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The epitope characterisation and the osteogenic differentiation potential of human fat pad-derived stem cells is maintained with ageing in later life

W.S. Khan [a,*], A.B. Adesida [a], S.R. Tew [a], J.G. Andrew [b], T.E. Hardingham [a]

[a] United Kingdom Centre for Tissue Engineering and Wellcome Trust Centre for Cell Matrix Research, University of Manchester, Oxford Road, Manchester M13 9PT, UK
[b] Department of Trauma and Orthopaedics, Bangor General Hospital, Wales LL5 2PW, UK

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ABSTRACT

Some clinical settings are deficient in osteogenic progenitors, e.g. atrophic nonunited fractures, large bone defects, and regions of scarring and osteonecrosis. These benefit from the additional use of bone marrow-derived mesenchymal stem cells, but these cells exhibit an age-related decline in lifespan, proliferation and osteogenic potential. Therapeutic approaches for the repair of bone could be optimised by the identification of a stem cell source that does not show age-related changes. Fat pad-derived stem cells are capable of osteogenesis, but a detailed study of the effect of ageing on their epitope profile and osteogenic potential has so far not been performed.

Fat pad-derived cells were isolated from 2 groups of 5 patients with a mean age of 57 years (S.D. 3 years) and 86 years (S.D. 3 years). The proliferation, epitope profile and osteogenic differentiation potential of cells from the 2 groups were compared. Cells isolated from the fat pad of both groups showed similar proliferation rates and exhibited a cell surface epitope profile similar but not identical to that of bone marrow-derived stem cells. The cells from both groups cultured in osteogenic medium exhibited osteogenesis as shown by a significant upregulation of alkaline phosphatase and osteocalcin genes, and significantly greater alkaline phosphatase enzyme activity compared to cells cultured in the control medium. The cells cultured in the osteogenic medium also showed greater calcium phosphate deposition on alizarin red staining. There was no significant difference between the osteogenic potential of the two age groups for any of the parameters studied.

The fat pad is a consistent and homogenous source of stem cells that exhibits osteogenic differentiation potential with no evidence of any decline with ageing in later life. This has many potential therapeutic tissue engineering applications for the repair of bone defects in an increasingly ageing population.

Introduction

Autologous cancellous bone grafting is currently the gold standard for the treatment of bone defects. The efficacy of bone grafts can be attributed to one or more of three core properties: osteoconduction, osteoinduction and osteogenic progenitor cells.[4,10] A sufficient number of osteogenic progenitors in the graft or at the graft site is important. Some clinical settings are deficient in osteogenic progenitors, e.g. atrophic nonunited fractures, large bone defects, and regions of scarring and osteonecrosis. These benefit from the additional use of bone marrow-derived mesenchymal stem cells, commonly aspirated from the iliac crest. These mesenchymal stem cells can be percutaneously injected at the lesion site or used in combination with demineralised bone as an adjuvant to bone grafting.[19,33]

Bone marrow-derived mesenchymal stem cells are multipotent with excellent osteogenic potential. The use of mesenchymal stem cells from the bone marrow does however have limitations. These cells are scarce and only 1 out of 18,000 nucleated bone marrow cells is a mesenchymal stem cell.[24] Harvesting of bone marrow is painful with donor site morbidity and risk of wound infection and sepsis.[27] Increasing the amount of bone marrow aspirated at each single aspiration does not coincide with a significant increase in stem cell number.[23] Most importantly, there is considerable variation in the concentration and prevalence of mesenchymal stem cells in the bone marrow. With increasing age there is a decline in bone marrow cellularity including bone marrow-derived stem cells.[24] Human bone marrow-derived stem cells from older donors have been shown to be fewer in number,[25] and have a reduced lifespan, proliferation[31] and osteogenic

* Corresponding author. Tel.: +44 797 119 0720; fax: +44 161 275 5752.
E-mail address: wasimkhan@doctors.org.uk (W.S. Khan).

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potential.[20,21] An ideal source of stem cells would be easy to obtain with a small risk of complications, with a sufficient cell yield and exhibit rapid proliferation and a strong differentiation potential.

Mesenchymal stem cells are present in almost every adult human tissue including adipose tissue[37] and the infrapatellar fat pad.[13,14,35] Culture conditions are important for promoting differentiation in these cells along specific pathways. Monolayer culture of mesenchymal stem cells with standard osteogenic medium containing β-glycerophosphate, dexamethasone and ascorbate has been shown to induce osteogenic differentiation.[5] A potential stem cell source that does not show age-related decline in proliferation and osteogenic differentiation is important in determining the optimal cell therapy for bone defects in an ageing population. Mesenchymal stem cell characterisation has previously been attempted using epitope profiling of Cluster of Differentiation (CD) markers and cell surface proteins.[8,27] A full characterisation of infrapatellar fat pad-derived cells is important to achieve a greater understanding of their origin and their repair potential. It is not known if the cell surface expression of these cells alters with age.

In this study infrapatellar fat pad-derived cells from two different older age groups were compared for their proliferation, epitope profile and osteogenic differentiation potential. The hypothesis was that the proliferation, epitope profiling and osteogenic differentiation potential between the two groups would not be significantly different.

Materials and methods

Cell isolation and culture

The infrapatellar fat pad was obtained with ethical approval and fully informed consent from two groups of five patients undergoing total knee replacement for osteoarthritis. Group 1 had a mean age of 57 years (S.D. 3 years) and group 2 had a mean age of 86 years (S.D. 4 years). Five grams of the tissue was dissected and cells were isolated with 0.2% (v/v) collagenase I (Invitrogen, Paisley, Renfrewshire, UK) for 3 h at 37 °C with constant agitation. The released cells were sieved (70 μm mesh) and washed in basic medium, Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20% (v/v) fetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin (all from Cambrex, Wokingham, UK), with L-glutamine (2 mM). The stromal cells were separated from the adipocytes (floating) by centrifugation at 300 × *g* for 5 min and were counted and plated at 100,000 cells/cm^2 in a T75 polystyrene tissue culture flask with basic medium. Although almost 90% of the plated cells were lost during media changes, passage 1 cells became confluent in almost 7 days yielding almost 2.5 million cells. Cultures were maintained at 37 °C with 5% CO_2 and normal oxygen (20%).

Cell proliferation rates

Passage 2 cells were plated at 10,000 cells/cm^2 in a T225 polystyrene tissue culture flask. Cells were trypsinised and collected at days 2, 4, 6, 8 and 10 after plating and the cell number was determined by counting with a cytometer. The viability of the cells was determined by staining with Trypan Blue.

Cell surface epitope characterisation and flow cytometry

In order to characterise the fat pad cells in these experiments, confluent passage 2 cells were stained with a panel of antibodies for cell surface epitopes. This included monoclonal antibodies against the following: CD13 (aminopeptidase N), CD44 (hyaluronan receptor), CD90 (Thy-1), LNGFR (low-affinity nerve growth factor receptor), STRO1 (marker for bone marrow-derived stem cell) and CD56 (neural cell adhesion molecule; NCAM) from BD Biosciences (Oxford, UK); CD29 (β1 integrin), CD105 (SH2 or endoglin) and CD34 (marker for haematopoietic cells) from Dako (Ely, UK); and 3G5 (marker for vascular pericytes) courtesy of Dr. A. Canfield (University of Manchester, UK). The cells were incubated for 1 h with the primary mouse antibodies (undiluted 3G5 and 1:100 dilution for others), washed, and then incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgM secondary antibody (1:40 dilution; Dako). For controls, nonspecific monoclonal mouse IgG antibody (Santa Cruz Biotechnology, Santa Cruz, USA) was substituted for the primary antibody. The cells were then rewashed before being incubated with 4′,6-diamidino-2-phenylindole stain (1:100 dilution) for 5 min, and images were captured with an Axioplan 2 microscope using an Axiocam HRc camera and AxioVision 4.3 software (all from Carl Zeiss Ltd, Welwyn Garden City, UK).

Cells from passage 2 were also analysed using flow cytometry. Cells in monolayer were detached with trypsin (0.05% with 5 mM EDTA), washed and incubated with primary mouse antibodies (undiluted 3G5 and 1:100 dilution for others) followed by FITC-conjugated anti-mouse IgM secondary antibody (1:40 dilution). The cells were rewashed, suspended at 1 million cells/ml and assayed in a flow cytometer (Dako cytomation cyan, Ely, UK).

Osteogenic differentiation

Cultured cells from passage 2 were placed at a concentration of 5000 cells/cm^2 in a six-well plate. The cells were cultured either in osteogenic medium or in control basic medium for 21 days with the medium changed every 2 days. Osteogenic medium consisted of DMEM with 10% FCS, β-glycerophosphate (10 mM), dexamethasone (10 nM) and L-ascorbic acid-2-phosphate (0.1 mM).

Gene expression analysis

Quantitative real-time gene expression analysis was carried out for alkaline phosphatase, β-actin and osteocalcin. Total RNA was extracted with Tri Reagent (Sigma, Poole, UK) from cells from both groups cultured with osteogenic and control media after 21 days. cDNA was generated from 10 to 100 ng of total RNA by using reverse transcription followed by poly(A) PCR global amplification.[1] Globally amplified cDNAs were diluted 1:1000 and 1 μl aliquot of the diluted cDNA was amplified by quantitative real-time PCR in a final reaction volume of 25 μl by using an MJ Research Opticon with a SYBR Green Core Kit (Eugentec, Seraing, Belgium). Gene-specific primers were designed within 300 base pairs of the 3′ region of the relevant gene with the use of ABI Primer Express software (Applied Biosystems, Foster City, CA, USA). Gene expression analyses were performed relative to β-actin and calculated using the $2^{-\Delta\Delta Ct}$ method.[15] All primers (Invitrogen, UK) were based on human sequences:

Alkaline phosphatase: Forward 5′–3′ CCCACAATGTGGACTACCT, reverse 5′–3′ GAAGCCTTTGGGGTTCTTC.
β-Actin: Forward 5′–3′ AAGCCACCCCACTTCTCTCTAA, reverse 5′–3′ AATGCTATCACCTCCCCTGTGT.
Osteocalcin: Forward 5′–3′ CATGAGAGCCCTCACA, reverse 5′–3′ AGAGCGACACCCTAGAC.

Alizarin red staining

Alizarin red staining was used to quantify calcium phosphate mineral formation in culture after 21 days. The cells cultured in six-well plates were rinsed in Dulbecco's Phosphate Balanced Solution (DPBS; Cambrex, UK) and then fixed in 4% formaldehyde for 10 min. This was followed by a wash in ultrapure water and staining with 1 ml of sterile-filtered 1% (v/v) alizarin red, made up