CELL REGENERATION

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Combined influence of basal media and fibroblast growth factor on the expansion and differentiation capabilities of adipose-derived stem cells

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Abstract

Background: Interest in adipose-derived stem cells (ASCs) has increased in recent years due to their multi-linage differentiation capabilities. While much work has been done to optimize the differentiation media, few studies have focused on examining the influence of different expansion media on cell behavior. In this study, three basal media (low glucose Dulbecco's modified Eagle's medium (DMEM), high glucose DMEM and DMEM-F12) supplemented with or without fibroblast growth factor 2 (FGF) were examined to assess their suitability for expanding ASCs.

Findings: Flow cytometry, colony-forming unit assays (CFU-Fs) and differentiation assays were utilized to study cell behavior. High glucose media CFU-Fs produced fewest colonies while the addition of FGF increased colony size. By passage 2, the majority of cells were positive for CD44, 45, 73, 90 and 105 and negative for CD14, 31 and 45, indicating a mesenchymal phenotype. A sub-population of CD34 positive cells was present among passage 2 cells; however, by passage 4 the cells were negative for CD34. FGF has a negative effective on passage 4 ASC adipogenesis and high glucose media plus FGF-enhanced osteogenic capacity of passage 4 ASCs. FGF supplemented basal media were most suitable for chondrogenesis. High glucose media plus FGF appeared to be the most beneficial for priming ASCs to induce a keratocyte phenotype.

Conclusions: These findings demonstrate the reciprocal effect FGF and basal media have on ASCs. This research has implications for those interested regenerating bone, cartilage, cornea or adipose tissues.

Keywords: Cell differentiation, Cartilage, Bone, Adipose, Cornea

Findings

Research hypothesis

The ability of adipose-derived stem cells (ASCs) to undergo prolonged periods of expansion and differentiation towards specific lineages has made them a valuable cell source for clinicians and researchers interested in tissue repair and regenerative medicine. These cells are considered to exhibit mesenchymal stem cell characteristics by their ability to form bone, cartilage and adipose tissues and by the presence or absence of specific CD markers [1]. Maintaining a suitable culture environment is essential for retaining the stem-like characteristics of ASCs. Commercially available media have been formulated to cultivate these cells; however, these are generally more expensive than standard culture media and their effectiveness over standard chemically defined supplemented media has been brought into question [2]. For these reasons, many researchers continue to use media consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal bovine serum (FBS), antibiotics and in some cases growth factors most commonly fibroblast growth factor 2 (FGF). Low glucose DMEM [3,4], high glucose DMEM [5,6] and DMEM plus nutrient mix F12 [7,8] are among the most commonly used basal media for expanding ASCs, although many publications do not state the media glucose concentration. There

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appears to be little consensus as to which of these media sources and growth factors are most suitable for ASC expansion. The aims of this study were to compare three of the most commonly used basal media for ASC expansion (low glucose DMEM, high glucose DMEM and DMEM-F12) and determine whether they had any advantages or limitations. The influence of adding FGF to each of these media was also examined. After expansion in each type of media, the cells were placed in chemically defined differentiation media to promote an adipogenic, osteogenic, chondrogenic or keratocyte phenotype. ASC differentiation was evaluated and used to determine the most effective expansion media for each specific lineage.

Materials and methods

Isolation and culture of adipose-derived stem cells

Subcutaneous adipose tissue was taken with consent from patients undergoing esophageal surgery. This study has received ethical approval from the St. James's Hospital Institutional Review Board. The tissue was then digested by using 300 unit/ml collagenase type I solution under constant agitation for 90 min at 37°C. Once digested, the tissue was poured through a 100 μm filter and centrifuged to separate the stromal vascular fraction from the adipocytes. The adipose stromal vascular fraction was then suspended in erythrocyte lysis buffer consisting of 155 mM NH_4Cl, 10 mM K_2CO_3 and 0.1 mM EDTA in H_2O at room temperature for 10 min. The cells were centrifuged, suspended in fresh media and poured through a 40 μm filter. The cells were then counted and split into six groups: 1) low glucose DMEM (LG) with a glucose concentration of 1,000 mg/l; 2) high glucose DMEM (HG) with a glucose concentration of 4,500 mg/l; 3) DMEM-F12 (F12); 4) low glucose DMEM supplemented with FGF (LG + FGF); 5) high glucose DMEM supplemented with FGF (HG + FGF) and 6) DMEM-F12 supplemented with FGF (F12 + FGF). All basal media were purchased from Thermo Scientific (Hyclone). All media were supplemented with 10% (*v*/*v*) FBS (Hyclone, Thermo Scientific), 100 U/ml penicillin (Gibco), 100 μg/ml streptomycin (Gibco) and 250 ng/ml amphotericin-B (Sigma-Aldrich). When FGF was added, it gave a final concentration of 10 ng/ml in media. Cells isolated from two separate donors were examined.

Colony-forming unit assay (CFU-F)

Freshly isolated cells from the stromal fraction were plated onto Petri dishes at a concentration of 50 cells/cm^2. These cells were cultured in one of the six media already mentioned (LG, HG, F12, LG + FGF, HG + FGF and F12 + FGF) for 14 days. The cells were then washed using PBS, fixed in 2% (*w*/*v*) paraformaldehyde and stained using 1% (*w*/*v*) crystal violet solution. The numbers of colonies formed on each dish were counted.

Flow cytometry

Passage 2 (P2) and passage 4 (P4) ASCs that had been cultured in all six media types were suspended in a buffer solution consisting of PBS with 2% FBS. The cells were labelled using fluorescently conjugated primary antibodies for CD14, 19, 31, 34, 44, 45, 73, 90, 105 and 146 (all eBioscience) in buffer solution as previously described [9]. The cells were then repeatedly washed and centrifuged to remove unbound antibodies. Compensation controls were performed using compensation beads (eBioscience). Flow cytometry was performed using a LSRFortessa flow cytometer (BD Biosciences). The data collected was analysed using Flowing Software version 2.5.1 (University of Turku, Finland).

Differentiation assays

For adipogenesis, 5×10^4 P2 or P4 ASCs cultured in each of the six media types were plated onto each well of a six-well plate and cultured in their respective expansion media at 37°C, 5% CO_2 until they reached 80% confluence. The cells were then cultured for a further 21 days in an adipogenic differentiation media. After 21 days in culture the cells were fixed using 4% (*w*/*v*) paraformaldehyde and then stained using 1% (*w*/*v*) oil red O solution. Images were recorded using a light microscope at six random locations in each well. The percentage surface area stained positive by the oil red O in each well was quantified using ImageJ software (NIH). Cells cultured in regular expansion media were used as a negative control. For osteogenesis, 5×10^4 P2 or P4 ASCs were plated onto each well of a six-well plate and cultured in their respective expansion media at 37°C, 5% CO_2 until they reach 80% confluence. The cells were then cultured for a further 21 days in an osteogenic differentiation media [9]. After 21 days, the cells were fixed using ice-cold ethanol and stained using 1% (*w*/*v*) alizarin red solution for 2 min. The plates were then washed until the negative control wells were clear. Images of the staining were recorded. The absorbance of each well was measured at a wavelength of 405 nm to determine the intensity of alizarin red staining. For chondrogenesis, P2 or P4 ASCs were centrifuged to form pellets each containing 250,000 cells. The pellets were cultured in a chondrogenic differentiation media [10]. The pellets were cultured at 37°C, 5% CO_2, 5% O_2 for 21 days. The pellets were then taken for biochemical analysis or fixed in 4% (*w*/*v*) paraformaldehyde. Fixed pellets were dehydrated using alcohol and zylene, embedded in paraffin wax. The pellets were then sliced (5 μm) and stained for GAG and cells using alcian blue and 0.1% (*w*/*v*) nuclear fast red solution. Pellets for biochemical analysis were digested using a papain digest for 18 h at 60°C. DNA was quantified using a Hoechst 33324 assay while sGAG was quantified using a DMMB assay as previously described [5].

For keratocyte differentiation, 250,000 P2 cells were seeded to 25-cm^2 culture flasks and cultured for 14 days