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Co Expression of CD14/45 and CD3/19 Markers is Unique Signature for Identification of Differentiated Chondrocytes from hADSC

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Abstract

Background: Stem cell based therapy is a new paradigm for treatment of many abnormal conditions such as cartilage lesions. Although this method has many beneficial therapeutic effects, the serious adverse event of stem cell transplantation such as tumorigenic capacity is not deniable. Thus, other strategy such as differentiated cells transplantation instead of stem cells transplantation has been proposed. Overall, before cell transplantation, in vitro cell isolation in order to raise the purity of transplanted cells is necessary. So, the aim of this study is to identifying a unique CD "signature" that could be ascribed specifically to the chondrocyte before cell transplantation.

Methods: Human adipose derived stem cells (hADSC) were isolated from lipoaspirate samples and were subjected to osteogenic and chondrogenic differentiation in culture medium which supplemented with specific materials. In addition, Human articular cartilages were obtained from shoulder joint and chondrocytes isolation was carried out. Finally, Flow cytometry technique was done in order to specify of CD expression in stem cells, human articular chondrocyte and differentiated cells.

Results: Our findings showed that hADSCs have the ability to differentiate into osteoblasts and chondrocytes. In addition, flow cytometry analysis indicated that 0.24 ± 0.31 percent of hADSCs expressed CD14/45 and 0.34 ± 0.11 percent of them expressed CD3/19 markers which are specific marker of chondrocytes. Whereas, one week after chondrogenic differentiation 99.92 ± 0.14 percent of differentiated cells expressed CD14/45 and 99.88 ± 0.12 of them expressed CD3/19 markers. Moreover, two weeks post differentiation; the expression of these markers not changed and was similar to human articular chondrocytes.

Conclusion: During chondrogenesis and before cell transplantation, CD14/45 and CD3/19 markers can be used to identify differentiated chondrocytes from undifferentiated stem cells.

Keywords: CD14/45; CD3/19; Chondrocyte; Tissue engineering

Introduction

Tissue engineering is a new strategy in the field of regenerative medicine with the purpose of regenerates, improves or replaces biological functions of damaged tissues [1]. This method refers to the practice of combination stem cells, growth factors and environmental factors into functional tissue. Mesenchymal stem cells (MSCs), an ideal cell source for tissue engineering, first described in 1970 [2], and characterized by their self-renewal ability and multi-potency as well as their phenotypic stability maintained after several passages [3-6]. Thus, these cells have the ability to create more stem cells and other specialized cells. Adipose-derived stem cells (ADSCs) also identified in 2001 [7] and are a kind of MSCs which have a much higher frequency in the adipose tissue. Fatty tissue can be access easily by a minimally invasive procedure. In addition, the content of MSCs in this tissue is approximately 500-fold more than in other source such as bone marrow [8,9]. Meanwhile, the results of several studies showed that ADSCs have ability to protect their specifications during sequential subculture. Moreover, in the proper conditions these cells are able to convert into other cells from different germ layers including neuronal, glial, muscle and special connective tissue cells [10-12]. Previous studies revealed that ADSCs express typical mesenchymal markers including CD9, CD10, CD13, CD29, CD44, CD49d, CD49e, CD51, CD55, CD59, CD71, CD73, CD90, CD105, CD166 antigens. Also, these cells are negative for others antigens such as CD11a, 11b, 11c, CD14, CD16, CD18, CD31, CD34, CD45, CD50, CD56, CD104 and CD106 [13-15]. The expression of these antigens seems to be dependent on duration and condition of cell culture. For example, during the cell culture, the expression of CD29, CD90, and CD166 markers was significantly increased [16], but the expression of other markers was decreases [16]. However, it is important to mention that, the change expression of these markers can lead to induce different cell signaling and cell fate.

Growth factors and scaffolds are other factors that are important in tissue engineering. Transforming growth factor beta (TGF-β) and their family members are the most common factors for cell differentiation which widely used in tissue engineering especially in cartilage repair. These factors through specific receptors and intracellular signaling have an important role in control of MSCs proliferation; cellular differentiation, provide competence for chondrogenesis, inhibit osteoblast maturation and regulate CD expression [17]. Recently, it has been reported that TGF-β1 via enzyme inhibition, is able to regulate CD133 expression [18]. In spite of this, inadequate studies have been conducted the effects of TGF-β on the expression of stem cell CD markers.

The various scaffolds including gelatin and hydrogel biomaterials can support the chondrogenic differentiation of ADAS cells. Among of these materials, alginate which is natural hydrogel biomaterial, has low

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price and is available. So, this scafold was used in various experiments as a good material for chondrocytes to regenerate cartilage tissue [19-22]. Recently, it has been reported that alginate is a suitable biomaterial for encapsulation of stem cell for cell based therapy because does not affect on cell main characteristics [23]. Unlike many studies which reported the beneficial potential of cell based therapy, the serious adverse events of stem cell transplantation such as tumorigenic potential have presented in some experimental studies [24,25]. The differentiation degree of the transplanted cells is important between several factors which are associated with tumorigenesis. Therefore, before cell transplantation in order to raise the purity of transplanted cells and improve the prognosis of treatment, the cell isolation using specific marker is necessary.

Thus, in order to identifying a unique CD "signature" that could be ascribed specifically to the chondrocyte before cell transplantation, we investigated the expression of CD14/45, CD3/19, CD44, and CD90 markers in natural and chondrocyte differentiated from human ADSCs during chondrogenic process.

Materials and Methods

Human ADSCs isolation and culture

All chemicals for cell isolation and culture (unless specified otherwise) were prepared from Sigma-Aldrich. Moreover, all methods were accepted by the Ethics Committee of Isfahan University of Medical Sciences. After received informed consent, human abdominal fat were collected from lipoaspirate samples from donors (age range 18–30 years) and cultured as previously described [16]. Briefly, obtained adipose tissue were washed twice with phosphate-buffer saline (PBS) in order to remove contaminating debris. In the following, the washed aspirates were treated with 0.075% type I collagenase for 30 min in standard condition. After this period, enzyme activity was neutralized and the infranatant centrifuged at 1400 rpm for eight minutes. The cellular pellet was resuspended and was cultured in Dulbecco's Modified Eagle Medium (DMEM/low glucose) solution. After 80% confluency, the cells were separated with 0.25% Trypsin/0.02% EDTA and were passaged.

Mesenchymal differentiation assays for human ADSCs

Osteogenic differentiation: In order to study of differentiation potential of hADSCs into osteoblasts, hADSCs within 3-5 passages, were cultured in DMEM medium supplemented with FBS10%, ascorbic acid (50 µg/ml), beta glycerophosphate (10 mM), and penicillin / streptomycin 1% for two weeks. Finally, mineralization is assessed by von kossa staining.

Chondrogenic differentiation of hADSCs: Human ADSCs within 3-5 passages were differentiated into chondrocytes with two protocols. In pellet protocol, after harvested hADSCs from the passage 3, these cells was resuspended in chondrogenic medium (DMEM-HG) with 1% insulin transferring selenium, 10 ng/ml TGF-ß3, 1/25 mg/ml BSA, 5 mg/ml Linoleic acid, 50 µg/ml ASP and 1% penicillin- streptomycin supplement and were cultivated in standard condition for 21 days. Eventually, after cell fixation, the samples were stained with toluidine Blue dye.

In second protocol, hADSCs 5×10^5 /ml was resuspended in 1.5% alginate (Sigma). Then, the alginate/cell suspension was dropeded through a 23-gauge needle into a 102 mM $CaCl_2$ solution (Merck). After 15 min, the alginate beads were washed twice in 0.9% saline solution and once in DMEM-HG (Gibco). Finally, 1 ml chondrogenic medium was added to each well. Chondrogenic culture medium (high glucose Dulbeco's modified Eagle medium (Gibco) supplemented with 10 ng/ml TGF-ß3, 1/25 mg/ml BSA, 5 µg/ml Linoleic acid, 100 nM dexamethasone, 6/25 ng/ml ITS, 50 µg/ml ASP and 1% penicillin-streptomycin. The medium was substituted every 3-4 days for 14 days.

Isolation and culture of human articular chondrocyte

Human articular cartilages were obtained from knee joint of male donors (age range 33–55 years) after received informed consent. The samples were washed thoroughly several times with PBS in order to remove contaminating debris. In the following, the cartilage tissue was placed into a petri dish and was diced into small pieces and bone fragments were completely separated. After this period, cartilage tissue were treated with type I collagenase (350 mg/ml) for 4 hours in a 37°C, 5% CO_2 humidified cell culture incubator and each half-hour, the tubes were shaken for 30 seconds. After incubation, enzyme activity was neutralized with an equal volume of DMEM/F12/FBS. The resulting cell suspension was centrifuged at 1400 rpm for ten minutes and cellular pellet was cultured in DMEM/F12 Medium supplemented with FBS10% and Penicillin/streptomycin 1%. The medium was replaced every 4 days.

Flow cytometry technique

In order to specify of hADSCs, human articular chondrocyte and differentiated cells, single-cell suspensions in PBS (1×105 cells/20 µl) were prepared and stained with 3 µl antibodies against CD14/45, CD3/19, CD44, and CD90 according to the manufacturer's instructions in dark environments at 4°C for 30 minutes. In addition, non- specific FITC-conjugated IgG was used for isotype control. After this time, the cells were washed 2 times with 0.5% BSA/PBS and were resuspended in 500 µl PBS. The percentage of fluorescent cells were analyzed thorough a FACScan flow cytometer.

Statistical analysis

The mean percent cells which expressed CD markers were obtained using one-way analysis of variance (ANOVA) (SPSS Inc., Chicago, IL, USA). All data were shown as mean ± standard error of the mean (mean ± SEM).

Results

hADSCs characterization

Human ADSCs in primary culture exhibit fibroblast-like cells morphology (Figure 1A). In addition, vankosa and toluidine blue staining results indicated that hASCs have the ability to differentiate into different lineages cells such as osteoblast and chondrocyte (Figure 2).

Finally, flow cytometry analysis of hADSCs within passage 3, revealed that (99.18 ± 0.29) percent of these cells were expressed CD90 and (99.21 ± 0.23) percent of them were expressed CD44 markers. Meanwhile, the low percent of hADSCs were expressed CD14/45 and CD3/19 (Figure 3 and Table 1). Figure 1 Phase contrast images of cell morphology. Morphological changes were observed in human adipose-derived stem cells (hADSCs) during chondrocyte differentiation. Cultured hADSCs in passage three (A), human articular chondrocyte (B), differentiated cells in alginate bead (C). Magnification in A = 40X, in B& C = 20X.

Figure 2 Multilineage differentiation potential of human adipose-derived stem cells into osteoblast and chondrocyte. Von kossa staining

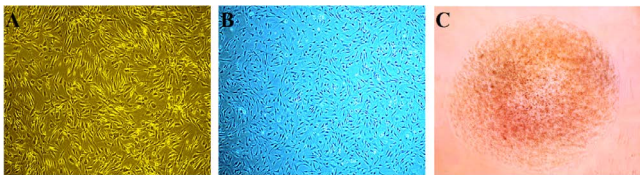

Figure 1: Phase contrast images of cell morphology. Morphological changes were observed in human adipose-derived stem cells (hADSCs) during chondrocyte differentiation. Cultured hADSCs in passage three (A), human articular chondrocyte (B), differentiated cells in alginate bead (C). Magnification in A= 40X, in B & C = 20X.

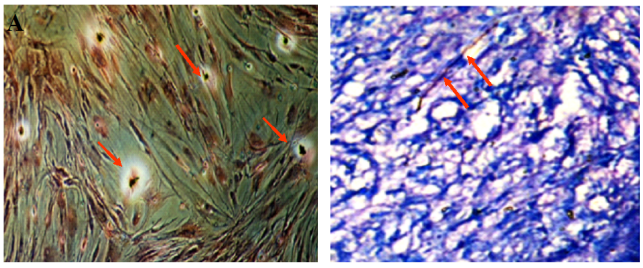

Figure 2: Multilineage differentiation potential of human adipose-derived stem cells into osteoblast and chondrocyte. Von kossa staining for prove osteogenic process (A) and toluidine blue staining for chondrogenesis (B) Magnification in A& B = 100X.

for prove osteogenic process (A) and toluidine blue staining for chondrogenesis (b). Magnification in A& B = 100X.

Flow cytometry dot plots show the CD marker expression including CD14/45 Co-expression, CD3/19 Co-expression, CD44 and CD90 in human adipose-derived stem cells (A), 7 day post differentiation (B), 14 day post differentiation (C) and in Human articular chondrocyte.

Human articular chondrocyte characterization

The isolated human articular chondrocyte after isolation and culture display a spindle-shaped morphology (Figure 1B). In addition, flow cytometry analysis of natural human chondrocyte revealed that high percent of these cells were expressed CD90 (98.50 ± 0.26), CD44 (96.90 ± 0.18), CD14/45 (99.10 ± 0.24) and CD3/19 (95.20 ± 0.19) markers (Figure 3 and Table 1).

Differentiated cell characterization

One and two week's post differentiation, cellular analysis which was performed thorough phase contrast microscopy revealed that differentiated cells in alginate had a round-shaped morphology (Figure 1C).

Meanwhile, flow cytometry analysis also indicated that one week after cell differentiation, high percent of these cells were expressed CD90 (99.20 ± 0.26), CD44 (96.3 ± 0.24), CD14/45 (99.92 ± 0.14) and CD3/19 (99.88 ± 0.12) markers. In addition, two week after cell differentiation, the percentage of differentiated cells which were expressed these markers, was almost the same as previous group (Figure 3 and Table 1).

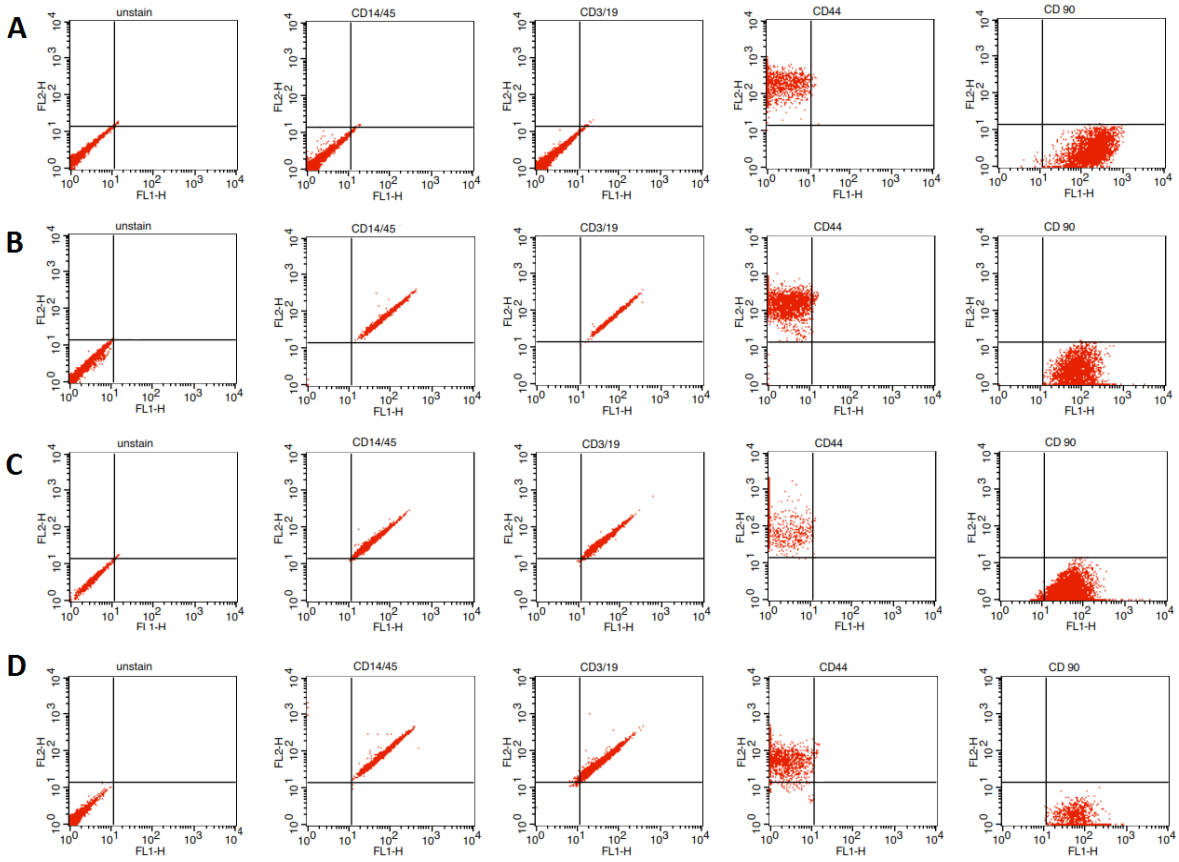

Figure 3: Flow cytometry dot plots show the CD marker expression including CD14/45 Co-expression, CD3/19 Co-expression, CD44 and CD90 in human adipose-derived stem cells (A), 7 day post differentiation (B), 14 day post differentiation (C), and in Human articular chondrocyte (D).

Cell/ CD Marker	CD14/45	CD3/19	CD44	CD90
Human ADSCs	0.24 ± 0.31%	0.34 ± 0.11%	99.21 ± 0.23%	99.18 ± 0.29%
Differentiated cells (7 day post differentiation)	99.92 ± 0.14%	99.88 ± 0.12%	96.3 ± 0.24%	99.2 ± 0.26%
Differentiated cells (14 day post differentiation)	99.82 ± 0.26%	99.87 ± 0.21%	98.89 ± 0.28%	97.15 ± 0.29%
Human articular chondrocyte	99.1 ± 0.24%	95.2 ± 0.19%	96.9 ± 0.18%	98.5 ± 0.26%

Table 1: The mean percentage of CD marker expression in different cells.

Discussion

Age-related diseases such as chronic and inflammatory diseases of joints are a major cause of disability in the middle-aged and the elderly which results in, enormous costs for health and social care systems. Arthritis has been explained as the most common type of degenerative joint abnormality which can lead to pain and severe physical disability [26]. Conventional therapies for arthritis are based on ant inflammation, but the current disease-modifying anti-arthritis drugs and any other treatments do not stop the ongoing progression of degeneration. The articular cartilage is largely avascular tissue which results in, decrease potential for regeneration [26,27]. So, stem cell-based therapies are proposed as a potential novel strategy for cartilage repair. A reason for these effects might be that these cells can inhibit cartilage degeneration progression through secretion of soluble anti-inflammatory factors which cause multiple anti-inflammatory and antioxidative effects [28-30]. In addition, after intra-articularly MSC transplantation, these cells are able to secrete liquid factors which have chondroprotective effects including regulation of chondrocyte viability and protection of cartilage matrix [31]. ADSCs seem to be a better cell source for cell based therapy than other sources due to several special characteristic including: the lack of the HLA-class II antigen on their surface and xenogeneic transplantation possibility [32]; the high migration capability via α4ß1expersion [33]; the high antioxidant and anti apoptotic activity as well as immunomoulatory and anti-inflammatory effects [34,35].

In a recent study, TGFβ3 was used for ADSCs differentiation and it has been demonstrated that this growth factor can promote ADSCs differentiation into chondrocyte and can improve the efficacy of ADSCs for cell based therapy in cartilage defect [36].

In similar experiment, ADSCs were seed in acellular cartilage matrices and then was used for transplantation into cartilage defect regions of rabbits. The results of this study showed that hADSCs can repair articular cartilage defects and proposed a promising cell-based procedure for repair [37].

In spite of this, it is important to mention that transplantation of undifferentiated stem cells can be induce teratomas [24,25], but the tumorigenic potential of these cells will be reduced if in vitro predifferentiation has done before cell implantation. Thus, pre-transplantation assessments, including detection, isolation and elimination of undifferentiated cells will have a better prognosis.

However, it is important to know that CD markers have a key role in multi-lineage differentiation potential of ADSCs and can be used for cell detection. It has been reported that the expression of CD90 marker will be elevated after human articular chondrocytes cultured which is consistent with our study [38]. In addition, another study's results demonstrated that CD90 is a main index of the chondrogenic differentiation [39].

Here, flow cytometry analysis showed that during chondrogenesis, the mean percent of cells which expressed CD90 and CD44 markers remains almost unchanged. So, it can be concluded that although these markers may have a fundamental role in differentiation of stem cells into chondrocytes, but these markers cannot be considered as unique markers for chondrocyte. Thus, before cell transplantation, these markers cannot be used for isolation of differentiated cells from undifferentiated.

As shown in Figure 3, the mean percent of hADSCs which expressed CD14/45 and CD3/19 markers, is very low, but one or two weeks after chondrogenic differentiation, the high percent of differentiated cells expressed these markers which is similar to human articular chondrocytes.

Since, the alginate does not affect on cell main characteristics, the increase expression of these markers may be related to TGF-β3 via enzyme inhibition. Meanwhile, due to the same expression of CD14/45 and CD3/19 markers in both differentiated cells and in human articular chondrocytes, it can be concluded that these markers can be considered as a unique CD "signature" for chondrocyte isolation before cell transplantation.

Conclusion

Overall, the results of this study showed that in both human articular chondrocytes and differentiated cells, CD14/45 and CD3/19 markers were express in high level. Therefore, during chondrogenesis and before cell transplantation these markers can be used to identify differentiated cells from undifferentiated cells.

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