Identification, sorting and in vitro study of human mesenchymal stem cells for use in tissue regeneration

Summary of the PhD thesis

Áron Szepesi

Eötvös Loránd University, Faculty of Science
PhD School of Biology, Immunology Program

PhD School leader: Dr. Anna Erdei
Program leader: Dr. Anna Erdei
Supervisor: Dr. Katalin Német

Hungarian Academy of Sciences, Research Centre for Natural Sciences,
Institute of Enzymology

Budapest
2016
Introduction

The human body harbours a wide variety of stem cells. They all share common features including the ability to self-renew and produce differentiated offspring. In particular, tissue stem cells referred to as mesenchymal stem cells (MSC) hold great promise for regenerative medicine due to their potential to differentiate, among others, into bone, cartilage, and adipose cells both in vitro and in vivo [1]. In addition to their differentiation capacity, MSCs by definition must meet further criteria such as the presence of MSC-specific cell surface proteins and the absence of hematopoietic markers [2]. Multipotent stem cells have been successfully isolated from virtually all tissues of the human body [3]; however, even when applying the same selection criteria, cells derived from different parts of the body exhibit vast heterogeneity. Thus, a thorough characterization of cells to be used in future therapies is imperative.

The limited availability of autologous bone grafts and the side effects associated with their retrieval create an unmet clinical need for in vitro engineered bone tissue. Bone produced in the dish could provide an unlimited supply of grafts to fill critical size defects [4] or repair periodontal lesions caused by aggressive periodontitis [5]. Because human osteoblasts with an immediate bone-forming capacity are difficult to access, more attention has been paid to mesenchymal stem cells capable of osteogenic differentiation.

Large grafts also require an adequate supply of nutrients to avoid necrosis of their interior. Vascularised grafts can be produced by the addition of cells with blood vessel-forming ability [5]. Ideally, MSCs derived from the same tissue of origin could play a dual role as both osteogenic and vasculogenic precursors.

Stem cells isolated from periodontal ligaments (PDL) are promising candidates for the repair of periodontal defects. PDLs consist of soft connective tissue and help immobilise teeth by connecting the outer cementum layer of their roots with the alveolar bone surface [7]. While PDL cells have been demonstrated to form both bone and cementum with reasonable success, sorting mixed PDL populations for cells with enhanced bone- and cementum-forming potential can further increase the efficacy of regeneration.

A widely accepted approach to selecting cells with superior stem-like qualities is sorting for the Hoechst 33342-low so-called “side population” (SP). Low accumulation of Hoechst 33342 in these cells is due to active extrusion of the DNA dye by the transmembrane transport pump ABCG2 [9]. In recent years, SP cells have been identified in many tissues as stem or progenitor cells. Although SP cells have been found in periodontal ligaments, too [10], no
function has yet been assigned to them. Also, selection of SP cells using a potentially mutagenic DNA dye would preclude their use in the clinic. Antibody-mediated immunosorting of ABCG2-positive cells could circumvent this problem and thus offer a therapy-compatible alternative.

The repair of large bone defects may require the surgical application of an adequately shaped implant. The incorporation of such an implant, whether made of synthetic or inorganic matrix, can be facilitated by seeding stromal cells on the implant surface [11]. Fast and firm adhesion of therapeutic stromal cells to the implant surface is a key limiting step in this process that can be promoted by physical or chemical pre-treatment of the surface. A commonly applied chemical treatment is biomimetic coating [12].

**Aims**

1) We aimed to compare mesenchymal stem cells with potential use in regenerative medicine derived from a) adipose tissue, b) Wharton’s jelly, c) periodontal ligament, d) bone marrow. Cells were isolated from human tissues and maintained in vitro. We intended to demonstrate the heterogeneity of the samples using multiple methods, and investigate their osteogenic and endothelial cell-forming capacity in vitro in detail. We were also planning to study adipose and chondrogenic differentiation, as well as the expression of pluripotency markers.

2) We aimed to find MSC surface markers suitable for the identification and sorting of therapeutically relevant stem-like subpopulations from heterogeneous samples.

3) Our collaborators have synthesised a cyclic RGD-motif-containing branched polymer and showed its utility in promoting the adhesion and survival of various cell types on plastic and glass cell culture surfaces. We set out to carry out MSC adhesion and differentiation experiments in 3D cultures using clinical implant materials such as titanium or Bio-Oss® bone mineral granules that were pre-coated with an improved version of the RGD polymer.
Methods

- Following isolation of MSCs from various tissues, heterogeneity of samples was investigated with respect to a) size and morphology by light microscopy; b) self-renewing capacity using a colony-forming assay; c) proliferative potential using the resazurin vital dye assay; d) expression of cell surface markers by flow cytometry.

- Endothelial differentiation was assessed in an in vitro angiogenesis system and evaluated based on selected phenotypic features of the capillaries formed.

- Expression levels of genes responsible for maintaining pluripotency were determined by real time quantitative PCR, immunocytochemistry, and flow cytometry.

- Cell samples isolated from periodontal ligaments were FACS sorted based on the expression of ABCG2 following immunolabeling with an ABCG2-specific monoclonal antibody.

- The in vitro osteogenic, adipogenic and chondrogenic potential of isolated cells upon appropriate induction was studied using histological stains and real time quantitative PCR determination of differentiation-specific gene transcripts.

- Adhesion of isolated cells to RGD-polymer-treated surfaces was assessed based on resazurin reduction of adhered cells or, when applicable, GFP signal intensity. In either case, signals were quantified using a multiwell plate reader.

Results

- Cells isolated from different sources exhibited remarkable heterogeneity of shape and size. The small, spindle-shaped MSCs derived from the bone marrow were comparatively uniform, whereas Wharton’s jelly-derived samples contained a wide variety of cell shapes and sizes ranging from small, elongated, fibroblast-like cell forms to extremely large and flat cells. Samples isolated from periodontal ligament and fat were dominated by rhomboidal and triangular cells.
In our samples we found a close correlation between proliferative capacity and colony-forming ability. Samples that proliferated faster also contained a higher proportion of cells capable of self-renewal. PDL and bone marrow samples had both shorter population doubling times and more colony-forming cells when compared to Wharton’s jelly and adipose samples.

Regardless to the tissue of origin, 100% of the isolated cells expressed the minimal defining set of MSC markers and showed negligible or no expression of haematopoietic markers. The surface expression of certain adhesion molecules such as CD106 or CD146 was variable across cells within the same sample, while the cell surface density of other antigens such as CD90, CD73 or CD29 varied according to the tissue source. Samples coming from different donors but derived from the same tissue clustered together in respect with surface marker expression.

Stem cells isolated from fat, PDL and bone marrow responded to osteogenic induction with intensive calcium accumulation, elevated alkaline phosphatase activity, and increased expression of the osteogenic master transcription factor RUNX2. Lipid production upon adipogenic induction was most pronounced in fat-derived MSCs.

While MSC samples from all tissues showed endothelial tubule formation following predifferentiation, PDL- and adipose-derived MSCs formed the most extensive capillary networks in Matrigel. Endothelial differentiation was also hallmarked by upregulation of PECAM1 mRNA.

MSC samples did not express any of the embryonic stem cell markers OCT4, SOX2, Nanog or hTERT.

Compared to MSCs of other origin, Wharton’s jelly-derived MSCs showed inferior performance in osteogenic, adipogenic and endothelial differentiation assays. In our interpretation, this behaviour might correlate with high expression of the pluripotency markers SSEA-4, GATA6 and α-SMA which was unique to Wharton’s jelly-derived cells.
The multidrug transporter ABCG2, reported by others to be present on certain types of stem cells, was highly expressed by a subpopulation of PDL-derived MSCs. Antibody-labelled and flow cytometry-sorted ABCG2-high PDL cells outperformed the unsorted PDL samples in osteogenic, adipogenic, chondrogenic and endothelial differentiation assays. The same cells responded to osteogenic stimuli with more intense calcium accumulation, as well as greater induction of genes marking osteogenesis (ALP, OSX, OCN) and cementum formation (CEMP1). Similarly, enhanced production of lipids and glucosaminoglycans was observed in the ABCG2-high population upon adipogenic and chondrogenic differentiation, respectively.

We have demonstrated that ABCG2 does not play a functional role in the osteogenic differentiation of PDL-derived stem cells.

Pre-treatment of titanium surfaces or oral surgery quality bone mineral granules with the cyclic RGD motif-containing biomimetic polymer significantly improved the adhesion of cells seeded on these scaffolds, and enhanced bone formation under osteogenic conditions.

Conclusions

In our experiments we studied tissue stem cells most commonly used for producing seeded bone grafts. Samples derived from various tissues of origin were found to differ markedly in respect with cell shape and size, proliferative and colony-forming capacity, as well as the expression of cell surface antigens. In our in vitro differentiation models, adipose tissue- and periodontal ligament-derived cells were equally competent in forming complex bone and endothelial structures. Based on both their anatomic location and the amount of cells retrievable, periodontal cells are primarily recommended for use in the repair of craniofacial defects. Irrespective of origin, none of our samples expressed any of the genes responsible for the maintenance of pluripotency (such as OCT4, SOX2, Nanog and hTERT). However, unlike the adult stem cells we examined, Wharton’s jelly-derived cells expressed SSEA-4, α-SMA and GATA6, indicative of a state closer to that of embryonic stem cells. This might be related to our observation that umbilical cord-derived cells, in comparison with adult tissue stem cells, displayed a far weaker propensity to terminally differentiate into multiple lineages upon in vitro induction.
The multidrug transporter ABCG2 has been proposed as a stem cell marker in multiple contexts. Out of all samples we studied we could only detect ABCG2 in a subpopulation of periodontal ligament cells. Antibody-mediated, flow cytometry-based sorting of this cell population offers simple access to a stem cell population characterised by superior bone- and cementum-forming ability. ABCG2-positive periodontal cells may therefore provide an ideal solution for the restoration of defects in the craniofacial region. The applicability of periodontal cells to the regeneration of large defects might be limited by the amount of cells retrievable from the root surface; this limitation, however, may be surpassed by expanding these cells in vitro. Periodontal cells have been successfully maintained in culture for prolonged periods without undergoing major phenotypic changes.

The cyclic RGD motif-containing polymer we tested significantly improved the adhesion of adipose-derived MSCs to clinically relevant implant materials, and promoted their osteogenic differentiation on these surfaces. These promising in vitro results grant further investigation of this compound in vivo.

References


Publications connected to the PhD thesis


Other publications

