Human Adipose Stem Cells in Chondrogenic Differentiation Medium without Growth Factors Differentiate Towards Annulus Fibrosus Phenotype In Vitro

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Summary: Intervertebral disc degeneration is the main cause of chronic back pain. Disc degeneration mainly leads to tearing of annulus fibrosus (AF), which is with current methods difficult to restore and impossible to regenerate. Stem cell technology offers a potential technique to repair the ruptured AF by enabling new matrix synthesis at the defect site.

Previous studies have shown that human adipose stem cells (hASCs) are able to differentiate towards AF phenotype when treated with suitable growth factors. There are concerns about the use of growth factors in clinical applications because of their short half-lives, high costs and low effectiveness. The main aim of this research project was to regenerate AF tissue *in vitro* using hASCs and serum free chondrogenic medium without supplementation of growth factors.

Differentiation of hASCs was induced by using the micromass culture technique. Human annulus fibrosus cell (hAFCs) cultured in commercial AF growth medium were used as positive control. Assessment of AF phenotype of hASCs and hAFCs was done at 14 and 21 days of culture.

Quantification of sulphated glycosaminoglycan (GAG) content showed that hASCs cultured in chondrogenic medium expressed similar levels of sulphated GAGs as hAFCs. qRT-PCR confirmed the similarity of the differentiated hASCs with AF phenotype. Several markers for AF phenotype (aggrecan, collagen type I and glypican-3) were expressed in both hAFCs and differentiated hASCs.

This is the first study that demonstrated that hASCs can be differentiated towards AF phenotype using serum free chondrogenic medium without growth factors. In a next step, scaffolds manufactured from biodegradable polymers will be used in combination with ASCs to find an optimal construct to repair AF defects.

Keywords: adipose stem cells; annulus fibrosus; intervertebral disc; tissue engineering

Introduction

Low back pain is the most common spinal disorder. It affects over 75 to 80% of people at some point in their life.[1] Intervertebral disc (IVD) degeneration is thought to be the primary cause of low back pain.[2] In an attempt to restore the biomechanical function of degenerated IVDs as shock absorber, replacement or regeneration of the inner nucleus pulposus (NP) will only be successful in the presence of an outer annulus fibrosus (AF) that is able to withstand the forces exerted from inside the disc.[2] A compromised AF is with current surgical methods difficult to restore and impossible

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to regenerate.[3] Stem cell technology offers a potential technique to repair the ruptured AF by enabling new matrix synthesis at the defect site.

AF cells have limited expansion and have shown to lose differentiation markers during monolayer expansion. Therefore, the use of stem cells in an engineered tissue seems to be more promising than culturing primary AF cells.[4] Human adipose stem cells (hASCs) are a group of mesenchymal stem cells (MSCs) derived from adipose tissue. After lineage-specific stimulation, hASCs have shown to differentiate into AF cells, among other lineages. In addition, they are relatively easily harvested from adipose tissue.[5,6]

Concerns have emerged about the use of growth factors in clinical applications because of their short half-lives, high costs and low effectiveness.[7] The main aim of this research project was to regenerate AF tissue *in vitro* using hASCs and serum-free chondrogenic medium without supplementation of growth factors. This study comprised the development of novel culture conditions for hASCs to efficiently differentiate them towards AF cells. Characterization of hASC phenotype at 14 and 21 days of culture was done using sulphated GAG assay and qRT-PCR.

Serum-free conditions were selected, because of concerns regarding the use of fetal bovine serum (FBS) for the culture of hASCs aimed for clinical therapy. Alternatives for FBS have been found in the use of autologous human serum (HS), but its use has been hindered due to variability and limited availability.[8] Therefore, the best option for clinical hASC culture would be a defined serum-free medium.[9,10]

Secondly, this study aimed to find specific markers to verify the AF differentiation of hASCs in order to distinguish it from articular cartilage and NP differentiation.

Materials and Methods

Adipose Stem Cell Isolation and Expansion

Human adipose tissue was received from 3 female donors (age 51 ± 7) in surgical operations performed at the Tampere University Hospital, Tampere, Finland. The study was carried out under approval of the ethical committee of Pirkanmaa hospital district and with informed consent from the donors.

Surgically obtained subcutaneous fat tissue was placed in a sterile petri dish, minced well and digested for a minimum of 1 h with 1.5 mg/ml collagenase type I (Gibco, Life technologies, Carlsbad, CA, USA) at 37°C in a shaking water bath. The digested tissue was then centrifuged at 1800 rpm for 5 min at RT, after which the fat phase, connective tissue phase and collagenase phase were removed. Red blood cells were lysed by adding 2 ml sterile water (Baxter, Deerfield, IL, USA) for 2 min. Human ASCs were suspended in maintenance medium (MM; Table 1) Undigested tissue was removed by filtering

Table 1.
Medium compositions used in the study.

Medium	Composition
Maintenance medium (MM)	DMEM-F12 (Gibco); 5–10% human serum (PAA Laboratories GmbH, Pasching, Austria); antibiotics (100 U/ml penicillin; 100 μg/ml streptomycin; Lonza Biowhittaker, Verviers, Belgium); 1x L-alanyl-L-glutamine (Glutamax I, Gibco)
Chondrogenic medium (CM)	DMEM-F12 (Gibco); ITS + 1 (6.25μg/ml insulin, 6.25μg/ml human transferrin, 6.25 ng/ml selenous acid 1.25 mg/ml bovine serum albumin, 5.35 μg/ml linoleic acid, BD Biosciences); antibiotics (30 U/ml penicillin, 30 μg/ml streptomycin, Lonza Biowhittaker, Verviers, Belgium); 1x L-alanyl-L-glutamine (Glutamax I; Gibco); 50 μg/ml L-Ascorbic acid 2-phosphate (Sigma); 55 μg/ml sodium pyruvate (Lonza); 23 μg/ml L-proline (Sigma)
Nucleus Pulposus Culture medium (NPCM)	NPCM basal medium (ScienCell), 2% vol FBS (ScienCell), 1% vol Nucleus Pulposus Cell Growth Supplement (ScienCell), 1% vol antibiotics (100 U/ml penicillin; 100 μg/ml streptomycin; ScienCell)

through 100 μm nylon cell strainers (BD Falcon, BD Biosciences, San Jose, CA, USA). Human ASCs were plated for primary culture in sterile culture flasks (Nunc, Roskilde, Denmark) for expansion. Cells were cultured in 10 ml MM at 37°C and 5% carbon dioxide (CO_2). Flasks were washed with Dulbecco's Phosphate-Buffered Saline (Gibco) the next day to remove non-adhering cells. Half of the medium was refreshed twice a week. When they were confluent, hASCs were subcultured by detaching with TrypLE Select (Gibco), centrifuging at 1000 rpm for 5 min and replating.

Adipose Stem Cell Characterization

After primary culture, hASCs were characterized by flow cytometry (FACSAria; BD Biosciences, Erembodegem, Belgium) to confirm the mesenchymal origin of the cells. Monoclonal antibodies against CD3-PE, CD14-PE-Cy7, CD19-PE-Cy7, CD45RO-APC, CD54-FITC, CD73-PE and CD90-APC (BD Biosciences); CD34-APC and HLA-DR-PE (Immunotools GmbH, Friesoythe, Germany) and CD11a-APC, CD80-PE and CD105-PE (R&D Systems Inc, MN, USA) were used. Analysis was performed on 10,000 cells per samples and positive expression was defined as the level of fluorescence greater than 99% of the corresponding unstained cell sample.

Human ASCs demonstrated high expression (>90%) of CD90 (Thy-1) and CD105 (endoglin), moderate (>65%) or high expression of CD73 (ecto 5′ nucleotidase) and no or low expression (≤2%) of CD3 (T cell marker), CD11a (lymphocyte function-associated antigen 1), CD14 (monocyte and macrophage marker), CD19 (dendritic cell marker), CD34 (hematopoietic progenitor and endothelial cell marker), CD45RO (pan-leukocyte marker), CD80 (B cell marker), CD86 (antigen presenting cell marker), and HLA-< PREFIX > DR </PREFIX > (HLA class II). The results showed that hASCs expressed several of the specific antigens that define human stem cells of mesenchymal origin according to the Mesenchymal and Tissue Stem Cell Committee of the ISCT.[11]

Stimulation of Adipose Stem Cell Differentiation Using Micromass Technique

Differentiation was induced by using the micromass culture technique modified from Ahrens et al. (1977).[12] A cell suspension with high cell density (1×10^7 cells/ml) was prepared. The cell suspension was added as 3 droplets of 10 μl at the centre of wells in 24-well plates (Nunc). Cells were allowed to attach for 3 h in the incubator (37°C, 5% CO_2). Being careful to avoid detachment of the micromasses, 700 μl chondrogenic medium (CM; Table 1) was gently added. Cultures were maintained in the incubator for 3 weeks and whole medium was changed 3 times a week.

Annulus Fibrosus Cell Culture

Human annulus fibrosus cells (hAFCs; ScienCell Research Laboratories, Carlsbad, CA, USA) were plated as passage 1 at 5000 cells/cm^2 in sterile culture flasks. Human AFCs were expanded in 20 ml nucleus pulposus culture medium (NPCM; Table 1) at 37°C, 5% CO_2. Whole medium was refreshed 3 times a week. When the cells were confluent, hAFCs were subcultured by detaching with trypsin solution (Gibco®), centrifuging at 1000 rpm for 5 min and replating at 5000 cells/cm^2.

After expansion, cells of passage 3–4 were plated as micromasses in 24-well plates (Nunc) as described in the previous section. Cells were allowed to attach for 3 h in incubator (37°C, 5% CO_2) before NPCM was added. Cultures were maintained in the incubator for 3 weeks and whole medium was changed 3 times a week. After 2 weeks and 3 weeks of culture, micromasses were collected for analysis.

Sulphated Glycosaminoglycan Assay

The total amount of sulphated GAGs in the papain lysate samples was analysed with a sulphated GAG assay (Blyscan, Biocolor Ltd, Carrickfergus, UK). In order to quantify the amount of sulphated GAGs,

chondroitin 4-sulphate sodium salt from bovine trachea was used as a standard. Dye reagent (Biocolor) was added to standards and papain lysate sample duplicates. Binding of the cationic dye (methylmethylene blue) to sulphated parts of GAG molecules was done for 30 min at room temperature. Samples were centrifuged at 11,000 rcf for 10 min. The unbound dye was then removed by inverting the sample tubes. Dissociation reagent (Biocolor) was added to release the bound dye into solution. The dye solution was added in duplicates to a 96-well plate (Nunc) and measured with Victor (PerkinElmer, Waltham, MA, USA) multiplate reader at 650 nm. Sulphated GAGs were quantified 3 times for hASCs each time using a different donor. Human AFCs cultured in commercial AF growth medium were used as a positive control. Since the commercially obtained hAFCs were derived from one donor, sulphated GAG assay was only performed once for hAFCs. Therefore no SD is presented for hAFCs and statistical analysis against the hASCs derived from 3 donors was not possible.

Quantitative Reverse Transcription PCR

Quantitative Reverse Transcription polymerase chain reaction (qPCR) was used to compare the relative expression of AF specific genes. Total RNA was isolated from micromasses after 3 weeks of differentiation using NucleoSpin® RNA II Total RNA isolation kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) according to the manufacturer's instructions. Nanodrop (Thermo Fisher Scientific, Wilmington, USA) was used to measure total RNA yield by optical density at 260 nm and to assure sample purity from the ratio of A260/A280.

A High-Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, Foster City, CA, USA) was used to prepare cDNA from the total RNA. Reverse transcription was performed in GeneAmp PCR System 2400 (Perkin Elmer, CT, USA).

Gene expression of aggrecan (AGG), collagen type I (ColI) and glypican-3 (GPC3) was analysed on the basis of mRNA levels by quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) method. Human acidic ribosomal phosphoprotein P0(RPLP0) was used as a reference gene, which has shown to have stable expression under several experimental conditions. The primer sequences (Oligomer Oy, Helsinki, Finland) are presented in Table 2.

The qRT-PCR reaction mixture contained a maximum of 50 ng cDNA, 300 nM forward and reverse primers and Power SYBR® Green PCR Master Mix (Applied Biosystems).

Reactions were carried out in duplicates in ABI Prism® 7300 Sequence Detection System, Applied Biosystem) with initial activation at 95°C for 10 minutes, followed by 45 cycles of denaturation at 95°C for 15 seconds and anneal and extend at 60°C for 60 seconds. Melting curve analysis was used to check the quality of the PCR products. Normalization was performed using a cycle threshold (C_t) value obtained from the exponential area of amplification curves for each sample and a slope value of a linear standard curve. The results were normalized to hRPLP0 expression according to a

Table 2.
Primer sequences.

Gene		Primer sequence	Product size
acidic ribosomal phosphoprotein Po	Forward:	5′-AAT CTC CAG GGG CAC CAT T-3′	70 bp
	Reverse	5′-CGC TGG CTC CCA CTT TGT-3′	
aggrecan	Forward:	5′-TCG AGG ACA GCG AGG CC-3′	85 bp
	Reverse:	5′-TCG AGG GTG TAG CGT GTA GAG A-3′	
collagen type I	Forward:	5′-CCA GAA GAA CTG GTA CAT CAG CAA-3′	95 bp
	Reverse:	5′-CGC CAT ACT CGA ACT GGA ATC-3′	
glypican-3	Forward:	5′-CTCTGCTGTTGACAATGGCTCTCT-3′	420 bp
	Reverse:	5′-TGGATGGCTGTATCTCCCAGTACT-3′	

mathematical model described by Pfaffl.[13] Analysis with qPCR was repeated 3 times for hASCs each time using a different donor. Human AFCs cultured in commercial AF growth medium were used as a positive control. Since the commercially obtained hAFCs were derived from one donor, sulphated qPCR was only performed once for hAFCs. Therefore no SD is presented for hAFCs and statistical analysis against the hASCs derived from 3 donors was not possible.

Results

Sulphated Glycosaminoglycan Assay

Micromasses were harvested for quantitative sulphated GAG analysis at 14 and 21 days of culture (Figure 1). Similar levels of sulphated GAGs were found in hASC and hAFC cultures at 14 days of culture. hASC micromasses showed an overall decrease of sulphated GAGs between 14 and 21 days of culture The sulphated GAG content of hAFC micromasses stayed on similar levels at both time points.

qRT-PCR

Expression levels of AF specific genes at 21 days of culture are presented relative to the expression level of the hAFC cultures (Figure 2). Means and standard deviations of the expression in hASCs are calculated from the average dCt values from 3 donor repeats. In case of hAFC, no standard deviation is shown, because only 1 donor was used for this cell type. Both hAFCs and differentiated hASCs showed expression of AGG, ColI and GPC3. Donor variation was detected between the 3 repeats. Expression of AGG and ColI was higher in differentiated hASCs compared to hAFCs. GPC3 was expressed at similar levels in hAFCs and hASCs.

Discussion

To the best of our knowledge, this is the first study to assess the differentiation of hASCs towards an AF phenotype using a defined serum-free medium without growth factors. This is also the first time that annulus fibrosus differentiation of hASCs was assessed up to 21 days of culture.

In order to direct ASCs to an annulus phenotype, several studies have assessed the effect of adding TGF-β3 in culturing of ASCs.[14,15] These studies show the importance of the isoforms of TGF-β in differentiation of ASCs towards a disc-like phenotype. In a future study, it would be interesting to compare the effects of TGF- β supplemented chondrogenic medium to chondrogenic medium without growth factors on hASC differentiation towards hAFCs.

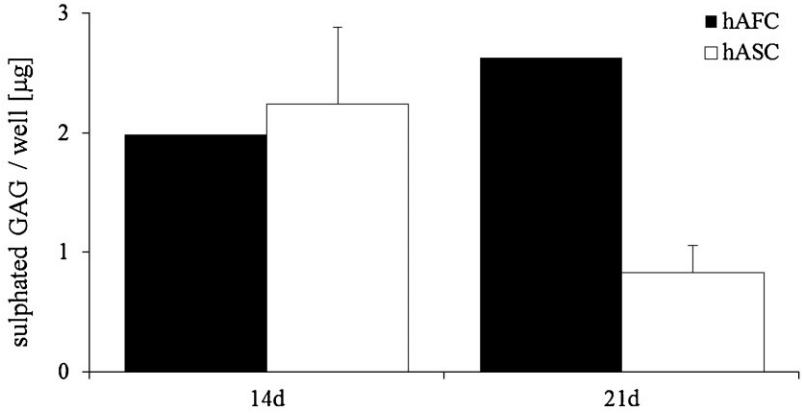

Figure 1.
Amount of sulphated GAGs at 14 and 21 days of culture. The results are expressed as mean ± SD.

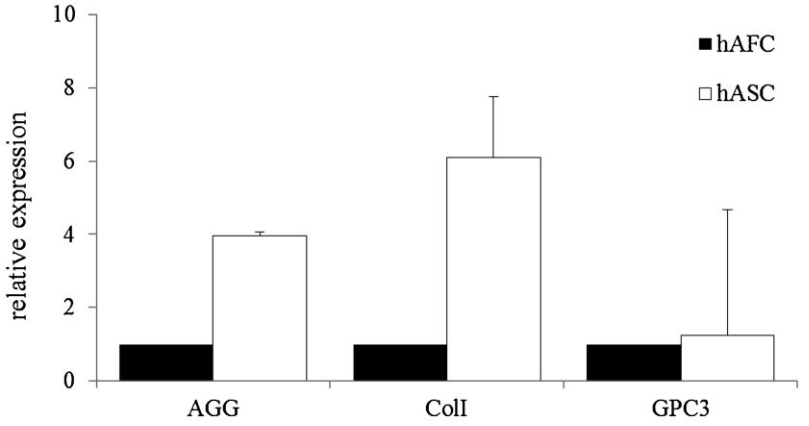

Figure 2.
Expression of AGG, ColI and GPC3 relative to hAFCs at 21 days of culture. In case of hASC, the results are expressed as means ± SD from 3 donors.

Human ASC micromasses condensed within a few days after plating. Cell condensation is directed by cell-cell and cell-matrix interactions as well as secreted factors. Cell-cell interactions are probably involved in triggering signal transduction pathways that initiate chondrogenic differentiation.[16] Contrary to the hASC micromasses, hAFC micromasses did not condense. Previous studies, have used micromass culture for the culture of hAFCs in serum containing medium.[17,18] These studies showed that hAFCs grow in micromass culture, but do not condense to the same extend as chondrocyte or hMSC micromasses.

Sulphated GAG content of the ECM was similar in hAFCs and differentiated hASCs at 14 days of culture. However, sulphated GAG content showed a decreasing trend after 21 days of culture compared to 14 days in case of the differentiated hASCs. The sustained expression of proteoglycan proteins and GAGs is essential for the successful regeneration of AF tissue. Proteoglycans attract and hold water, distributing the force around the circumference of the AF.[15] It is for this reason that studies attempting to direct ASCs towards an AF phenotype have focussed on the production of an ECM rich in proteoglycan and GAG content.[14,15] These studies showed that incorporation of TGF-β1 or TGF-β3 support the production of an ECM with high sulphated GAG content. However, in these studies, sulphated GAG content was only determined at 14 days of culture. Therefore, it is not known if sulphated GAG content is sustained until 21 days of culture even in presence of TGF-β. Chondrogenic differentiation of MSCs takes approximately 3 weeks and accumulation of sulphated GAGs takes place until the last phase of chondrogenesis.[19]

Gene expression of AGG, ColI and GPC3 was analysed on the basis of mRNA levels by qPCR. AGG was analysed, being the most abundant proteoglycan in AF tissue. ColI was analysed as another abundant ECM component.[20] GPC3 was examined as a specific marker for AF differentiation.[21] AGG, ColI and GPC3 were expressed in both hAFCs and differentiated hASCs. AGG and ColI were expressed at higher levels in differentiated hASCs compared to hAFCs. Condensation of hASC micromasses may have induced the expression of ColI and AGG by promoting cell-cell interactions.[16] The expression of GPC3 was similar in hAFCs and hASCs at 21 days of culture, although high donor variation was observed between the 3 repeats. Previously it has been shown that expression of GPC3 is higher in AF, compared to articular cartilage and NP.[21,22] To the best of our knowledge,

this was the first time that GPC3 was used as a marker for the AF differentiation of MSCs. For the successful regeneration of annulus fibrosus tissue, it is important that differentiated stem cells show sustained expression of AF phenotype markers, therefore we chose to use the 21 d time point in the qRT-PCR method. In following studies the expression of specific markers for annulus fibrosus phenotype will be analysed at several time points during differentiation. Also, additional functional studies will be performed, including the evaluation of collagen production and orientation, to prove the successful differentiation of hASCs towards hAFCs. Furthermore, the qRT-PCR primers will be optimized in order to create products with similar size to improve the sensitivity of the qRT-PCR method.

Having optimized culture conditions for the differentiation of hASCs towards an AF phenotype, the next step in the regeneration of AF tissue is to find an optimal biomaterial construct to repair AF defects. The use of an appropriate biomaterial scaffold will provide immediate closure of the defect and restore the biomechanical properties of the disc. The use of scaffolds manufactured from biodegradable polymers is a promising strategy. For example, MSC differentiation towards NP-like cells has been demonstrated on slowly degrading poly-L-lactic acid (PLLA) scaffolds.[23] Vadalà et al. demonstrated that bovine AFCs cultured on PLLA electrospun scaffolds functionalized with TGF-β1 produced an ECM rich in GAGs and collagen.[24] The porous structure of the biodegradable scaffold should replicate the internal structure of the AF, thereby enabling synthesis of an ECM similar to that of native AF tissue. The newly formed ECM will gradually take over the function of the biodegradable scaffold. For complex tissues such as AF, scaffolds have failed to match the native internal structure. In a study by Mauck et al.,[25] nanofibrous scaffolds were manufactured by electrospinning poly(ε-caprolactone) (PCL). These scaffolds were seeded with bovine BMSCs to generate multi-lamellar tissues that replicate the native internal structure of the AFs. Although the tissue generated in this study replicated the form and function of the AF, there remain several challenges that must be addressed before clinical implementation. Considering its flexibility and elasticity, together with its biocompatibility and biodegradability, poly(trimethylene carbonate) (PTMC) could be a suitable scaffold material for AF tissue engineering.[26] In future studies, hASCs will be seeded on the biodegradable scaffold and differentiated towards an AF phenotype using our novel differentiation conditions.

Conclusion

This study showed that hASCs can be differentiated towards an AF phenotype using serum-free chondrogenic medium without growth factors. This was the first time that AF differentiation of hASCs was assessed up to 21 days of culture.

Our results show that sulphated GAG content of the ECM was similar in hAFCs and differentiated hASCs at 14 days of culture. However, the sulphated GAG content level of hASCs was not maintained after 21 days of culture.

Markers for AF differentiation were expressed in differentiated hASCs at 21 days of culture. Expression levels of AGG and ColI were higher in hASCs compared to hAFCs. The expression level of GPC3 in differentiated hASCs was similar to hAFCs. This was the first study to show that GPC3 can be used as a marker for AF differentiation of MSCs.

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