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Research article

Comparative epigenetic influence of autologous versus fetal bovine serum on mesenchymal stem cells through in vitro osteogenic and adipogenic differentiation

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ABSTRACT

Mesenchymal stem cells (MSCs) derived from bone marrow (BM) represents a useful source of adult stem cells for cell therapy and tissue engineering. MSCs are present at a low frequency in the BM; therefore expansion is necessary before performing clinical studies. Fetal bovine serum (FBS) as a nutritional supplement for in vitro culture of MSCs is a suitable additive for human cell culture, but not regarding subsequent use of these cells for clinical treatment of human patients due to the risk of viral and prion transmission as well as xenogeneic immune responses after transplantation. Recently, autologous serum (AS) has been as a supplement to replace FBS in culture medium. We compared the effect of FBS versus AS on the histone modification pattern of MSCs through in vitro osteogenesis and adipogenesis. Differentiation of stem cells under various serum conditions to a committed state involves global changes in epigenetic patterns that are critically determined by chromatin modifications. Chromatin immunoprecipitation (ChIP) coupled with real-time PCR showed significant changes in the acetylation and methylation patterns in lysine 9 (Lys9) of histone H3 on the regulatory regions of stemness (*Nanog, Sox2, Rex1*), osteogenic (*Runx2, Oc, Sp7*) and adipogenic (*Ppar-γ, Lpl, adiponectin*) marker genes in undifferentiated MSCs, FBS and AS. All epigenetic changes occurred in a serum dependent manner which resulted in higher expression level of stemness genes in undifferentiated MSCs compared to differentiated MSCs and increased expression levels of osteogenic genes in AS compared to FBS. Adipogenic genes showed greater expression in FBS compared to AS. These findings have demonstrated the epigenetic influence of serum culture conditions on differentiation potential of MSCs, which suggest that AS is possibly more efficient serum for osteogenic differentiation of MSCs in cell therapy purposes.

1. Introduction

Mesenchymal stem cells (MSCs) are multipotent adult stem cells found in the bone marrow (BM) and possibly the stroma of numerous other organs [1,2]. These cells have the capability to differentiate into multiple mesoderm-type cells such as osteoblasts, adipocytes and chondrocytes. Due to their stable growth and differentiation potential, MSCs are ideal candidates for use in regenerative medicine and cell transplantation protocols. Given the therapeutic potential of MSCs for a variety of conditions, in particular bone and cartilage defects, it is important to continue to explain the accurate mechanisms that influence MSCs fate. Autologous stem cell transplantation therapies have been proposed for their therapeutic potential and low immunogenicity. However, many of these proposed therapies rely on prior in vitro expansion of the cell populations. Fetal bovine serum (FBS) is commonly used in cell culture medium for expansion of MSCs in vitro as well as for human clinical trials [3]. However, use of FBS may have undesirable effects in therapeutic applications and an increased risk of contamination by pathogens or transmission of xenogeneic proteins that can cause immune responses in humans [4]. Nevertheless, alternative sources of growth supplements are being investigated. Replacement of FBS with pooled allogeneic AB blood group serum [5,6], thrombin-activated platelet-rich plasma [5], human platelet lysate [6–9], bovine fibroblast growth factor [10], autologous platelet-rich plasma (PRP) [11] and chemically defined medium containing a low percentage of human serum [12] supports equal or greater proliferation and/or multilineage differentiation of MSCs [4]. Researchers have stated that BM-MSCs

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expanded in the medium that contains autologous serum (AS) proliferate faster, differentiate less rapidly [13] and possess greater mineralization capacity in osteogenic culture of cells than those cultured in FBS-supplemented medium [14].

Stem cell behavior is largely mediated by sequential changes in the expression pattern of genes involved in development. There are multiple levels of expression regulation apart from the genetic outline that includes posttranscriptional, translational, post-translational, and epigenetic regulatory processes. Epigenetic processes are heritable modifications of DNA and chromatin that affect gene expression without altering the genomic sequence [15,16]. It is well recognized that hyperacetylation of histones, specifically acetylation of Lys (K) 9 of histone H3 (H3K9ac), is highly associated with gene activation, whereas methylation of lysine 9 (Lys 9) of histone H3 (H3K9me) is considered a hallmark of inactive chromatin [17].

Epigenetic modifications of histones are basically controlled by a large family of histone modifying enzymes that include histone acetyl/methyl transferases and deacetylase or demethylase enzymes [18]. Theoretically, any bioactive component or condition that can affect the enzyme activity of these modifiers can potentially cause epigenetic alterations in its related biological system. An epigenetic study has shown that BM-MSCs display a more stable gene expression profile in AS compared to FBS [19]. In this regard, Zippo and colleagues were able to propose a mechanism of gene activation by identifying the histone-modifying enzymes that be found in serum [20]. On the other hand, it was shown by investigators that RNA Pol II recruits a deacetylase inhibitor in serum, and cause to an increase in the level of H3K9 and H4K16 acetylation [21]. However, as discovering the exact role of serum in gene expression has been complicated by finding crosstalks among modifications, there is to date no side-by-side study on how FBS and AS affect genomic and epigenomic stability of MSCs during their extended culture.

Differentiation of MSCs is a complex process tightly controlled by epigenetic regulation of numerous developmental genes [22]. In this study, we have compared histone modification of the promoter regions of marker genes involved in stemness (*Sox2, nanog, Rex1*), osteogenesis (*Runx2, Sp7, Oc*) and adipogenesis (*Ppar-γ, Lpl, adiponectin*) in BM-MSCs expanded in FBS or AS. The DNA methylation status of BM-MSCs expanded in media supplemented with AS and FBS has been previously analyzed and compared by investigators [23], but to our knowledge this is the first time that differentiation-sensitive alterations in an epigenetic state of involved genes have been compared in these culture conditions.

2. Materials and methods

2.1. Animals

Ten male Wistar rats, 8–10 weeks of age, were used. This experiment was approved by the Ethical Committee Royan Institute. The animals were raised under conventional, but permanently controlled conditions and fed with standard food and water ad libitum. They were anesthetized with intramuscular doses of ketamine and xylazine, their chests were opened under anesthetic, and the whole peripheral blood was collected with a needle inserted through the wall of the heart into the left ventricle. Bone marrow from rat long bones was harvested by flushing through the marrow with 5 ml Dulbecco's modified Eagle's medium (DMEM, Sigma, USA) containing 100 U/ml penicillin (Gibco, UK) and 100 IU/ml streptomycin (Gibco, UK).

2.2. Autologous serum (AS) preparation

All blood samples were allowed to clot for 4 h at 4–8 °C and centrifuged at 2500 rpm for 10 min. Serum was collected and filtered through a 0.2 μm membrane and aliquots were stored at −20 °C. The AS was inactivated at 56 °C for 30 min prior to use.

2.3. Isolation and culture of bone marrow mesenchymal stem cells (BM-MSCs)

MSCs were isolated from rat's BM according to a previously described procedure [14]. Cells were plated overnight in DMEM that contained either 15% AS or FBS and antibiotics. On day 3, the medium was replaced with fresh medium that contained 15% AS or FBS. Cells were subcultured by trypsinization at 70% confluency and re-seeded at a density of approximately 5000 cells/cm^2. Viable cells were passaged 3 times and the culture medium was replaced every 2–3 days.

2.4. Flow cytometry analysis

Flow cytometric analysis was used to characterize the isolated cells cultured in media supplemented with FBS and AS with respect to their surface antigen profiles. For this purpose, approximately 250,000 cells/tube of passage-3 (P3) rMSCs were incubated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE)-conjugated monoclonal MSC marker antibodies against CD11-b, CD44, CD90 and CD45 (BD) with their IgG isotypes for 30 min at 4 °C in the dark. After the incubation time, 100 μl PBS or FACS dilution solution was added to each tube and the tubes were centrifuged at 1200 rpm for 5 min in the dark. Supernatants were removed and pellets re-suspended in a 100 μl PBS dilution in the dark. We evaluated the data with a Becton Dickinson FACSCalibur using Flowing software.

2.5. Mesodermal lineage differentiation

Multipotency was characterized by in vitro differentiation of MSCs to adipogenic, chondrogenic and osteogenic lineages. Differentiation of AS- and FBS-supplemented cultures towards osteogenic and adipogenic cell lineages were estimated by establishing previously described induction cultures [14] and both the groups were differentiated in the same time duration of 21 days. For osteogenic differentiation, DMEM medium supplemented with 50 mg/ml ascorbic 2-phosphate (Sigma, USA), 10 nM dexamethasone (Sigma, USA) and 10 mM β-glycerolphosphate (Sigma, USA) was added to confluent P3 cultures which were subsequently cultured for another three weeks. Cultures were fixed in methanol for 10 min and stained with alizarin red solution for 2 min to reveal the deposition of a mineralized matrix. At the end of the differentiation period, the amount of Alizarin Red staining in two group in AS and FBS cultures were quantified using an Osteogenesis Quantification Kit (ChemiconInternational, Hofheim, Germany). For adipogenesis, DMEM medium that contained 100 nM dexamethasone (Sigma, USA) and 50 mg/ml indomethacin (Sigma, USA) was used to induce differentiation in a confluent culture of P3 cells. Three weeks later, the culture was fixed with 4% formalin at room temperature, washed with 70% ethanol and stained with oil red solution in 99% isopropanol for 15 min. Quantitation of lipid was performed by oil red O extraction in triplicate wells by lipid staining kit (BioVision, USA). Cartilage differentiation was induced by a micro-mass culture system. P3 cells (2.5×10^5) were pelleted at 1200*g* for 5 min and cultured in a DMEM medium supplemented with 10 ng/ml transforming growth factor-β3 (TGF-β3) (Sigma, USA), 10 ng/ml bone morphogenetic protein-6 (Sigma, USA), 50 mg/ml insulin transferrin selenium+premix (Sigma,