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Original article

The effect of ketorolac and triamcinolone acetonide on adipogenic and hepatogenic differentiation through miRNAs 16/15/195: Possible clinical application in regenerative medicine

Sedigheh Fekri Aval[a,b], Nosratollah Zarghami[a,b,c,d,*], Effat Alizadeh[b], Seyed Abolghasem Mohammadi[b,e]

[a] Umbilical Cord Stem Cell Research Center (UCSRC), Tabriz University of Medical Sciences, Tabriz, Iran
[b] Department of Medical Biotechnology, Faculty of Advanced Medical Sciences, Tabriz, Iran
[c] Immunology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
[d] Hematology and Oncology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
[e] Department of Plant Breeding and Biotechnology, Faculty of Agriculture, University of Tabriz, Tabriz, Iran

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ABSTRACT

Objective: The role of miR-16/15b/195 and complementary compounds in the commitment of mesenchymal stem cells (MSCs) to different lineages is unknown. The aim of this work was to investigate the effect of ketorolac and triamcinolone acetonide on adipogenic and hepatogenic processes, respectively. Also, miR-16/15 and miR-195 expression levels were evaluated under different induction conditions.

Methods: MSCs were isolated, expanded and directed using adipogenesis medium or medium supplemented with ketorolac, and also subjected to hepatogenic differentiation using a cocktail of hepatocyte growth factor (HGF) and Oncostatin M (OSM) either with or without triamcinolone acetonide. Periodic acid-Schiff (PAS) staining, Oil red O staining, albumin and adiponectin protein secretion were evaluated. MiR-16 family expression level was assessed using qRT-PCR in four induced groups as compared to non-induced cells.

Results: The expression levels of miR-16 and miR-15 increased significantly from adipose derived stem cells to adipocytes ($p < 0.01$). Positive stimulatory effects of ketorolac and triamcinolone on adipogenic and hepatogenic induction, respectively, were observed. Ketorolac stimulated upregulation of miR-16/15b in the adipogenic induced stem cells. Interestingly, triamcinolone caused upregulation of miR-15b and miR-195 in hepatic commitment.

Conclusions: Two highly effective stimulators were identified. Ketorolac is similar to indomethacin and affects adipogenic differentiation. Triamcinolone is involved in hepatogenic commitment of stem cells like differentiation process of macrophages, adipocytes and osteocytes. The alteration of miR-16 family members' expression indicates that this family may play a possible role in directing stem cell fate.

1. Introduction

Transplantation of damaged organs and tissues is always one of the main aims of regenerative medicine. However, there are some barriers in this regard, which include immune system response [1]. In this area, self-stem cells, especially MSCs can be differentiated into desired cells. MSCs have been isolated from different organs such as the bone marrow, adipose tissue, and peripheral blood. As a result of the ease of harvest, lack of ethical concerns, potential autologous application and relative abundance of progenitors, adipose tissue derived stem cells (ADSCs) are the most widely used source. ADSCs are fibroblast like cells with multipotential capability which make them promising for regenerative medicine [2–5]. Substantial progress in regeneration field is needed to identify implicated epigenetic factors like miRNAs [6]. MiRNAs play an important role in different processes associated with obesity, adipocyte differentiation, lipid storage, hepatic triglyceride metabolism and liver metabolism [7]. Undoubtedly, they are the essential determinants of cell differentiation. However, accurate conclusions require detailed illustration of miRNAs expression pattern.

In the field of transplantation, the use of optimized culture compositions improves the biological processes, especially cell differentiation [8]. Ketorolac is a nonsteroidal anti-inflammatory drug (NSAID)

* Corresponding author at: Stem Cell Research Center, Tabriz University of Medical Sciences, Tabriz, Postal code: 51666-14756, Iran.
E-mail addresses: Fekrise@tbzmed.ac.ir (S.F. Aval), Zarghami@tbzmed.ac.ir (N. Zarghami), Alizadehe@tbzmed.ac.ir (E. Alizadeh), Mohammadi@tabrizu.ac.ir (S.A. Mohammadi).

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that is more active against cyclooxygenase-2 (COX-2). The efficiency of COX-2 in adipose tissue differentiation has been proven [9,10]. Also, studies show that glucocorticoids are involved in differentiation process, and triamcinolone is a long- acting glucocorticoid [11].

Recent studies show that upregulation of miR-16 contributes to the differentiation of MSCs towards myogenic phenotypes. MiR-15 and miR-16 arrest cells in G0/G1 stage by silencing multiple cell cycle genes [12]. MiR-195a is involved in obese fat tissues and downregulation of miR-195 significantly increase adipogenesis [13]. MiR-122 is highly expressed in the embryonic and adult livers making it liver–specific miRNA. Studies show that miR-16 and miR-122 are overlapping and miRNAs might share conserved functions [14,15]. MiR-16 is associated with the regulation of cell proliferation and regulates the expression of Cyclin D1 (CCND1) [15]. Differentiation of cells into cell types is always accompanied by an arrest of the cell proliferation [16]. According to these findings, the high level of miR-16 family expression can trigger the commitment of pluripotent stem cells.

Despite the vital effect of miR-16 family on cell proliferation, there is no report on the implication of this family in production of different cells. In this study, first, the possible effect of ketorolac and triamcinolone was investigated. Then, miR-16 family expression pattern was assessed in both the standard and induction differentiation conditions.

2. Materials and methods

2.1. Reagents

All the reagents were purchased from Gibco Life Technologies (Invitrogen, UK) unless otherwise specified. Tissue culture plastic ware was from SPL Life Sciences (Pocheon, Korea). All experimental procedures were repeated thrice.

2.2. Isolation and expansion of adipose derived MSCs

Subcutaneous adipose tissue specimens were obtained from five male donors undergoing partial thoracic surgery. Adipose tissue was washed three times with sterile phosphate buffered saline (PBS), and extracellular matrix was digested with 1% collagenase type I at 37 °C on a shaker to release the cellular fraction. The enzyme was neutralized with Dulbecco's modified Eagle's medium (DMEM) containing 10% Fetal Bovine Serum (FBS). After centrifugation, floating adipocytes, lipids and liquid were aspirated to obtain stromal vascular fraction (SVF) pellet. The cell pellet was resuspended in low glucose DMEM supplemented with FBS, L-glutamine and penicillin/streptomycin. Osteogenic induction media consisted of Alpha-Minimum Essential medium (α-MEM) supplemented with 10 mmol/L β-glycerol phosphate and 0.1 μmol/L dexamethasone from Sigma (St. Louis, MO, USA) and 10% FBS. Extracellular calcium was quantified on by Alizarin red staining (Sigma-Aldrich) according to the manufacturer's guideline.

2.3. Phenotypic analysis of ADSCs by flow cytometry

Third passage cells were analyzed for surface antigens by incubating the adherent cells with monoclonal antibodies. Cells were washed twice with PBS, and subsequently detached using trypsin/EDTA solution for 5 min at 37 °C. ADSCs were adjusted to 106 cells/mL and then stained with FITC-conjugated mouse anti-human CD14, CD19, CD73, CD45, CD90, CD105 and PE-conjugated mouse anti-human CD34 (Pharmingen, San Diego, CA, USA) antibodies (diluted 1:100), washed twice in PBS, fixed with 4% paraformaldehyde (PFA) and analyzed using a Becton, Dickinson (BD) and Company flow cytometer (FACScalibur™, San Jose, CA, USA).

2.4. Induction to adipocytes

Cells were seeded in 6-well plates and left to reach 60% confluency. The cells were treated with an adipocyte induction cocktail containing FBS, 3-isobutyl-1-methylxanthine (IBMX 0.5 mM), indomethacin (0.2 mM), insulin-transferrin-selenium-ethanolamine (ITS) (1 μg/mL) and dexamethasone (0.1 μM) in DMEM. The other group was treated with the same cocktail as well as 100 μM ketorolac from Sigma-Aldrich (St. Louis, USA). The final volume was 1.5 mL media per well/sample for a six-well plate. The cells under differentiation were harvested at the 7th, 14th, 21st and 28th days for total microRNA extraction. Then cells were fixed and assessed with Oil Red O (1-([4-(Xylylazo)xylyl]azo)-2-naphthol) using manufacturer's instruction (Bioidea, Iran). For this purpose, cells were fixed in 4% formalin at room temperature for 1 h. The fixed cells were stained for 15 min at room temperature and washed with 70% ethanol and distilled water.

2.5. Induction of hepatocyte-like cells

MSCs were cultured in H-DMEM containing l-ascorbic acid (20 μL), HGF (10 ng/mL, Gibco, USA) and OSM (10 ng/mL, Sigma, USA), while the other group was treated with the same cocktail as well as 0.01 mg/mL of triamcinolone acetonide (Hexal AG, Holzkirchen, Germany). After 10 days, 0.1% Dimethyl sulfoxide (DMSO, Merck, and German) was added to the induction medium. The final volume was 1.5 mL media per well/sample for a six-well plate. PAS staining was performed for hepatocyte-like cells monitoring (Sigma-Aldrich). The cells were harvested at the 7th, 14th, 21st, and 28th days for total microRNA extraction.

2.6. Adiponectin and albumin quantification

The culture media were gathered from wells during differentiation and stored at −70 °C. After completion of the process in each triple group, obtained supernatants were centrifuged for 10 min to remove debris. Albumin and adiponectin protein secretion were measured by enzyme linked immunosorbent assay (ELISA, Invitrogen, Thermo Scientific™ Pierce™) from supernatants in two 96-well microplates which were pre-coated with adiponectin or albumin antibody, according to the manufacturer's guideline by microplate reader (Awareness Technology, USA) at 450 nm.

2.7. Evaluation of miRNA expression level

RNA was isolated from the cells under induction and MSCs with miRCURYTM RNA isolation kit- Cell and Plant (Exiqon, Denmark, Cat No. #300110). The RNA concentration was determined using NanoDrop; ONEC Spectrophotometer (Thermo Scientific, MA, USA) with 1 μL of each sample. Complementary DNA (cDNA) was synthesized using Universal cDNA synthesis kit (Exiqon, Denmark, Cat No. #203301), in the total volume of 10 μL reaction mixture. The product was loaded with 1:100 dilution for PCR reaction by miRCURY LNATM Universal RT microRNA PCR SYBR master mix (ExiLENT, Exiqon, Denmark, Cat No. #203403). The reaction parameters for the quantitative PCR analysis were 95 °C for 10 min followed by 45 amplification cycles at 95 °C for 10 s, 60 °C for 1 min and ramp-rate 1.6 °C/S. The relative quantitative levels of each miRNA were calculated by the $2^{-\Delta\Delta CT}$ method. Locked nucleic acid (LNA) based reverse- transcriptional microRNA primers are listed in Table 1.

2.8. Statistical analysis

The data were analyzed by IBM SPSS Statistics 22 and GraphPad Prism 6 Demo (GraphPad Software, San Diego, CA). Student's *t*-test and one-way ANOVA analyses were used for comparing different groups. All variables were expressed as Mean ± SEM. A value of $p \leq 0.05$ was considered significant, whereas $p \leq 0.001$ was highly significant.