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Full Length Research Paper

Cell surface protein expression of stem cells from human adipose tissue at early passage with reference to mesenchymal stem cell phenotype

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Research is now being focused on stem cell therapy for repair of diseased and damaged tissues. Various sources for mesenchymal stem cells (MSCs) have been identified. The ideal source of MSCs is still unclear. An ideal source for MSCs should be one which could be easily accessed, causes minimal patient discomfort and also gives a high yield of cells with characteristic cell surface protein expression of MSCs at early passage. Thus, this study analysed the cell surface protein expression of human adipose stem cells (ASCs) at early passage with reference to mesenchymal stem cell phenotype and differentiation into cells of mesodermal origin. Adipose tissue was obtained as waste material from patients undergoing certain elective surgical procedures, after taking informed consent. Flow cytometry analysis of ASCs showed both positive and negative cell surface protein expression, highly characteristic for MSCs as early as passage 0 or 1 (early passage). Under appropriate culture conditions ASCs showed osteogenic differentiation evidenced by presence of calcium phosphate identified by alizarin red staining and adipogenic differentiation evidenced by lipid rich vacuoles within the cells, recognized by oil red O staining. Since adipose tissue is available in large quantities and has a high yield of stem cells, availability of high percentage of characteristic MSCs at early passage will reduce the time for expansion in culture, the costs involved and the risk of cell contamination. Thus adipose tissue is a promising source of stem cells for tissue engineering and regenerative medicine.

Key words: Adipose stem cells, mesenchymal phenotype, early passage.

INTRODUCTION

Stem cell therapy offers promise in the repair of diseased and damaged tissues (Zuk et al., 2001). The practical use of embryonic stem cells is limited due to ethical constraints (Zuk et al., 2001). Various sources of mesenchymal stem cells (MSCs) include bone marrow, adipose tissue, umbilical cord blood (Erices et al., 2000), amnion (De Coppi et al., 2007), placenta (Fukuchi et al., 2004), dental pulp (Gronthos et al., 2002), dermis (Young et al., 2001) and peripheral blood (He et al., 2007). MSCs show the ability of self renewal and differentiation into various lineages and therefore are being researched extensively as a treatment option for repair and regeneration of damaged or diseased tissues in animal models and humans. MSCs also suppress immune response and thus may be beneficial for patients with autoimmune disorders and transplant rejection (Popp et al., 2009). Over the past many years, MSCs isolated from bone marrow have been the subject of interest and research. Like bone marrow, adipose tissue is a mesodermally derived organ containing stem cells (Strem et al., 2005). Extensive research is being done on human adipose stem cells (ASCs). Studies have been carried out on both the initial stromal vascular fraction cell population isolated from adipose tissue as well as the plastic adherent stem cell subpopulation during multiple

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stages of passage (Mitchell et al., 2006).

A minimum criterion has been proposed by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy in order to define MSCs (Dominici et al., 2006). The criterion states that MSCs must be adherent to plastic in culture, high percentage of cells must express CD73, CD90 and CD105; MSCs must lack hematopoietic lineage markers for example, CD 45 expression on their cell surface (2% or less cells), MSCs must have the ability to differentiate into osteogenic, adipogenic and chondrogenic lineages (Dominici et al., 2006). The best source for stem cells is yet to be established (Strem et al., 2005). There is evidence that stem cells are found in numerous sites. An ideal source for MSCs should be one that can be easily accessed without causing much pain and gives a high yield of stem cells highly characteristic of MSCs at early passage. This will reduce the time for expansion, the costs involved and the risk of contamination of cell cultures (Zuk et al., 2001). This study analysed the cell surface protein expression of human ASCs at early passage (passage 0/1) with reference to mesenchymal stem cell phenotype and differentiation of ASCs into cells of mesodermal origin. Tissues which contain a high percentage of cells with characteristic phenotype of MSCs at very early passage will be a promising source.

MATERIALS AND METHODS

Human ASCs isolation and expansion

All chemicals except those specifically mentioned were purchased from Sigma. All protocols were approved by the institutional review board of Christian Medical College, Vellore. After taking informed consent, human subcutaneous adipose tissue and infrapatellar fat pad were obtained as waste material from patients undergoing elective surgical procedures (n = 5) and processed independently. Stem cells were isolated from adipose tissue according to previously published protocols with slight modifications (Aust et al., 2004). The adipose tissue was transported in a sterile container. The adipose tissue was minced and washed many times with phosphate buffer saline (PBS, Hyclone) till there was no trace of blood. The tissue was then digested with equal volume of 0.1% Collagenase Type 1 (Worthington) solution (prepared in PBS with 1% bovine serum albumin) on a shaking water bath at 37°C for 60 min. The digested tissue was centrifuged at 1200 rpm for 5 min at 26°C. The supernatant containing adipocytes was removed using a pipette and stromal medium was added to the pellet. The stromal medium consisted of Dulbecco's modified Eagle's medium (DMEM/F-12), 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin, all purchased from Hyclone. The pellet with the stromal medium was mixed and allocated in T75 culture flasks at a density of 0.16 ml of initial tissue per cm^2 (Mitchell et al., 2006).

The culture flasks were kept in 5% CO_2 incubator at 37°C for expansion and culture. The cells were washed after 24 h with PBS and stromal medium was added. The cells were maintained with stromal medium until they reached about 90% confluence, after which the cells were harvested (passage 0) by digestion with 0.05% trypsin (Hyclone). At the time of passage, cell viability was determined by trypan blue exclusion method and cell count was also determined using a haemocytometer. The cells were used for characterization and differentiation or again expanded in T75 culture flasks at a seeding density of 4000 cells per cm^2 until passage 1.

Adipogenesis

The trypsinized cells were plated on a 12 well culture plate at a seeding density of 30,000 cells per cm^2 with stromal medium. When the cells reached 80 to 90% confluence, the stromal medium was changed to adipocyte induction medium composed of DMEM/F-12, 3% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin, 1 µM dexamethasone, 500 µM isobutylmethylx-anthine (IBMX), 33 µM biotin, 5 µM rosiglitazone, 100 nM insulin and 17 µM pantothenate . After 3 days the adipocyte induction medium was changed to adipocyte maintenance medium which was similar to the adipocyte induction medium without IBMX and rosiglitazone. The cells were maintained for 9 to 10 days with media change once every third day. The cells were washed with PBS and fixed in 10% formalin. Differentiation of cells into adipocyte lineage was assessed by staining the cells with oil red O.

Osteogenesis

The trypsinized cells were plated on a 12 well culture plate at a seeding density of 30,000 cells per cm^2 with stromal medium. When the cells reached 80 to 90% confluence, the stromal medium was changed to osteogenic induction medium composed of DMEM/F-12, 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin, 10 nM dexamethasone, 10 mM glycerophosphate and 50 µg/ml ascorbate 2-phosphate. The cells were maintained for 9 to 10 days with media change once every third day. The cells were washed with 0.9% saline and fixed in 70% ethanol. Differentiation of cells into osteogenic lineage was assessed by staining the cells with alizarin red.

Characterization

The trypsinized cells, either at passage 0 or 1 (early passage) were characterized for mesenchymal stem cell surface protein expression by flow cytometry using a BD FACS Calibur and data analysis was performed using Cell Quest Pro software. All antibodies were purchased from BD Biosciences unless otherwise specified. The cells were washed with PBS by centrifuging at 1000 rpm for 2 min. Appropriate fluorochrome conjugated mouse monoclonal antibody was added to each tube. 50 µl cell suspensions (≈1 x 10^6 cells) was added to each tube and incubated for 15 min at room temperature in dark environment. The monoclonal antibodies were either coupled to phycoerythrin (PE) or fluoresceinisothiocyanate (FITC). The monoclonal antibodies used were PE labeled anti-CD 73, PE-Cy5 labeled anti-CD 90, FITC labeled anti-CD 105 (AbD Serotec) and FITC labeled CD 45. PBS was added to all tubes to make it 2 ml. The tubes were centrifuged at 1000 rpm for 2 min.

The supernatant was decanted and 200 µl PBS added to all tubes. Flow cytometry analysis was done for expression of cell surface markers CD 73, 90, 105 and 45. Isotype control antibody for CD 105 (AbD Serotec) and a control of cells with no antibodies were also analyzed.

RESULTS

Cell yield

The average time in culture for the adherent cells to

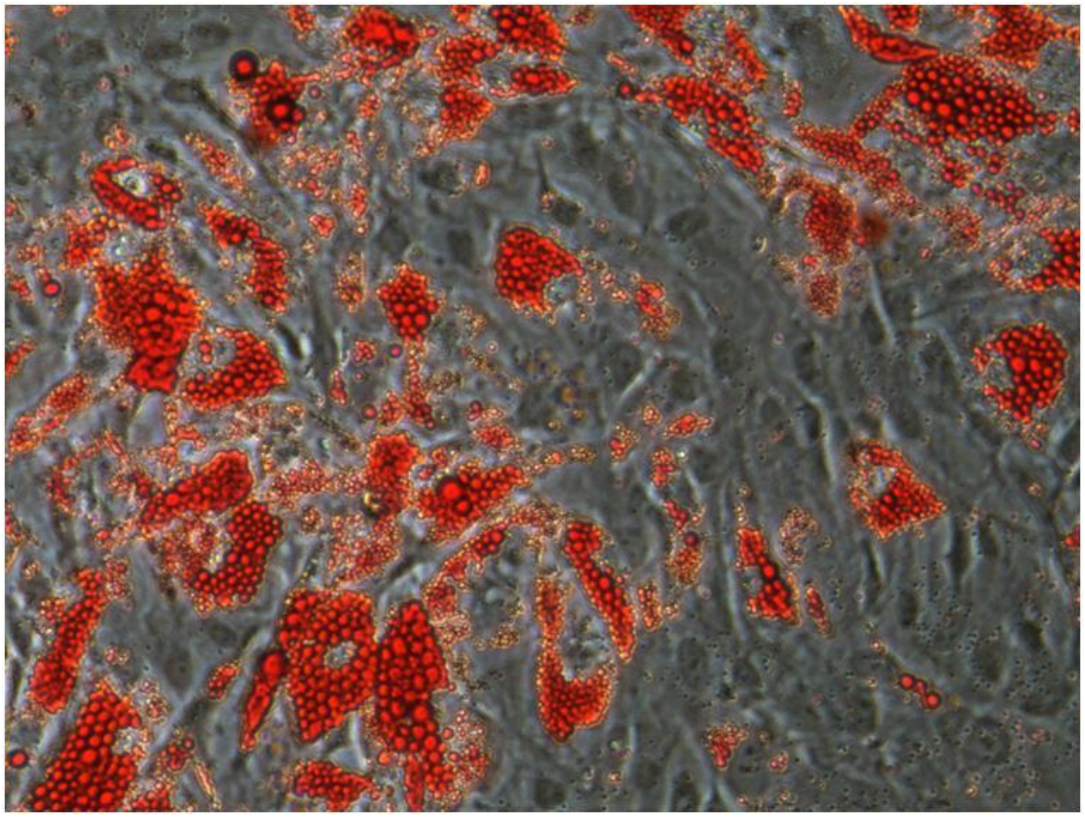

Figure 1. Adipogenic differentiation of adipose stem cells – Oil red O staining. Adipose stem cells (ASCs) differentiated with adipogenic medium showing characteristic staining for neutral lipids with Oil red O (20×).

reach 90% confluence after initial plating was 10.6 (± 3) days. The cells were harvested at this point by trypsin digestion. The average yield of adherent cells (ASCs) was 0.9 (±0.37) × 10^6 cells per ml of minced adipose tissue. Cell viability at the time of passage was nearly 100%.

Differentiation

The ASCs were differentiated along the adipogenic and osteogenic lineages. ASCs differentiated with adipogenic medium showed characteristic staining for neutral lipids with Oil red O (Figure 1). The ASCs treated with osteogenic medium showed characteristic staining for calcium phosphate with Alizarin red (Figure 2).

Characterization

The trypsinized cells were characterized for mesenchymal cell surface protein expression at early passage using flow cytometry (n = 5). The results are summarised in Table 1. The cell surface protein expression was highly characteristic of MSCs, even at early passage. A representative flow histogram is shown in Figure 3.

DISCUSSION

Though several studies report cell surface protein expression of ASCs, few studies report cell surface protein expression of ASCs with relation to mesenchymal phenotype at early passage (Mitchell et al., 2006; Zuk et al., 2002; Katz et al., 2005; Aust et al., 2004). The stromal vascular fraction and cells at early passage from adipose tissue have been studied for markers of endothelial cell lineage (Miranville et al., 2004; Planat-Benard et al., 2004; Rehman et al., 2004). In this study, the ASCs showed plastic-adherent property in culture, differentiation potential into osteogenic and adipogenic lineages and expression of cell surface proteins highly characteristic of MSCs at very early passage (passage 0/1). About 98% of ASCs expressed the MSC positive CD markers CD 73, 90 and 105 while only 1.8% of cells expressed the negative marker CD 45 at early passage. The results of this study are consistent with a similar study that has been published recently (Yu et al., 2010). Two previous studies showed a lower percentage of ASCs expressing MSC positive CD markers at early

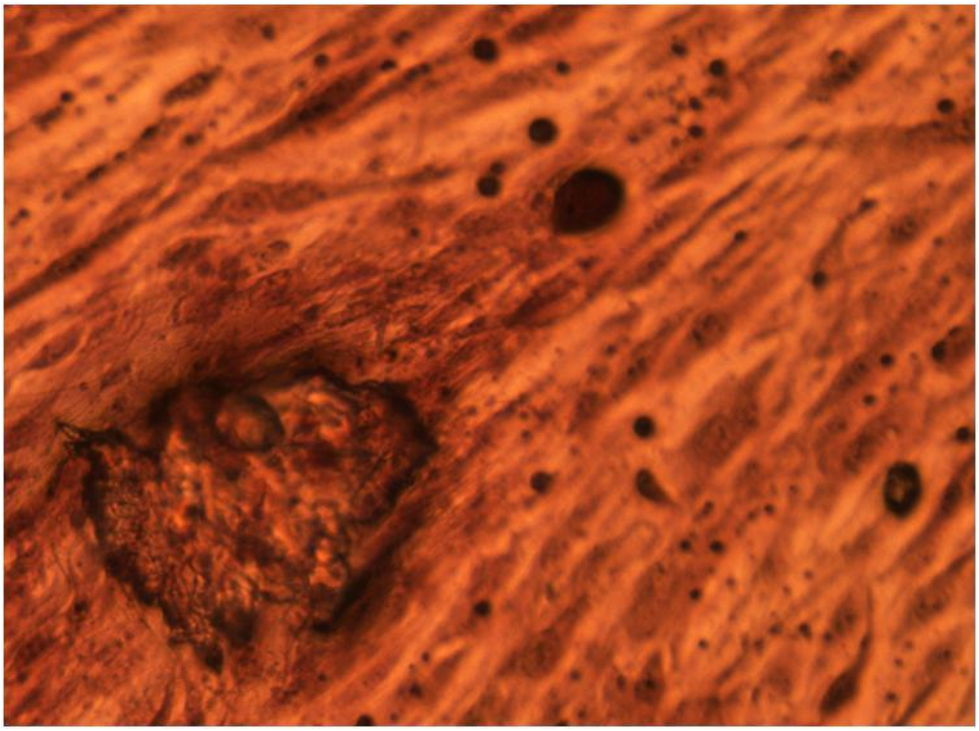

Figure 2. Osteogenic differentiation of adipose stem cells – Alizarin red staining. Adipose stem cells (ASCs) differentiated with osteogenic medium showing characteristic staining for calcium phosphate with Alizarin red (20×).

Table 1. Cell surface protein expression of adipose stem cells with reference to mesenchymal phenotype at early passage using flow cytometry (n = 5).

Antigen	Mean % positive cells (± SD)
CD 73	98.0 (±1.7)
CD 90	97.7 (±1.2)
CD 105	98.8 (±0.5)
CD 45	1.8 (±2.6)
CD 105 isotype control	3.6 (±5.3)

Adipose stem cells (ASCs) showing high percentage of cell surface protein expression for mesenchymal stem cell markers at very early passage (passage 0/1).

passage compared to the present study (Mitchell et al., 2006; Zuk et al., 2002). Consistently high percentage of ASCs expressing MSC markers were shown at passage 3 or 4 (Mitchell et al., 2006). The MSC markers were present in more than 90% of the ASCs by passage 4 (Mitchell et al., 2006). A pattern of increased percentage of MSC markers with serial passages is seen with bone marrow MSCs (Pittenger et al., 1999). Two other studies showed 98% of ASCs express CD 90 in early passage (passage 0/1/2) (Katz et al., 2005; Aust et al., 2004). A study comparing MSCs from different sources which included bone marrow, synovium, periosteum, skeletal muscle, and adipose tissue showed that at passage 3, CD 45 expression from all sources was less than 2% while CD 90 and 105 expression was more than 80% (Sakaguchi et al., 2005). High percentage of cells expressing cell surface proteins highly characteristic of MSCs at early passage could prove to be a huge advantage as this reduces time for expansion of cells, costs involved and risk of cell contamination.

The number of MSCs in adipose tissue is about 100 times more than in bone marrow and also shows greater ability to proliferate (Lee et al., 2004). The differentiation potential and immunomodulatory property of ASCs is similar to bone marrow MSCs (Zuk et al., 2001, 2002; Puissant et al., 2005). Stem cells from adipose tissue can be obtained in large numbers and can be extracted in large quantities with minimal morbidity (Strem et al.,

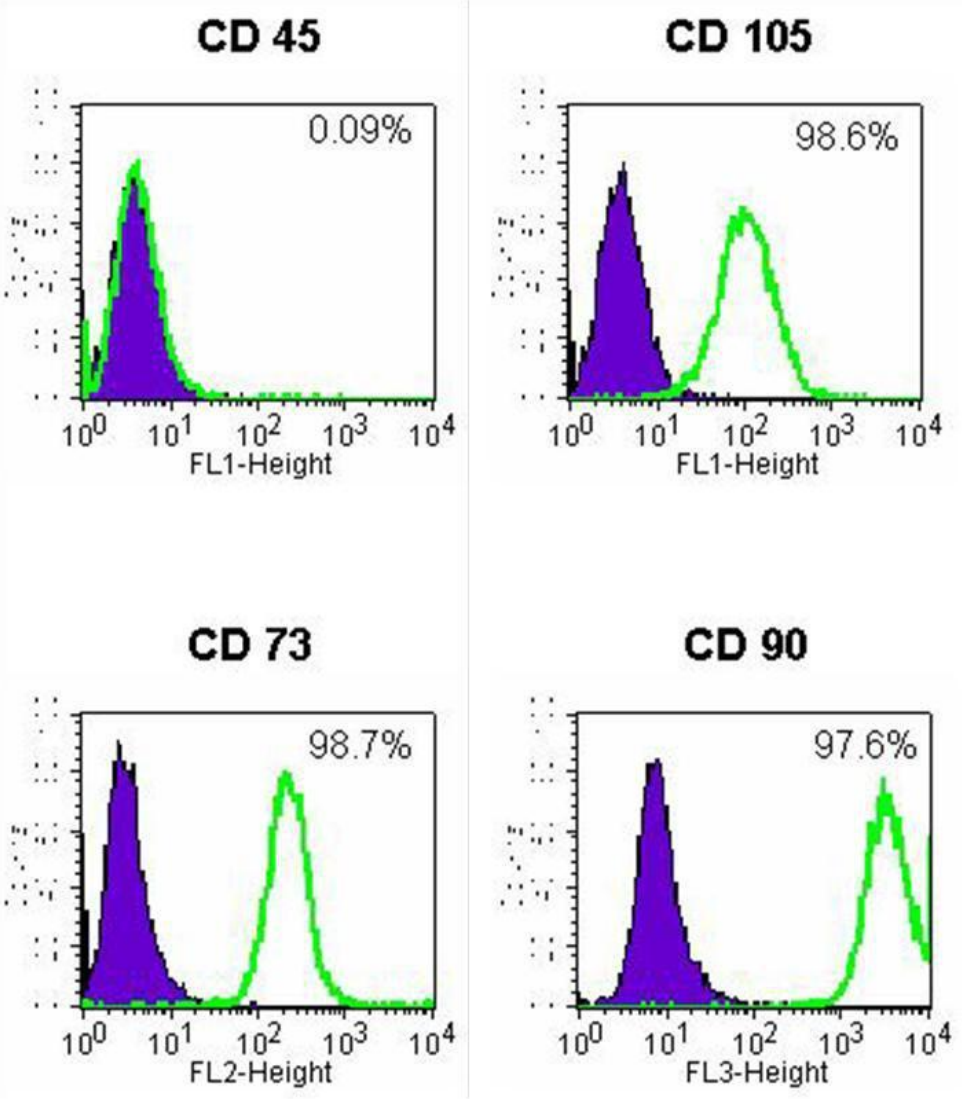

Figure 3. Representative flow cytometry histogram of adipose stem cells (ASCs) at passage 1. The purple shaded area represents the control while the green curve represents the cells staining positive for the CD marker. The adipose stem cells show a high percentage expression of cell surface proteins highly characteristic of MSCs at very early passage.

2005; Parker et al., 2006). A modest tissue harvest of 100 g of adipose tissue may yield as many as 20 to 40 million cells (Parker et al., 2006). A high yield of cells with characteristic phenotype of MSCs at early passage is an ideal source and adipose tissue shows this potential. This could prove vital in considering the source of MSCs for various stem cell therapies.

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