Acta Biomaterialia 53 (2017) 59–69

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Full length article

Identification of the mechanisms by which age alters the mechanosensitivity of mesenchymal stromal cells on substrates of differing stiffness: Implications for osteogenesis and angiogenesis

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ARTICLE INFO

Article history:
Received 29 November 2016
Received in revised form 8 February 2017
Accepted 15 February 2017
Available online 16 February 2017

Keywords:
Mechanosensitivity
Children MSCs
Ageing
Stem cells
Osteogenesis
JNK3
MSCs

ABSTRACT

In order to identify the mechanisms by which skeletal maturity alters the mechanosensitivity of mesenchymal stromal cells (MSCs) and, the implications for osteogenesis and angiogenesis during bone formation, we compared the response of MSCs derived from children and skeletally-mature healthy adults cultured on soft and stiff collagen-coated polyacrylamide substrates. MSCs from children were more mechanosensitive, showing enhanced angiogenesis and osteogenesis on stiff substrates as indicated by increased endothelial tubule formation, PGF production, nuclear-translocation of YAP, ALP activity and mineralisation. To examine these mechanisms in more detail, a customised PCR array identified an age-dependent, stiffness-induced upregulation of NOX1, VEGFR1, VEGFR2, WIF1 and, of particular interest, JNK3 in cells from children compared to adults. When JNK3 activity was inhibited, a reduction in stiffness-induced driven osteogenesis was observed – suggesting that JNK3 might serve as a novel target for recapitulating the enhanced regenerative potential of children in adults suffering from bone degeneration.

Statement of Significance

We investigated the age-associated changes in the capacity of MSCs for bone regeneration involving the mechanosensitive signalling pathways, which reduce the ability of adult cells to respond to biophysical cues in comparison to cells from children, who are still undergoing bone development. Our results offer new insights into the mechanobiology of MSCs and sheds new light on age-altered mechanosensitivity and, on why children have such an immense capacity to regenerate their skeletal system. We have identified the mechanisms by which skeletal maturity alters the mechanosensitivity of mesenchymal stromal cells and an age-dependent, stiffness-induced upregulation of a number of prominent genes including, most notably, JNK3 in children cells, thus suggesting its potential to promote enhanced bone repair.

1. Introduction

Mesenchymal stromal cells (MSCs) are multipotent, non-hematopoietic cells with multi-lineage potential to differentiate into various tissues of mesodermal origin. MSCs can be easily isolated from the bone marrow (BM) and present an extensive capacity to adhere to tissue culture polystyrene and to proliferate *in vitro*. Bone marrow-MSCs are progenitors for several connective tissues, including bone [1], cartilage [2], vasculature [3] and fat [2]. MSCs also possess unique biological characteristics suitable for enhancement of tissue repair, treatment of degenerative diseases or even amelioration of dysfunction associated with normal ageing [4,5]. However, the cellular ageing process has profound

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http://dx.doi.org/10.1016/j.actbio.2017.02.031
1742-7061/

physiological effects that cause a decrease in the unique regenerative capacity of MSCs [6,7], which may contribute to age-related degenerative conditions, including osteoarthritis and osteoporosis [8]. Previously reported age-related changes in MSCs include loss of differentiation [9] and reduced proliferation potential [10], increase in senescent cell numbers and loss of *in vivo* bone formation [11]. More recently, age-associated bone loss has been linked to decreased osteogenic ability of MSCs [12], which is in line with previous studies that found reduced osteogenesis in MSCs isolated from aged individuals [9,11,13,14] and consequent delayed fracture healing [15].

Children have a renowned potential to repair fractured bones quickly. While previous studies suggest that MSCs derived from younger/child donors have a greater ability to regenerate bone tissue compared to MSCs derived from older/adult donors [11,12], the underlying cellular signalling mechanism influencing the reduced bone-healing ability of aged MSCs remains unclear. Within the native cellular microenvironment, MSCs are presented with a myriad of cues that are responsible for directing their behaviour. As MSCs can both generate forces and sense physical properties of the matrix through adhesion, the question arises as to whether the decline in MSC functionality with age is a result of the intrinsic ageing of cells or the deterioration of signalling factors provided by the ageing tissue environment [4]. This has been investigated by culturing MSCs from marrow of old and young mice on extracellular matrix of young and old donors, with findings showing remarkable defects in self-renewal and bone formation capacity of old MSCs, that were corrected by provision of a young extracellular matrix [16]. The use of extracellular matrix from foetal donors has also been shown to rejuvenate adult MSCs, by optimising their proliferation ability and maintaining their differentiation potential [17]. These findings suggest that there is an important role for cell-matrix interactions in overcoming growth-inhibitory mechanisms that occur with age [18].

Although there may be a partial biochemical basis for the cell-matrix interactions that govern differentiation of MSCs, biophysical cues could also have a profound effect on directing the behaviour and rejuvenation of these cells. For example, previous studies have demonstrated that multipotent progenitor cells isolated from older donors lose their sensitivity to changes in the stiffness of polyacrylamide substrates, while cells from younger donors exhibit a lineage dependent response to stiffness [19]. The molecular mechanisms associated with this age-dependent mechanosensitivity were found to hinge upon activation of Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif, known as Tafazzin (TAZ). YAP/TAZ has a well-established role in mechanotransduction of MSCs, in which the YAP/TAZ changes its location depending on the stiffness of the substrate and consequently mediates MSC differentiation. Specifically, if MSCs are cultured on soft substrates, YAP/TAZ is activated in the cytoplasm and promotes adipogenesis, whereas when cultured on stiff substrates YAP/TAZ is located in the nucleus and mediates osteogenic differentiation [20]. Given the previously identified change in YAP/TAZ translocation, this may provide a molecular mechanism for the age-associated decline in the stiffness-induced osteogenic differentiation of MSCs. Additionally, it may be the basis to provide mechanistic understanding at the molecular level of how matrix stiffness directs cells differentiation and influences long-term gene expression and cell fate [21].

Despite the known effects of biophysical cues on MSC osteogenesis [22–26], and the observed decrease in the ability of cells to respond to biophysical cues with age [19], it is not known how age impacts upon the stiffness-induced osteogenesis of MSCs and whether such an alteration in the sensitivity of MSCs to physical stimuli may provide a mechanism for understanding the reduced capacity for bone regeneration with skeletal maturity, which is reduced further in the ageing population. We hypothesise that the age-associated changes in the capacity of MSCs for bone regeneration involve mechanosensitive signalling pathways, which reduce the ability of adult cells to respond to biophysical cues in comparison to cells from children, who are still undergoing bone development. The current work aimed to further elucidate the effect of age on cell mechanosensitivity by comparing the response of human MSCs derived from 11 to 12 year old child donors (C-MSCs) with cells harvested from skeletally mature healthy 20–30 year old adult donors (A-MSCs) to variations in stiffness (10 and 300 kPa) of type I collagen-coated polyacrylamide (PAA) substrates. In addition to evaluating the activation of osteogenic and angiogenic markers related to bone regeneration, we investigated the age-associated alterations in mechanotransductive pathways of MSCs using a customised PCR array. The understanding of the mechanisms behind the age-altered mechanosensitivity of MSCs may open new avenues to identify possible therapeutic targets for recapitulating the enhanced osteogenic and angiogenic potential of children in adults suffering from bone degeneration and disease.

2. Methods

2.1. Cell isolation and characterisation

In order to study the effect of age in the MSCs response to stiffness we isolated bone marrow derived mesenchymal stromal cells (BM-MSCs) from the iliac crest of adults 20–30 years old (Lonza Biologics PLC) and from children 11–12 years old. The cells from young donors were collected from the bone marrow of children undergoing alveolar bone graft surgery, which is a procedure that involves the removal of bone marrow from the iliac crest to repair the cleft palate defect and, residual bone marrow was used to extract the cells from children used in this work. The extraction of bone marrow derived MSCs from the iliac crest of young donors was performed after parents' consent and with ethical approval from the Children's Hospital Temple Street, Dublin. In order avoid the effect of ageing by passaging in the potentiality of stem cells; the same passage of children-derived MSCs (C-MSCs) and adult-derived MSCs (A-MSCs) was used in each individual experiment. Assays were performed with cells at passage 4–5. Detailed protocols for cell isolation and characterisation are described in Supplemental Experimental Procedures S1 and S2.

2.2. Collagen-coated polyacrylamide gels fabrication and cell seeding

To assess the changes in the mechanosensitivity of cells from child and adult donors, polyacrylamide (PAA) gels were produced by mixing different ratios of 40% acrylamide and 2% bis-acrylamide monomer concentrations (Sigma-Aldrich, Ireland) in $_{dd}H_2O$, and inducing free radical polymerisation using ammonium persulfate (APS) and tetramethylethylenediamine (Sigma-Aldrich, Ireland), following a previously described methodology [27]. PAA substrates of 10 kPa and 300 kPa were produced and collagen type I solution from rat tail at a concentration of 50 μg/mL (Sigma-Aldrich, Ireland) was covalently bound to the surface of the gels (fabrication and characterisation procedures described in Supplemental Experimental Procedures S3). The 10 and 300 kPa substrates were seeded with C-MSCs and A-MSCs at different densities depending on the assay. All the cells were seeded using growth medium (GM) for 24 h to allow adhesion before replacing with fresh GM or osteogenic medium (OM).