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<td>2012-08-01</td>
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<td>Publication information</td>
<td>Current Stem Cell Research &amp; Therapy, 7 (5): 319-328</td>
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<tr>
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<td>Bentham Science</td>
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Isolation and Phenotypic Characterisation of Stem Cells from Late Stage Osteoarthritic Mesenchymal Tissues

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Abstract: Introduction: Osteoarthritis (OA) represents an increasing health issue worldwide. Regenerative medicine (RM) has raised the hope for introducing revolutionary therapies in clinical practice. Detection of autologus cell sources can improve accessibility to RM strategies.

Objectives: to assess the presence and biological potential of mesenchymal stem cells in three tissues (subchondral bone, synovial layer, periarticular adipose tissue) in late stages osteoarthritic patients.

Material and Methods: samples were collected from subjects undergoing total knee replacement (TKR). MSCs were isolated and cultured in complete MEM with β FGF. Cell morphology and growth potential was assessed. Flow cytometry was used for detection of several relevant cell surface markers. Quantitative and qualitative assessment of differentiation potential towards three mesenchymal lineages (osteogenesis adipogenesis chondrogenesis) was performed. Time lapse life cell imaging of nondiferentiated cells over 24 hours period was used to determine cell kinetics.

Results: mesenchymal cells derived from all donors and tissue types showed morphology, growth and surface cell markers associated with stemness. All cell types underwent differentiation toward three mesenchymal lineages with significant differences between tissues of origin, not between donors. Cell kinetics, as derived from life imaging records, was variable with tissue of origin, significant higher for adipose derived MSCS.

Conclusion: Human late stage OA mesenchymal tissues, contain progenitors with proliferative and differentiation potential of MSCs. These populations can be used for research and autologus regenerative therapies. Further comparative studies with age matched non OA samples has the potential of contributing to deepening knowledge about disease occurrence and progression.

Keywords: osteoarthritis, mesenchymal stem cells, chondrogenesis, regenerative medicine

INTRODUCTION

Osteoarthritis, the „progressive degenerative distruction of articular cartilage” represents the major cause of motor disability in both the developed and developing world [1]. With population aging, as well as with increased younger population physical activity, the prevalence of osteoarthritis is increasing worldwide [2]. Joint reconstructive surgery, the ultimate treatment for advanced stages of joint destruction, is succesful in restoring mobility and alleviating pain in elderly [3]. Taking advantage of cutting edge research in the field of cell and developmental biology, molecular genetics, nano and comosite biomaterials, regenerative medicine (RM) promotes biological treatments „focused on the repair, replacement and regeneration of cells, tissue or organs” [4]. RM aims not only symptom relief or mechanical substitution but to anatomically and functionally recompose living structures. The main actors of regenerative strategies are living cells, sought to be used in different technical contexts in order to recompose the lost, disabled or degenerated tissues or organs. Autologus chondrocyte implantation (ACT) and matrix assisted chondrocyte implantation (MACI) [5, 6] are RM therapies addressing focal cartilage defects. Several drawbacks are encountered using adult chondrocytes as cell source: limited number of cells, donor site pathology [7], failure of cultured cells to attain an adequate level of differentiation. Stem cells with higher proliferative potential and phenotypically stable can be used for cartilage regeneration [8]. For reasons of biological potential and accessibility, mesenchymal stem cells (MSC) are valuable sources for regenerative therapies [9]. Bone marrow derived stem cells (BMMSC), the first isolated and described [10] were the first to be tested for musculoskeletal regeneration [11].

Ubiquitous mensenchymal tissues contain MSC with comparable proliferative and differentiation potential with BMMSCs [12] and have similar regenerative potential [13]. Adipose derived MCSs (ADSCs) are easy to obtain with less donor site pathology, are more abundant within the tissue, therefore regarded as important cell source for regenerative strategies [14, 15].

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Stem cells derived from synovial layer [16], trabecular bone [17, 18] have been reported to be suitable for cartilage regeneration. Progenitor cells within osteoarthritic cartilage and other mesenchymal osteoarthritic tissues have been described in terms of their potential for in situ regeneration [19–21]. It has been presumed that OA occurrence and progression could be a process of uncoordinated growth [22, 23]. In this respect, articular and periarticular progenitor cell phenotype characterization is as important for disease pathogenic elucidation as it is for detecting autologous cell sources.

Recently described MSC anti-inflammatory and immunomodulatory properties justified their use in clinical trials for the treatment of graft versus host disease [24, 25]. Autologous MSCs cell therapy could be designed for controlling joint inflammatory processes.

In the quest for easy accessible autologous MSCs to be used in different RM techniques, aim of this study was to isolate and characterize MSCs from articular and periarticular tissues of adult subjects in late stages of knee OA. Isolated cells were assessed in terms of proliferative potential, surface cell markers and three lineage differentiation (osteogenesis, adipogenesis and chondrogenesis).

MATERIAL AND METHODS.

Tissue sample collection, tissues were collected from patients undergoing total knee replacement (TKR, Merlin General Hospital, Galway, Ireland) after institutional ethical committee approval and patient informed consent. Inclusion criteria selected a serial of elderly subjects. Mean patient age criteria were osteoarthritis of the knee, primary TKR procedure, absence of recorded metabolic disease (diabetes melitus). Synovial tissue (Syn) from nonfatty synovium was minced with a scalpel and enzymatically digested (collagenase type I Sigma Aldrich) 1.5mg/ml/g, 6 hours on a shaker (Heidolph Titramax, Heidolph Instruments, Schwabach, Germany) at 300 rpm in incubator (37°C, 5% CO2). Digested tissue was filtered twice (100 µm and 70 µm cell strainers, BD Falcon, Bedford, MA) mononuclear cells counted and plated as primary culture. Adipose tissue (Adipo) was collected from subcutaneous fat, enzymatically digested (1mg/ml/g tissue) 6 hours using the same protocol as described for synovium. Trabecular bone was collected from the subcondral bone. Mechanical cell extraction was performed by centrifugation 5,000 rpm, 8 min, 4°C). Mononuclear cells extracted were plated as P0 (BS cells). Bone chips were further digested using collagenase type I, 3mg/ml, 3 hours, mononuclear cells plated as P0 (BC cells).

Colony Forming Unit (CFU-F) assay, 10,000 cells from each sample and donor were plated in T75 flasks until appairation of visible colonies (> than 100 cells/colony). Colonies were counted in the culture flask, using an optical microscope at x4 magnification and a grid.

Cell culture. Primary culture (P0) was plated at 50,000 cells/cm² in complete αMEM with 4µl/100 ml media β recombinant human Fibroblast Growth Factor, (BFGF, Prepotech, London, UK). Subcultures were plated at 5,000 cells/cm².

Cumulative population doubling. Cell population doubling (PD) calculation was performed using the formula PD= (LOG (N2)-LOG (N1))/LOG (2) N1 being the number of cells at confluence prior to re-plating and N2 number of re-plated cells.

Flow cytometry A Guava flowcytometer (Guava Easycyte) was used for thawed cells in P2, P5 and P4, results interpreted with Guava ExcelUti5.2 software. FITC/PE antibodies for CD105 (Invitrogen) CD73 and CD90 (BD Biosciences), were used as positive surface markers for stemness; CD34 CD45 (BD biosciences), negative surface markers, HLA-Dr (Invitrogen), γ1, γ2a (BD Biosciences), negative controls and γ12b (BD Biosciences) as isotype control.

Differentiation assays: osteogenic and adipogenic differentiation. Assays were performed on P2 and P5 cells, in 24 well plates (2,000 cells/cm²). Chondrogenetic differentiation was performed on P2 and P5 cells using pellet culture method, 250,000 cells/ pellet. (Composition of differentiation media used in Table I supplementary material)

Evaluation of differentiation Reagents, if not otherwise mentioned, are from Sigma Aldrich. For qualitative assessment of osteogenesis, cells were stained with alizarin red; calcium quantified using Stanbio Kit (StanbioTotal Calcium LiquiColor®, Stanbio, and Boerne Texas). For adipogenesis qualitative evaluation, cells were stained with Oil Red O. Qualitative evaluation of chondrogenesis was performed on pellets stained with Safranin O. For quantitative chondrogenesis evaluation, total amount of GAG (glycozaminoglycans) was assessed using chondrotin-6-sulphate DMMB (1, 9 Dimethylmethylene blue) method; results were normalized for total DNA content using Quant-iT Pico Green dsDNA assay (Invitrogen).

Life imaging and cell movement characteristics. P4 thawed cells were plated at 2,000 cells/cm² in 60 cm² Petri dishes, exposed to life imaging microscope (Olympus IX51 Inverted Fluorescent Microscope) for 24 hours. Cell velocity, acceleration and distance was calculated using Image Pro software (Image Pro 6.0, Media Cybernetics Inc.)

Statistical analysis. Unless otherwise stated, data obtained are given as means ± SD. Between-group and within-group differences were considered significant at p < 0.05 (n = 4). Multiple one and two-way analysis of variance (ANOVA, OriginLab PRO version 8.0) with group factor and repeated measures followed by Bonferroni post hoc analysis.

RESULTS

Patient demographics and cell yield. The inclusion criteria selected a serial of elderly subjects. Mean patient age was 69±8 years; (n=4), range 59 – 78; female - male ratio; 3:1. Wet weight of all processed tissues was mean 3.28 gr for all tissues collected. Maximum amount of tissue was collected from trabecular bone (5.4gr) and minimum (0.7 gr) from subcutaneous adipose tissue with no statistical significant differences between donors or tissue type (Table 1).
Total cell yield. 1x10^6 of four different cell types from each donor were re-plated at the end of each passage, remainder cells were cryopreserved. Thawed cell were partially used for flow cytometry, differentiation assays and life imaging. Highest cell yield per tissue weight (mean 19.29 x10^6 cells) resulted from expansion of adipose derived cells and lowest from bone (BS, 5.2x10^6 cells). No significant difference between the cell yield/tissue unit from different patients or tissue types was found. Remarkable, the number of Adipo cells/gr tissue from one donor was 2.38 times higher than mean Adipo cells from all donors (Table 2A).

Cell morphology and CFU Cells derived from the three tissue types were morphologically distinct in culture (Fig. 1). BS and BC cells have spindle shaped cellular body, form distinct colonies, slower to reach confluence. Syn cells have rectangular cellular bodies, form rapidly confluent colonies; Adipo cells are polygonal, with broad cellular bodies,

Table 1. A: weight (gr) of collected samples/patient: BS trabecular bone mechanical separation; BC trabecular bone collagenase digestion (BC); Syn synovial tissue; Adipo subcutaneous fat pad periarticular; bold = maximum, bold italic = minimum values/tissue type/patient. B: CFU- colony forming units. C: Cell yield / gram harvested tissue P1 to P6. D: fold variation versus mean /tissue type/patient, highlighted highest fold values.
forming confluent colonies. Cultured cells from same tissue type showed remarkable morphological similarity regardless the donor. Number of colonies formed per tissue type and donor was not significantly different. Time in culture to apparition of visible colonies was significantly higher for BS and BC cells compared to Adipo and Syn. (Table 2B).

**Growth kinetics.** BS and BC were slower to grow in primary culture (10-14 days) compared to Adipo and Syn cells (5-6 days). After passage 2, time in culture was similar for all tissues and donors. Population doublings PD and CPD was significant higher for passage 3 and 4 compared to 1 and 2 for all donors and tissues. Remarkable, the only significant donor dependent PD0 and CPD variability was recorded for Adipo cells (p<0.05) (Table 3, Table 2 Supplementary material).

**Flow cytometry analysis.** All cells were highly positive for Cluster of Differentiation (CD) 73 (L-VAP-2, lymphocyte vascular adhesion protein-2) and CD 90 (THY-1
cell surface antigen) negative for CD45 (PORC-PI-1, Common Leukocyte Antigen) and CD34 (Cell Adhesion Factor Associated with Hematopoietic Progenitors). All cells were positive for CD105 (ENG, Endoglin) excepting Adipo cells from two donors. CD146 (MCAM, Melanoma Cell Adhesion Molecule) associated with endothelial progenitor cells was expressed in low percents in early passages BS and BC but not expressed in Adipo or Syn cells. Early passage (P2) BS cells showed presence of CD34 (Table 3 Suppl.

Differentiation potential. All cell types from all donors underwent three lineage differentiation. Osteogenesis, quantified as amount of calcium deposited during 14 days of osteogenic media exposure, was higher for P2 BS and BC (mean 96.25 μg/well BS) and lowest for P2 Syn (mean 5.95 μg/well). However, for assays conducted using later passage (P5), the amount of deposited calcium decreased for all cell types. No statistically significant differences could be found between amount of calcium produced by cells from different tissues or donors in the same passage. Significant higher amount of calcium deposition was detected for cells in P2 compared to P5 (Table 4).

Adipogenetic differentiation BS and BC accumulated smaller droplets disposed in clusters, Syn and Adipo cells formed larger intracellular deposits within larger cells (Suppl.material Fig. 1), quantitative spectrophotometer assessment of Oil Red O stained lipids showed a significant higher amount of lipids accumulated within Adipo comparing to Syn cells (Table 4 Suppl. material). Chondrogenetic differentiation was quantified normalizing the amount of GAGs accumulation to pellet DNA content. Syn GAGs/DNA ratio was significant higher compared to BS, BC and Adipo cells. No significant differences were found between donors (Table 5). Histological examination of Safranin O stained pellets revealed a more organized fibber network at the peripheral part of Syn samples. Pellets

Fig. (1). Cell morphology: a )BS, bone derived cells mechanical separation ;b) BC bone derived cells enzymatic digestion; c)Adipo, subcutaneous adipose derived cells: d) Syn, synovial layer derived cells (a, b, c, d, donor S001, Passage 2, day 5 in culture; e, f donor S002 P2 day5 in culture adipose respective synovial derived cells, Olympus 1X71inverted microscope,10x, scale bar 200μm).
produced by BS and BC cells had a more random collagen fiber orientation (Fig. 2). Differentiated cells displayed histological aspect of adult chondrocyte (Fig. 3).

**Cell movement characteristics** (distance, velocity, acceleration) were significantly higher for Adipo compared to Syn but not significantly different compared to BS cells (Table 5, 6 Supplementary material).

**DISCUSSION**

In this study, mesenchymal articular and periarticular tissues from elder donors were demonstrated to contain MSCs. Isolated cells were capable of proliferation and displayed stemness surface markers [26]. Exposed to specific media, isolated cells underwent three mesenchymal lineage differentiation. This is, to our knowledge, the first report of...
successful isolation of MSCs from three different articular and periarticular mesenchymal tissues collected from same donor. No significant variation between donors was found for this category regarding number of cells extracted per tissue unit. A consistent number of cells could be isolated and expanded from relatively limited amount of available tissue. Cells displayed a good viability after medium term cryopreservation (96-98% viable cells Trypan blue exclusion for thawed cells after 4-6 months liquid nitrogen storage, data not shown). Isolated cells showed remarkable similarities in terms of days in culture and population doublings between different donors suggesting similar proliferative capabilities of MSCs derived from same tissue of origin regardless the donor. Significant increase of CPD for the same donor in successive passages for all tissue types demonstrates a sustained in vitro growth potential. MSCs derived from adipose tissue were the only ones to display significant donor related growth parameters and cell yield. Number of Adipo MSCs as well as PD0 from one donor was significantly higher. Syn, BS and BC MSCs yield and PD0 was homogenous among donors. It is possible the distinct growth potential of adipose derived stem cells to be related to the presence of adipokines within the OA joint and periarticular tissue. Lipid metabolism and the presence of leptin within osteoarthritic joints have been positively correlated with the progress of the disease [27, 28]. Currently there are no available data regarding proliferative potential of cells derived from periarticular adipose tissue in different stages of osteoarthritis. Abnormal fat accumulation is recognized as being responsive of painful periarticular deformities (“lipoarthrosis”), empirically associated with cartilage degradation. Whenever this tissue contains cells

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with a particular phenotype which could be correlated with the occurrence or progression of disease, it is not yet known. Adipose tissue is recognized as a valuable cell source for regenerating different musculoskeletal [29, 30] or non mesenchymal tissues [31]. One of the main features of adipose derived MSCs is their proliferative capability and high cell yield. Whenever this characteristic is related to the metabolic status of the donor it is not yet clear. Comparison between proliferative capabilities of periarticular derived adipose stem cells from different donor groups based on their BMI could hold important answers in elucidating the link between obesity, leptin presence within OA joints and OA progression.

In this study BS and BC were slower to grow in primary culture and slower to form colonies. However, after the first passage the time in culture was similar for all cell types, suggesting that the initial cells isolated from bone were more deeply quiescent than their Adipo and Syn counterparts.

Cells were morphologically different depending on the tissue of origin but similar between same tissue types from all the donors. Cell surface markers associated with stemness (CD 105, CD 73, and CD 90) were present in all cell types and donors, except Adipo cells which showed heterogeneity in expression of CD 105, similar with reported data for ADSCs of young donor [32]. BS and BC cells showed expression of CD 146 in early passages (P2, P3, data not showed), but not in later passages (P5). CD 146 negative separation could be a method of obtaining homogenous population of bone derived cells starting from P0.

Expression of CD 34 in earlier passages (P2) by BS cells indicates an initial mixture of hematopoietic progenitor. All cell types from all donors displayed osteogenetic and chondrogenetic potential significantly higher in earlier passages (P2) than in later passages (P5). Syn cells retained higher potential in both passages for chondrogenesis not for osteogenesis. Late passages Syn cells could be used for cartilage regeneration. Freshly isolated MSCs or early passages can be used for bone augmentation procedures based on higher osteogenic potential. Syn cells showed the highest chondrogenic potential, compared to BS, BC and Adipo cells, with no significant differences between donors. Lower quantitative chondrogenesis (GAGs/DNA/pellet) obtained in this study compared to previous reports [33] could be age related. Syn MSCs cell harvest could be modulated using different cell seeding density [34]. It is possible differentiation capability could be modulated using similar strategies, to be further investigated.

Following a distinct characteristics movement of MSCs of different cells sources, cell movement in extended time lapse life cell imaging was tracked. Cell velocity, acceleration and distance were significant higher for Adipo than for Syn derived cells. There is an interesting parallel between cell kinetics and growth kinetics (CPD) of cells in same passage and similar tissue type. We found there is a direct correlation between recordable life tracking of non stained cells and their proliferative potential (Table 4, 5 Supplementary material). The correlation between cell motility and cell cycle progression [35] is to be further investigated. Being the fact that many components of the

Fig. (2). Chondrogenesis assay, pellet culture Safranin O staining digitally stitched (Tissue FAXS software, TissueGnostics GMBH, Austria, Vienna) image 20X (Zeiss Observerl microscope A) BC trabecular bone derived MSCs, enzymatic digestion; B) trabecular bone derived MSCs, mechanical separation C) Adipo, adipose derived MSCs, D) Syn, synovial tissue derived MSCs; scale bar 200μm.
molecular machineries responsible for cell kinetics, cell proliferation and cell differentiation are shared, the potential of life imaging in prediction of cell individualities and fates under the integrative basis of their kinetic parameter analysis is yet to be explored.

We report the successful isolation of stem cells from three different mesenchymal adult human osteoarthritic tissues. Isolated cells have morphology, surface markers, proliferative and differentiation potential characteristic of MSCs. Mesenchymal tissues derived from late stage osteoarthritic donors were found to contain MSCs with distinct phenotypes. Trabecular bone, synovial and extraarticular adipose tissue resulting from total joint reconstructive procedures can be considered as cell sources for autologous regenerative strategies.

The principal limitation of this study is the number of biological replicates. Studies including larger number of donors are needed to validate present findings. Further comparison with age matched tissues obtained from non OA joints could derive important insights about disease pathogeny. Further investigation is needed as well to address the cause of donor related variability observed in adipose derived mesenchymal stem cells from OA subjects. The history of progression of OA in the operated joint, the presence of OA in other joints or the lifetime of the implanted prosthesis could be in correlation with bone and lipid metabolism.

Differentiation assays conducted in this study had the purpose in demonstrating the differentiation capability towards three mesenchymal lineages. If considered for regenerative cartilage therapies, there is a need to optimize differentiation protocols for obtaining hyaline cartilage. MSC capability of undergoing differentiation when seeded on scaffolds is another important question to be answered.

Characterization of OA MSCs has the potential of generating new targets for disease prevention and treatment. MSCs derived from OA mesenchymal tissues during joint reconstructive procedures can be used as accessible cell source for research and future RM therapies.

CONFLICT OF INTERESTS

There are no conflict of interest from any of the authors of this manuscript.

ACKNOWLEDGEMENTS

The study was supported by 2010 OARSI scholarship and REMEDI Regenerative Medicine NUI Galway Ireland institutional support. The authors are grateful to Marry Murphy PhD for the thorough counseling and logistical support, to Mihaela Zlei PhD for flow cytometry assistance, to David Connolly for life microscope imaging set up and data recording and to Costel Silincu for histological preparation of samples.

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