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Functional heterogeneity of mesenchymal stem cells in non-inflammatory and inflammatory microenvironment

Ph.D. thesis

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Introduction

Mesenchymal stem cells (MSCs) are resident in many adult tissues. These cells are capable to differentiate into mesodermal lineages such as adipocytes, osteoblasts and chondrocytes, moreover they produce a plethora of bioactive molecules. Due to their differentiation into various cell types, production of soluble factors including those with immunoregulatory functions and pro-angionic activity, MSCs have been implicated in medical applications such as regenerative medicine and treating immunological disorders. Hence, the importance of mastery of heterogeneity of MSC population has emerged. Different levels of heterogeneity could be and has to be investigated: 1) MSCs from different individuals can be distinguished; 2) MSCs from various tissues of one individual are different regarding the cell morphology, growth and differentiation abilities; 3) MSCs from a given tissue of one animal may exert clonal heterogeneity. Our work aimed to examine the clonal heterogeneity of mouse bone marrow MSC.

Aims

To get deeper insight into the functional heterogeneity of MSCs and to understand how the inflammatory microenvironment affects the activities of single cell derived MSC clones, the following aims were envisaged:

1) Which parameters can characterize the heterogeneity of the MSC population?
2) MSC clones are compared for immunosuppressive effects and involved factors.
3) How does inflammatory microenvironment modify the *in vitro* and *in vivo* immunosuppressive activity and *in vitro* differentiation potential of single MSC clones?

Methods

Mesenchymal stem cells were isolated from bone marrow of C57BL/6 mouse and expanded and maintained in tissue culture. Limiting dilution was used as cloning technique to generate MSC2-MSC6 clones enrolled in this study. Differentiation into osteoblast and adipocytes was induced by special factors in the cell culture medium and validated using histochemical staining methods (Alisarin Red and Adipored™, respectively). Pro-angiogenic effect of MSC clones was examined in an *in vitro* pre-vascular structure test co-culturing each MSC clones and endothelial cells for 3 days, and the length of pre-vascular structures formed by the two cell types were measured using CellR Imaging software.

Immunosuppressive property of the clones was assessed in *in vitro* co-culture of MSCs and T-cells. Prior to initiation of co-cultures, T-cells were stimulated by ConA and labelled with CFSE fluorescent dye, then proliferation was evaluated by flow cytometry. The production or activity of

immunosuppressive factors, nitric oxide (NO), prostaglandin E2 (PGE2) and indolamine-2,3-dioxygenase 1 (IDO1) were blocked in MSC-T-cell co-cultures by specific inhibitors (L-NMMA, indomethacin and 1-methyl-tryptophane, respectively). Gene expression was analyzed by quantitative real-time polymerase chain reaction (qRT-PCR).

For licensing of MSC clones with inflammatory cytokines, the cells were pre-treated with IFN-γ and TNF-α for 24 hours prior to a given assay.

Delayed type hypersensitivity reaction (DTH) was initiated in C57BL/6 mice by subcutaneous injection of ovalbumin with intraperitoneal injection of an MSC clone or PBS and evoked with a secondary injection of ovalbumin into the footpads. DTH reaction was evaluated by measuring the thickness of the footpads.

Results

- **Morphology, proliferation rate, cell surface marker profile and differentiation potential of MSC clones.**
  - MSC clones have a fibroblast-like morphology and similar proliferative capacity. Immunophenotype of the MSC clones were also identical: $CD45^-$, $CD11b^-$, $CD29^+$, $CD44^+$, $CD73^+$, $CD106^+$, $CD119^+$ and Sca-1^+ corresponding to the criteria of MSCs.
  - In the presence of inducing factors MSC clones differentiate into osteoblasts and adipocytes. Based on histochemical staining, MSC2, MSC4 and MSC5 exhibited strong while MSC3 and MSC6 weak osteoblast differentiation. Cytofluorimetric lipid quantification showed similar lipid accumulation in MSC clones during adipogenic differentiation.
- **Pro-angiogenic effect of the MSC clones is similar.**
  - MSC clones pre-seeded with endothelial cells support formation of pre-vascular structures to a similar extent as confirmed with similar length of the structures.
  - Gene expression levels of pro-angiogenic factors, VEGF (*Vegfa*), angiopoietin (*Angpt1*) and galectin-1, respectively were similar in each MSC clones.
- **MSC clones vary in inhibiting T-cell proliferation. NO is a key mediator.**
  - In *in vitro* proliferation assay each MSC clone significantly inhibit proliferation of ConA-activated T-cells. Nevertheless, the degree of proliferation inhibition was remarkably different since the blocking effect of MSC2, MSC4, and MSC5 is strong while that of MSC3 and MSC6 is low. Based on this, the clones can be set in the following order regarding inhibition: MSC2≥MSC5>MSC4>MSC3>>MSC6.

- Anti-proliferative function of the MSC clones well coincides with the relative NO synthase 2 (*Nos2*) mRNA expression, as it is expressed at higher levels in MSC2, MSC5 and MSC4 than in MSC3 and is undetectable in MSC6. Production of NO is specifically inhibited by L-NMMA and as a consequence the anti-proliferative effect of MSC2 is diminished. Blocking PGE2 production with indomethacin results in partial suspension of inhibition of T-cell proliferation by MSC2.

- **Pro-inflammatory cytokines abolish the differentiation and pro-angiogenic activity of MSC clones.**
  - MSC2 can be induced to differentiate into osteoblasts with appropriate factors. Imitating inflammatory environment with IFN-γ/TNF-α pre-treatment, MSC2 fails to differentiate toward osteoblasts. In accordance, after pre-treating MSC2 and MSC6 with IFN-γ and TNF-α, the expression of osteoblast differentiation marker genes (*Runx2*, *Bglap*, *Spp1*) is downregulated.
  - Upon licensing with pro-inflammatory cytokines, supportive effect of MSC clones on *in vitro* angiogenesis is abolished confirmed with lack of pre-vascular structures.
- **Licensing by IFN-γ and TNF-α enhances the anti-proliferative activity of MSC clones. NO is a crucial component of this process.**
  - Following licensing, all MSC clones become strongly suppressive on T-cell proliferation. The elevation of the inhibitory activity is the most prominent in the cases of MSC3 and MSC6 clones, which exert poor inhibitory activity without cytokine treatment.
  - Expressions of genes involved in inflammation, *Nos2*, *Ptgs2* and *Ido1* are elevated upon pre-treatment with IFN-γ and TNF-α.
  - Anti-proliferative effects of MSC2 and MSC6 are equalized after induction with IFN-γ/TNF-α and inhibition of NO production blocks the activity both of these clones. Blocking PGE2 production and IDO-1 with indomethacin and 1-MT results in partial and no inhibition of the effect of MSC clones, respectively.
- **Effect of MSC clones on the *in vivo* delayed type hypersensitivity reaction.**
  - The most and least T-cell anti-proliferative MSC2 and MSC6 clones, respectively, act similarly *in vivo* as *in vitro*. Hence, MSC2 inhibits the DTH reaction while MSC6 did not affect it.
  - Pre-stimulation with pro-inflammatory cytokines extinguishes the difference between MSC2 and MSC6 as both clones diminish the inflammatory reaction in DTH response.

Summary

Summary of the presented results: bone marrow MSC population is heterogeneous regarding osteogenic differentiation, pro-angiogenic properties and more explicitly immunosuppressive activity. Anti-inflammatory cytokines, IFN-γ and TNF-α, polarize MSC functions since they shift MSC clones

from differentiation toward anti-inflammatory activity. Licensing MSC clones by the above cytokines abolishes osteogenic differentiation and pro-angiogenic activity meanwhile the immunosuppressive function is remarkably elevated.

Based on these results, it may be reasonable to consider that licensing of MSCs prior to therapy of immunological disorders and contrarily, reduction of inflammation using anti-inflammatory drugs systemically prior to usage of MSCs in regenerative medicine could enhance the effectiveness of MSC therapy.

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Publications related to the subject of the thesis:

1. Szebeni, G. J., Kriston-Pál, É., Blazsó, P., Katona, R. L., Novák, J., Szabó, E., Czibula, Á., Fajka-Boja, R., Hegyi, B., Uher, F., Krenács, L., Joó, G., and Monostori, É. (2012) Identification of Galectin-1 as a Critical Factor in Function of Mouse Mesenchymal Stromal Cell-Mediated Tumor Promotion. *PLoS ONE* **7**, e41372, IF(2012): 3.730

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