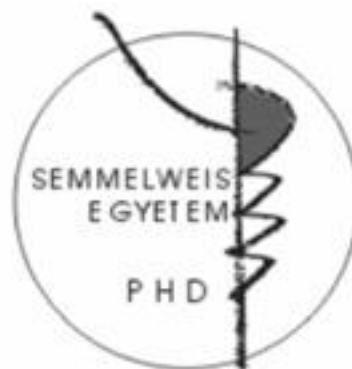


Comparative analysis of murine mesenchymal stem cells isolated from different organs and tissues

Ph.D. Thesis

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Budapest
2013

Introduction

Recent years stem cell biology became one of the most „hot” area in modern medical research. Stem cells have unique properties that make them suitable for tissue regeneration and also replacing dead cells. Initially research focused on embryonic stem cells. However, due to ethical and technical difficulties nowadays adult stem cells were getting into the focus of regenerative medicine researchers. Adult stem cells are the multipotent hematopoietic stem cells (HSCs) and mesenchymal stem cell or stromal cells (MSCs). Our investigations are connected to the latter one.

MSCs are non-hematopoietic stem/progenitor cells originally isolated from the bone marrow are characterized *in vitro* by their fibroblast-like morphology. In order to more accurately identify the cells, in 2006 ISCT (International Society for Cellular Therapy) defined three main criteria describing human MSCs. This means that cells called mesenchymal stem or stromal cells must simultaneously meet the following criteria:

1. adherence to plastic surface;
2. must be positive for CD105, CD73 és CD90 but negative for hematopoietic and endothelial marker molecule CD45, CD34, CD14, CD11b, CD79, CD19 and negative for HLA-DR;
3. have the potential to differentiate into adipocyte, osteoblast, chondrocyte lineages, in response to appropriate stimuli *in vitro*.

Recently it has been recently shown that in addition to the bone marrow, MSCs or MSC-like cells with similar features reside in virtually all murine and human organs. Researchers trying to find an explanation for the fact why MSCs can be found almost in all tissues.

MSCs has large number of favorable properties that make them particularly suitable for curing many diseases. Their role in tissue regeneration is obvious since they are able to replace dead cells or they can promote the restoration of damaged tissues through soluble molecules (trophic and immunomodulatory effect). These molecules can prevent overactivity of the immune system, reduce inflammation and scar

formation, inhibit apoptosis, promote angiogenesis, and the proliferation of endogenous tissue stem cells.

However, despite intensive investigations so far very little is known about their ontogeny, developmental origin and true biological function in situ. Without this knowledge the therapeutic application of the cells might be a risk. To utilize the probably essential role of these cells in the body it would be necessary to get more knowledge about their formation and development during ontogeny.

In the literature there are very few data on the origins of the cells, and they are also controversies. Basically there are three theory. One research group suggests that MSCs are pluripotent stem cells remaining from the early embryonic period. According to others stromal cells obtained from different anatomical locations consist of a unified system and cells are developing from the primary mesenchyme after the first EMT (epithelial-mesenchymal transition) following gastrulation. Third theory is that MSCs are the descendants of one homogenous cell population and the differences evolve later in the tissue environment they settle or they correspond to parallelly developing cell types which participate in the early tissue formation and then serve as somatic stem cells for tissue renewal.

Classical lineage tracing method is not suitable in case of stromal cells since there is no marker molecules specific for MSCs alone. In order to find the origin of the cells we have to use an indirect method, namely the examination of molecules expressing by cells. For this purposes, we performed the comparative analysis of MSCs isolated from various organs and tissues in gene and protein expression level as well.

Objectives

Mesenchymal stem or stromal cells (MSCs) have been implicated in tissue maintenance and repair and regulating immune effector cells through different mechanisms.

These functions were primarily investigated in bone marrow-derived MSCs. The question was that is there any differences between MSCs from other sources (adipose tissue, spleen, thymus and aorta wall) and MSCs from bone marrow and what might be the significance of any differences in the future cell therapies?

(i) First, we generated 6 adherent stromal cell lines from different organs of adult (2-3 months old) and juvenile (14 days old) C57BL/6 mice. The question is that based on cell surface markers and in vitro differentiation ability are these cells really considered to be MSCs? If yes, do the cultures have identical cells surface antigen profile and differentiation capacity, or are there minor differences?

(ii) Next, we examined the gene expression profile of MSCs derived from different organs. Our particular interesting was the possible positional memory-encoding Hox genes and other transcription factor encoding (also homeotic selector domain containing) genes which play a key role during ontogeny. On this basis does cells have a positional memory (they remember what was their originally anatomical location *in vivo*) which they retain during long-term cultivation?

(iii) From our results is it possible to draw conclusions for the developmental origin and ontogeny of MSCs? Does the cells express special transcription factors essential for the development of the tissue where they originally isolated from?

Methods

Animals

Juvenile (14 days of age) C57Bl/6 and adult (10–12 weeks of age) C57Bl/6 (National Institute of Oncology, Budapest, Hungary) mice were used for all experiments in possession of ethical permission emitted by Animal Health and Food Control Station (Budapest, Hungary).

Isolation and culture of MSCs

Isolation and culturing was performed using the protocol of Piester et al. with some modifications by our laboratory. Mice were sacrificed by cervical dislocation and the femurs, thymus, spleen, aorta and visceral adipose tissue were removed. BM cells were collected by flushing femurs and tibiae with complete medium (CM) containing DMEM/Ham's F-12 medium (Invitrogen, Carlsbad, CA, USA), 10% fetal bovine serum, 5% horse serum (Invitrogen), 50 U ml⁻¹ penicillin, 50 µg ml⁻¹ streptomycin (Sigma-Aldrich, St Louis, MO, USA) and 2 mM L-glutamine (Invitrogen) and supplemented with heparin at a final concentration of 5 U ml⁻¹.

Cells from the thymus, spleen and aorta were obtained by mechanical disruption as follows: the dissected organs were washed with PBS, transferred into CM, cut into smaller fragments and subsequently minced with needles. The samples were then washed and filtered through a 60-µm nylon mesh filter to remove debris. Cells were then washed twice in Hanks' balanced salt solution, plated in a 25-cm² flask (BD Falcon, Bedford, MA, USA) at a density of 2–4 x 10⁶ cells cm⁻² in CM and cultured in a humidified 5% CO₂ incubator at 37°C for 72 h. Non-adherent cells were removed by sequential changes of the medium twice a week.

Abdominal and inguinal fat were removed; after washing with PBS, fat was digested with 0.1% collagenase (Sigma-Aldrich) in PBS for 30 min on a 37_C shaker at 250 rpm. Mature adipocytes and connective tissues were removed by two or three centrifugation steps at 600 rpm for 8 min. The pellet (SVF-stromal vascular fraction) was resuspended and washed again with PBS at 1,200 rpm for 10 min. Resuspended cells were seeded and cultured under the same conditions as the other stromal cells.

Confluent primary cultures were washed with PBS and lifted by incubation with trypsin– EDTA at 37°C for 5 min. Cells were then washed again, diluted at a ratio 1:5 and seeded into a 75-cm² flask (BD Falcon). Subsequent passages were performed similarly. Since cultures are morphologically heterogeneous and contain hematopoietic cells of BM origin until passage 6 or 7, therefore adherent cells were used between passages 10 and 25 in all experiments.

PCR-based examinations

On the one hand PCR method was carried out for the simultaneous examination of gene groups using PCR Array system. 84-84 genes were analyzed using RT² Profiler PCR Array System, Mouse Mesenchymal Stem Cell PCR Array and Mouse Homeobox Genes PCR Array (SA Biosciences). Each MSC samples were established independently from three groups of animals to ensure true biological parallels of the PCR array analysis. Total RNA was extracted using RT² qPCR-Grade RNA Isolation Kit (SA Biosciences, Frederick, USA) following the manufacturer's instructions. 1.5 µg of total RNA from each MSC samples was reverse transcribed to cDNA using the RT² First Strand Kit (SA Biosciences) and then subjected to SYBR® Green based real-time PCR using RT² SYBR Green qPCR Master Mix (SA Biosciences). CDNA amplification was measured on Roche Light Cycler 480 instrument (95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min).

Threshold cycle values obtained from the PCR Array measurements were partly validated by quantitative real-time PCR method using specific primer pairs. CDNA was reverse transcribed from 0.5 µg of total RNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems; Foster City, CA) according to the manufacturer's recommendations. Real-Time PCR was performed on Eppendorf Mastercycler ep realplex4. Cycling conditions were as follows: enzyme activation at 95°C for 10 min, followed by 40 cycles of 95°C for 20 sec, and 60°C for 65 sec.

Statistical analysis was carried out with Kruskal-Wallis method using the SPSS 13.0 software. P values <0.05 were considered significant.

Confirmation of the gene expressions on protein level

Protein levels of cell surface molecules (CD31, CD34, CD44, CD73, CD90.2, CD45R/B220) were measured by flow cytometry.

Detecting the presence of transcription factors (Mkx, Pitx1, Tbx5, En2, Tlx1) was carried out using immunofluorescence labeling.

For the detection of Tbx5 protein Western blot technique was used.

Further, in the supernatant of the six confluent MSC culture we measured the following cytokines by ELISA using the Mouse Inflammatory Cytokines Multi-Analyte ELISArray Kit (SABiosciences): Il-1a, Il-1b, Il-2, Il-4, Il-6, Il-10, Il-12, Il-17A, IFN- γ , TNF α , G-CSF and GM-CSF.

Plasticity of the adherent stromal cells

Osteogenic differentiation

Osteogenic differentiation was induced by DMEM supplemented with 10% FCS, 10 mM β -glycerophosphate (Sigma-Aldrich), 50 $\mu\text{g ml}^{-1}$ ascorbic acid (Sigma-Aldrich) and 0.1 μM hydrocortisone (Sigma-Aldrich). Differentiation was carried out with changing the medium twice a week. After 14 days we observe extracellular calcium deposition using Alizarin Red S (Sigma-Aldrich) staining.

Adipogenic differentiation

Adipogenic differentiation was carried out using DMEM supplemented with 10% FCS, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX; Sigma-Aldrich) and 10^{-7} M dexamethasone (Sigma-Aldrich). Differentiation was carried out with changing the medium twice a week. After 14 days cells were fixed and stained with Oil Red O (Sigma-Aldrich) to visualize lipid droplets for microscopic analysis.

Examination hematopoiesis-supporting capacity of the aorta wall-derived MSCs

Dexter-type culture

GFP⁺ aorta-derived cells were cultured with a 5-10-fold excess of freshly prepared adult bone marrow cells in Dexter-like conditions as described by Dexter and Testa. After 14 days, cultures were analyzed for co-expression of GFP and leukocyte markers (CD45R, CD11b, and Gr1) by FACS analysis.

Differentiation towards osteoclasts

Aorta wall-derived MSCs were treated with 10^{-8} M $1,25(\text{OH})_2\text{D}_3$ vitamin (Supelco, Bellefonte, PA) for 6 days and the differentiation of JAo-MSC into osteoclast cells were examined. To detect TRAP (tartrate resistant acid phosphatase) positive osteoclast cells, Acid Phosphatase Leukocyte Kit (No. 386) (Sigma-Aldrich) was applied according to the manufacturer's instructions.

In vivo hematopoietic repopulation ability

Transplantation assay was used to evaluate the in vivo repopulating ability of aorta-derived cells. Recipients (adult C57Bl/6 mice) were irradiated with a single dose of 9Gy (80 cGy/min) from a ^{137}Cs γ -ray source in the Radiobiology and Radiation Health Research Institute. Following this mice were injected intravenously with 2×10^6 aorta-derived cells or 10^6 freshly prepared bone marrow cells as a positive control or 50 μ l PBS as a negative control.

Results

Detection of cell surface antigens

In the identification of MSCs an important criteria is the surface molecule profile of the cells. First we examined these molecules in MSC populations derived from different tissue sources. According to the flow cytometry MSCs were positive for Sca-1 and CD44, excluding cells from aorta wall (JAo-MSc), which expresses less of Sca-1, and thymus-derived cells (JThy-MSc) which expresses less of CD44. CD73 expression was high in juvenile bone marrow MSCs (JCsv-MSc) and JAo-MScs, while the CD105 was dominant in JCsv-MSc, JLP-MSc (young mouse spleen of origin) and JAo-MSc. The presence of the CD90.2 was striking in stromal cells derived from adipose tissue of adult animals (Zs-MSc), while was hardly expressed in MSCs from other organs. In addition to the protein expression results, we wanted to get more information about the absence or presence of MSC „markers” in gene level as well. We found that the results obtained with PCR Array were well reflected the data of flow cytometry measurements.

Furthermore none of the examined MSC populations expressed hematopoietic markers such as CD34, CD3e, CD45R/B220, CD11b, Ly-6G and TER-119 protein and panendothelial marker CD31.

Cell plasticity analysis

Adipose differentiation medium direct stromal cells to accumulate intracellular lipid droplets, while during osteoblastic differentiation cells generate extracellular, calcium-rich mineralized matrix. These observations demonstrate the multipotency of MSC cultures. Differentiation capabilities of MSCs from different sources varied as MSCs from BM, thymus and spleen differentiated readily, whereas aorta-derived MSCs differentiated weakly into adipocytes. To find explanation on gene level we used PCR method to measure the level of mRNA transcripts involved in adipogenesis (Pparg) and osteogenesis (Runx2 and osteocalcin/ Bglap1). We found that Runx2 and Pparg were similarly expressed in all MSC types. Notably, osteocalcin (Bglap1) transcript was more abundant in JCsv-MSc than in any other MSCs tested.

MSCs do not express pluripotency-associated genes

Recent studies have indicated that somatic stem cells, including MSCs express pluripotency-associated genes (*Oct-4*, *Nanog* and *Rex-1*) regulating the maintenance of undifferentiated state of embryonic stem cells. However, the expression of these transcription factors was undetectable using Mouse Mesenchymal Stem Cell PCR Array in the different MSC lines analyzed. To further confirm the failure of expression the above transcription factors, single set quantitative RT-PCR was carried out on mRNA prepared from the different MSC lines and a murine ES cell line (R1/E) as control. These measurements confirmed our previous results, namely that none of the MSC cultures contain pluripotency gene expressing cells.

Further examination of aorta wall cells

Based on the results of PCR Array low level of telomerase reverse transcriptase (*Tert*) and leukemia inhibitory factor (*Lif*) gene expression was detected in the various MSC samples except JAo-MSCs in which *Tert* and *LIF* specific mRNA levels were 9- and 15-fold higher, respectively than in the other MSC samples. Moreover, JAo-MSCs formed unique cell clusters in over-confluent cultures that gradually organized into compact spheroids resembling a less mature “mesoangioblast-like” phenotype. The name “mesoangioblast” was chosen to denote a common progenitor for vascular and extravascular mesodermal derivatives and can be easily isolated from aorta wall of juvenile mice. These cells are less mature and able to give rise to hematopoietic cells in contrast to mesenchymal stem cells. To ascertain real identity of these cells we carried out some *in vitro* (Dexter-type culture, differentiation towards osteoclast cells) and *in vivo* experiments (bone marrow-repopulating ability). Based on our results we suggest that there are no hematopoietic cells in aorta-derived cell cultures.

Expression of mesodermal marker genes in MSCs

Mesodermal origin of the various MSC lines was analyzed by detecting expression of most common connective tissue proteins. The determination of mRNA level of type I collagen α -chain (*Colla1*), vimentin (*Vim*) and α -smooth muscle actin (*Acta2*) was carried out with PCR Array and qRT-PCR. The control cell type JCsv-MSCs expressed high levels of *Colla1*, *Vim* and *Acta2*. MSCs from other tissue sources

showed similar expression levels of *Coll1a1* and *Vim* as JCsv-MSCs, however, *Acta2* expression was lower in JAo-MSCs.

Next, the expression of three transcription factor (*Gata4*, *Gata6*, and *Nkx2.5*) coding genes, also involved in the specification and differentiation of cells with mesodermal origin, was evaluated by quantitative RT-PCR. These data imply that all MSC populations can be classified as mesenchymal cells, likely deriving from the mesoderm.

Positional memory of MSCs

Homeotic selector (Hox) genes encode a family of evolutionary conserved transcription factors that specify embryonic positional identity along the anterior-posterior and secondary axes in animals, and their expression in adult cells constitutes a form of positional memory.

Thus, to assess a possible role for Hox genes in tissue specific differences between MSCs from distinct sources, Hox expression profiles were generated from each MSC samples. The primary basis of these experiments was the Mouse Homeobox (HOX) Genes PCR Array, including in total 84 Hox-specific primer pairs.

Among the 4 classical Hox gene family (Hox a,b,c,d) we compared the level of 24 genes in the MSC cultures with different tissue origin. Based on PCR results, consistent expressions of 5 Hox genes (*Hoxa1,7*, *Hoxb3,4*, and *Hoxd9*) and no expression of 6 other transcripts (*Hoxb1, 9*, *Hoxd1,3, 12,13*) were detected in all MSC lines. 13 Hox genes (*Hoxa9*, *Hoxb2,7,8*, *Hoxc6,8,9,10,11,12,13*, and *Hoxd4,8*) were differently expressed in MSCs isolated from distinct sources. Thus, we can say the MSCs have individual “Hox code” that may reflect their anatomical origin.

Furthermore other homeodomain-containing transcription factor-encoding genes – cut-like homeobox 1 (*Cux1*), distal-less homeobox 1 (*Dlx1*), Mohawk (*Mkx*), and sine oculis-related homeobox 4 (*Six4*) – shared similarly high expression levels in all MSC cultures. Based on literature we know that for example Mohawk transcribed in cell lineages derived from the somites, similarly *Six4* gene expressed in somites and limb buds.

Most importantly, we identified such transcription factors that were specific for only one MSC population while they were absent from the others. T-box 5 (*Tbx5*), involved in the formation of the forelimb and heart was expressed at an especially high level in JThy-MSCs. Engrailed homeobox 2 (*En2*) in JAo-MSCs, T-cell leukemia homeobox 1 (*Tlx1*), participating in the early patterning and proliferation of the splenic primordium in JLP-MSCs, and paired-like homeodomain transcription factor 1 (*Pitx1*), implied in the development of hindlimb in the femoral bone marrow-derived samples (JCsv-MSCs and Csv-MSCs). Results in protein level confirmed the expression of the particular gene products in the proper MSC lines.

Conclusions

In the mirror of the results we have the following conclusions:

(i) On the basis of the main MSC characteristics cells derived from the bone marrow, adipose tissue, spleen, thymus and aorta wall of 14 days old and 10-12 weeks old mice should be considered mesenchymal stem cells.

Similar to bone marrow other stromal cells, greater or lesser amounts but also express CD44, CD73 and CD90 cell surface antigens and do not show the molecular characteristics of hematopoietic and endothelial cell molecules. At the same time all of 6 cell lines are able to differentiate into adipocytes and osteoblasts, although slightly different extent.

The reason could be that tissue microenvironment lesser or greater extent but is able to influence the characteristics and function of the cells.

(ii) We suggest that the examined cells develop from the mesoderm, as all MSC populations express large amount of mesodermal and mesenchymal molecules. Furthermore, each MSC population is characterized with individual HOX gene pattern which gives a positional information regarding the anatomical location of the cells.

Mkx1 and Dlx1 genes, associated with the development of somites are highly expressed in MSCs derived from any tissues. This observation supports the ancestry of all MSC populations from the mesoderm, presumable from the somites.

(iii) MSCs derived from different tissues are distinct in various body sides.

In case of each MSC population we specify such transcription factors that were specific for only one MSC population and were absent from the others. Thus, Pitx1 showed high expression in bone marrow-, Tbx5 in thymus-, Tlx1 in spleen- and En2 in aorta wall-derived stromal cells.

Our hypothesis is that the developmental origin of the cells is uniform, the place and time of their development could be started from the postsegmentation mesoderm, presumable from the somites. Later, when somite segmentation takes place, MSC migration starts and cells become established in the tissues of different body segments. Then stromal cells, in addition to retaining their original genetic memory, adapting to

the new environment's gaining new characteristics as well. This memory is long-lasting, as cells retain it even keeping them in long-term culture conditions, as we showed it in our experiments.

Our findings are of great importance to human therapeutic use including for regenerative medicine, since so far we thought that MSCs from any region of the body may also substitute each other. The reason is that so far our understanding of the MSC phylogenesis was poor. We hope that our work could greatly promote new and existing genetic research and clinical trials.

Publications

Publications of the Ph.D. thesis:

Journal articles:

1. Beáta Hegyi, Bernadett **Sági**, János Kovács, Judit Kiss, Veronika S. Urbán, Gabriella Mészáros, Éva Monostori, and Ferenc Uher: *Identical, Similar or Different? Learning about Immunomodulatory Function of Mesenchymal Stem Cells Isolated from Various Mouse Tissues: Bone Marrow, Spleen, Thymus, and Aorta Wall*. *International Immunology* 22:551-559. (2010) (IF=3.301)
2. Urbán S. Veronika, Hegyi Beáta, **Sági** Bernadett, Kudlik Gyöngyi, Uher Ferenc: *A diabetes mellitus őssejterápiája – az endokrin pancreas regenerációja*. *Diabetologia Hungarica* 19:279-286. (2011)
3. Bernadett **Sági**, Pouneh Maraghechi, Veronika S. Urbán, Beáta Hegyi, Anna Szigeti, Roberta Fajka-Boja, Gyöngyi Kudlik, Katalin Német, Éva Monostori, Elen Gócza, Ferenc Uher: *Positional Identity of Murine Mesenchymal Stem Cells Resident in Different Organs is Determined in the Post-Segmentation Mesoderm*. *Stem Cells and Development* 21:814-828. (2012) (IF=4.791)
4. Hegyi Beáta, **Sági** Bernadett, Kudlik Gyöngyi, Uher Ferenc: *A mesenchymalis őssejtek szerepe a gyulladásos- és immunfolyamatok szabályozásában*. *Immunológiai Szemle* IV:4-10. (2012)

Other publications:

1. Urbán S. Veronika, Benevolenskaya Elisabeta, Kiss Judit, **Sági** Bernadett, Hegyi Beáta, Uher Ferenc: *A genetikán is túl - Az epigenetika előretörése és orvosi vonatkozásai*. *Orvosi Hetilap* 153:214-221. (2012)

2. Tátrai Péter, **Sági Bernadett**, Szigeti Anna, Szepesi Áron, Szabó Ildikó, Bősze Szilvia, Kristóf Zoltán, Markó Károly, Szakács Gergely, Urbán István, Mező Gábor, Uher Ferenc, Német Katalin: A novel cyclic RGD-containing peptide polymer improves serum-free adhesion of adipose tissue-derived mesenchymal stem cells to bone implant surfaces. *Journal of Materials Science- Materials in Medicine* 24:479-488. (2013) (IF=2.141)