terms of their isoelectric point and molecular weight, by 2-D gel electrophoresis.

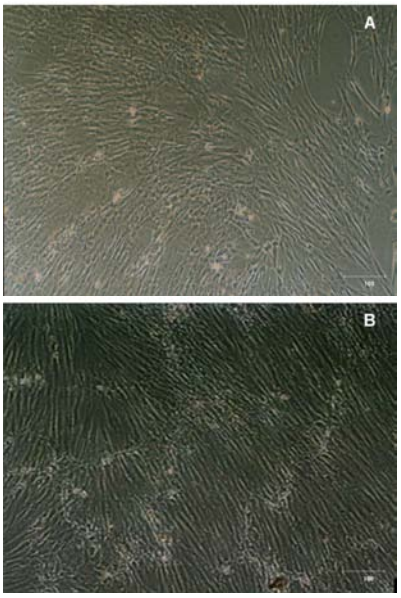

Figure 1: Bone marrow stromal cells in culture. (A) Bone marrow stromal cell culture at passage 0, day 7, 40X (100 microns). (B) Passage 2 of bone marrow stromal cells. Phase contrast image was captured at day 7, 40X (100 microns).

2-D gel electrophoresis optimization

The first-dimension Isoelectric Focusing (IEF) was performed by using PROTEAN IEF system (Bio-Rad Laboratories, USA). 60 µg (extracted from 200,000 cells) of protein lysate mixture was supplemented with 60 µl sample buffer solution (9 M urea, 0.5% v/v Triton X-100, 2% v/v IPG buffer pH 3-10 and 60 mM DDT), and left at room temperature (20°C) for 30 minutes. A rehydration solution was added to the mixture (8 M urea, 0.5% v/v Triton X-100, 0.5% v/v IPG buffer pH 3-10, 12 mM DDT and 0.002% of Orange G), to make final volume of 200 µl for 11 cm IPG Strip gel, pH 3-10 (Bio-Rad Laboratories, USA), respectively [12,13]. The IPG strips were then rehydrated with the sample mixture in the Immobiline DryStrip Reswelling Tray. The reswelling tray and IPG strips were rehydrated at room temperature for 16 hours.

Isoelectric focusing (IEF) was performed under the following conditions: 300 V for 30 minutes, 3500 V for remaining hours, till reached 12000 V/hr. Upon completion of IEF, the strips were equilibrated in buffer (6 M urea,1.5 M Tris-HCl, pH 8.8, 30% v/v Glycerol, 2% SDS, 0.002% bromophenol blue, 0.06 M DTT), containing DTT for 15 minutes and for another 15 minutes in the same buffer containing 240 mM iodoacetamide. The second dimension separation was carried out at 16°C on 12.5% SDS slab gels using 2-D gel electrophoresis system (Bio-Rad Laboratories, USA), with the IPG strips sealed on the top of the gels with 0.5% agarose. SDS-PAGE was run for 40 mA/gel at 50 V for the first 30 minutes. The voltage was subsequently increased to 600 V, until the bromophenol blue marker reached the bottom of the gel. Protein spots were visualized by silver staining [14]. Images of stained 2-D gels were acquired with Platinum Image Master Scanner (Amersham Biosciences), and stored as TIF file. All samples were analyzed in triplicate.

Results

Protein expression analysis

Figure 2 shows the 2-D protein maps of freshly isolated mononuclear cells and cultured BMSCs (P0). 2-D gel electrophoresis analyses were optimized at pH 3-10 with 60 µg protein loading. The separation of the proteins from cultured BMSCs (P2) samples were also optimized at pH 3-10, with 60 µg protein loading (Figure 3). Consistency and reproducibility were achieved by standardizing the procedures for sample preparations of cells cultured, first and second dimension of electrophoresis, gel staining and image acquisition. Under these conditions, proteins could be visualized without much background noise and sacrificing gel resolution. Thus, we have established a robust and reliable protocol for the 2-D gel electrophoresis analysis of the protein profile of human bone marrow stromal cells.

Discussion

It is important to have an optimized system for developing 2-D protein map of fresh bone marrow and cultured bone marrow derived mesenchymal progenitor cells. High serum, salt and albumin content of the samples posed a great obstacle in 2-D gel electrophoresis. In our study, the problem was resolved by using appropriate buffers as mentioned in methodology section [12,13].

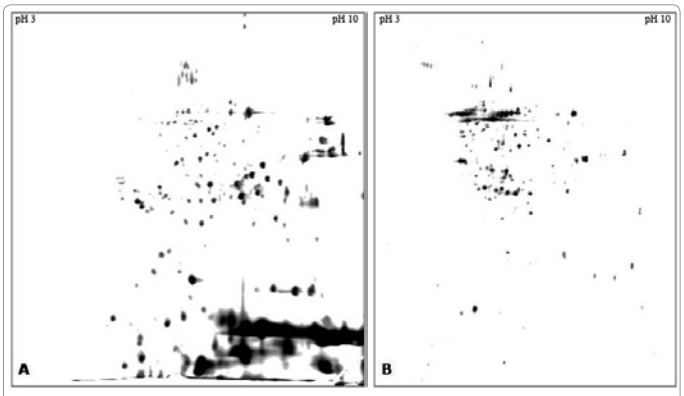

Figure 2: Representative 2-D gel image. (A) Representative protein map of freshly isolated mononuclear cells from bone marrow aspirate. (B) Representative protein map of cultured bone marrow stromal cells at P0. Protein mixtures were loaded at 60µg for IPG Strip 11cm, pH 3-10.

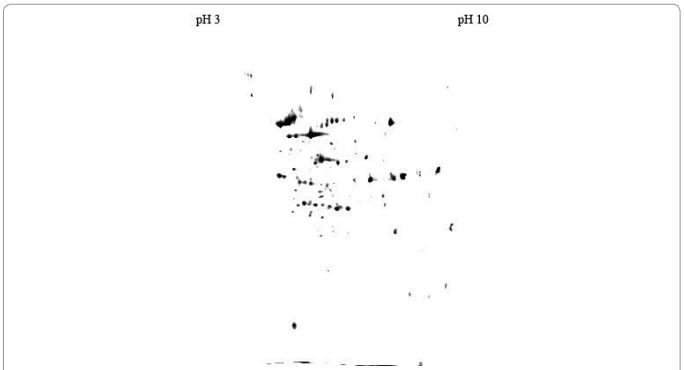

Figure 3: 2-D protein mapping of cultured BMSCs at P2. Protein lysate mixtures were loaded at 60 µg for IPG Strip 11 cm, pH 3-10.

Ng et al. [15] previously characterized the bone marrow stromal cells, which were isolated to possess the various characteristics of mesenchymal stem cells (MSCs), including positive expression of CD13, CD90, CD105 and telomerase activity, while negative for CD45 and partially expressing CD34. The osteogenic potential of these cells have also been demonstrated [16]. By establishment of the reliable and reproducible protocol to generate 2-D protein map, further continuation of this study could be undertaken to evaluate the protein profiling during the osteogenic differentiation of the cultured BMSCs, under the influence of cytokines and steroids.

In conclusion, the procedure used generated a highly reproducible reference 2-D protein mapping of freshly isolated mononuclear cells from the bone marrow aspirate and bone marrow stromal cells in culture. 2-D protein mapping could further be used, along with identification of differentially expressed protein by mass spectrometry for a better understanding of mesenchymal cells, thus, enabling widespread use of such cell type in both clinical and experimental investigations

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