The role of mesenchymal stem cells in the regulation of immune responses

Doctoral Thesis

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Introduction

Mesenchymal stem or stromal cells (MSCs) are multipotent tissue-resident stem cells that can be found throughout the whole body and play an important role in the maintenance and regeneration of multiple organs/tissues and affect haematopoiesis and immune responses as well.

The therapeutically most promising property of MSCs is their immunosuppressive capability, since they can inhibit the maturation, activation, proliferation and/or differentiation of the cells of both the innate and the adaptive immune system, thus MSCs can turn them into a functionally unresponsive state. This is accompanied by hypo-immunogenicity and the ability to support endogenous tissue regeneration of cells damaged as a result of inflammatory processes as well. These properties made MSCs promising candidates for the treatment of various autoimmune diseases, such as graft versus host disease, certain types of diabetes, systemic lupus erythematosus or multiple sclerosis. Based on the encouraging results of *in vitro* and *in vivo* experiments and clinical trials, it can be stated that in the coming decades the therapeutic use of MSCs has a good chance of bringing a breakthrough in the treatment of autoimmune diseases.

But first it would be very important to map and understand in depth the underlying molecular mechanisms of immunosuppression and promotion of tissue regeneration as well as the potentially arising risk factors (such as promotion of tumor growth and metastasis formation). We would like to contribute to this progress with our work.

Objectives

We would like to get answers to the following questions:

1) Can we isolate stem cells similar to bone marrow derived MSCs from other parts of the body, such as abdominal adipose tissue, thymus, spleen and aorta as well?
2) Is the immunosuppressive activity of MSCs derived from different organs similar or different?
3) What kind of soluble factors play a role in the immunosuppressive activity of MSCs?
4) How can the most important inflammatory cytokines - TNF-α and IFN-γ - influence prostaglandin E2 (PGE2) production of the mesenchymal stem cells?
5) What kind of other mechanisms play role in the regulation of the PGE2 production of mesenchymal stem cells?
6) Whether MSCs are capable of treating experimental autoimmune encephalomyelitis or not?
7) How can MSCs affect the morphology, phagocytosis, cytokine production and antigen-presenting ability, i.e. activation state (polarization) of microglial cells?
8) How does the presence of bacterial endotoxin (LPS) affect the impact of MSCs on microglia?
9) What kind of signaling pathways play a role in the interaction of mesenchymal stem cells and microglial cells?

Materials and methods

Isolation and characterisation of mesenchymal stem cells

We generally isolate mesenchymal stem cells according to their plastic adherence. C57Bl/6 mice were sacrificed, their femurs were isolated then the bone marrow was flushed out with a syringe. The cell suspension was plated in 25 cm^2 culture flasks in cell counts 2-5x10^6 per square centimetre. The abdominal and lumbar fat was digested with PBS containing 0.1% collagenase at 37˚C for 30 minutes and the obtained cells were suspended in complete medium as described above. The cultures were incubated at 37 °C in CO_2 incubator and the non-adherent cells were removed by replacing the medium twice a week.

The confluent cell layer was digested with 0.25% trypsin/EDTA solution and then cells were re-seeded in 75 cm^2 cell culture dishes. Subsequent passages were performed similarly. Experiments were performed with 8-15 times passaged MSCs.

To characterise MSCs their cell surface markers were determined with specific monoclonal antibodies by flow cytometry. Then MSCs were differentiated into adipocytes and osteoblasts in the presence of appropriate inductor media.

Inhibition of T cell proliferation

To test their immunosuppressive capability, we added 2x10^5 splenocytes or isolated T cells to MSCs in the presence or absence of 5 μg/ml concanavalin A (ConA). The mixed lymphocyte reactions (MLR) were prepared with 2x10^5 responder and 2x10^5 stimulator spleen cells in the presence or absence of MSCs. After 2 (or in case of MLR 4) days of incubation we pulsed the cells with 1 μCi ^{3}H-thymidin then the proliferation was determined as counts per minute

which was measured in a beta counter. In trans-well experiments the two cell types were spatially separated from each other using a 1 μm pore-size semipermeable membrane in the cultures.

Detection of secreted proteins in cell culture supernatants

The amounts of cytokines in the previously collected cell culture supernatants were analyzed with specific quantitative ParameterTM ELISA Kits according to the manufacturer's instructions.

In vitro stimulation of mesenchymal stem cells

$2x10^5$ MSCs were seeded into 24-well plates in 1 ml complete medium. After 24 hours proinflammatory cytokines (TNF-α and IFN-γ), enzyme inhibitors (Indomethacin, L-NMA and methyl-tryptophan), nitric oxide donor molecule (NOC-18) and/or lipopolysaccharide (LPS) were given to the cells and cultures got incubated for an additional 48 hours. We collected samples from the supernatants and stored them frozen at -80 ºC.

The induction of experimental autoimmune encephalomyelitis (EAE)

12 weeks old C57Bl/6 female mice were vaccinated subcutaneously with 1:1 mixture of complete Freund's adjuvant (CFA) containing 5 mg/ml of killed Mycobacterium tuberculosis and PBS containing 1 mg/ml MOG_{35-55} peptide. The animals also got 330 μg Pertussis toxin intraperitoneally on the day of induction and 2 days later. On the 7th day every second randomly selected animal was treated with intraperitoneal administration of $2x10^6$ mesenchymal stem cells. The experiment lasted 28 days and during this time the health status of the animals was monitored daily. Disease severity was scored on a 5-point scale as follows:

0 point: healthy animal;

1 point: the tail of the animal is paralysed;
2 points: the movement of the animal's hind legs became uncertain or one of the hind limbs is paralyzed;
3 points: two hind limbs are paralyzed;
4 points: the movement of the animal's forelegs also became uncertain or one of the forelimbs is paralyzed;
5 points: all of the limbs are paralysed or the animal has died.

The animals characterized as 4-point were over-anesthetized. The experiments were performed according to the rules established by the Animal Care Committee of the National Medical Center and the recommendation of the European Union 86/609/EC.

Isolation of microglia

The brain of newborn (P1-P3) CD1 mice was isolated, the meninges were removed and after mechanical trituration we digested them enzymatically with PBS containing 0.05% trypsin/EDTA solution. The obtained cells were seeded into poly-L-lysine coated Petri dishes in complete medium. After 24 and 48 hours the non-adherent cells were removed by thorough washing of the surface. The cultures were incubated for 3 weeks while the media was changed twice a week.

On the 21th day we isolated microglia. We incubated the cultures in EDTA-free 0.05% trypsin solution for 2 hours then we removed the floating astroglia monolayer. The remaining adherent cells were incubated for an additional 10 minutes in 0.25%s trypsin/EDTA solution. The thus selectively isolated microglial cells were seeded onto PLL coated surfaces.

Morphological analysis of microglial cells (immunocytochemistry)

10^5 microglia were incubated in the presence or absence of 10^4 bone-marrow derived MSCs and/or 10 μg/ml LPS at 37˚C for 48 hours. After fixation in paraformaldehyde, the plasmamembrane of the cells was labelled with biotinylated Isolectin B4 overnight at 4 ˚C then Alexa488 conjugated avidin was added to them. We took photos of 15 independent random field of views for each sample (n = 4) with 20x objective of Nikon A1R confocal laser-scanning microscope. The area of each cell was measured in the pictures by Zeiss AxioVision 4.8 software.

Phagocytosis of microglia

$5x10^6$ heat-inactivated yeast cells were added to the cultures containing microglia and/or MSCs and incubated for 1 hour at 37˚C. After three washes the preparations were fixed and stained with Giemsa solution. We took photos of 10 independent random field of views and counted the number of engulfed yeast particles per microglia (n = 100).

To study the phagocytosis of apoptotic thymocytes we isolated and mechanically triturated the thymus of adult C57BL/6 mice. We added 1 μM dexamethasone to the suspension containing $5x10^6$ thymocytes/ml then incubated it for 12 hours at 37 °C.

To investigate the cytokine production after phagocytosis we added $5x10^6$ yeast or apoptotic thymocytes to the microglia- and/or MSC-containing cultures and then incubated them for 1 hour at 37˚C. After three thorough washes with PBS we cultured the samples for further 48 hours, then supernatants were collected and stored frozen at - 80 ºC until the subsequent detection of secreted proteins.

Measurement of the antigen-presenting capability of microglia

To obtain ovalbumin-specific T cells we vaccinated 10-12 weeks old C57Bl/6 mice with 1:1 mixture of complete Freund adjuvant and 2 mg/ml ovalbumin solution. After one week the lymph nodes of pre-treated animals were isolated and triturated mechanically in Hanks solution. After centrifugation the resuspended T lymphocytes were added to the microglia- (antigen presenting cells) and MSC-containing cultures. After 4 days of incubation we pulsed the cultures with 1 μCi ^{3}H-thymidin for 6 hours and we determined the proliferation as counts per minute with a beta counter.

Quantitative real-time PCR

In the 5th hour of the LPS treatment, we collected samples with Trizol reagent from microglia- and/or MSC-containing cultures. We isolated total RNA with GeneAid mini Kit and checked the purity of the preparations with Nano Drop Spectrophotometer. RNAs were converted to cDNA with High-Capacity cDNA Reverse Transcription Kit. Real-time PCR was performed on ABI StepOne instrument with Power SYBR Green PCR Master Mix according to the manufacturer's instructions. The primer sequences were designed with Primer Express 3.0 software. GAPDH message was used as internal control. The melting curve analysis and the evaluation of gene expression data had been performed with Software v.2.0 of StepOne instrument.

Statistics

The significance of the results was determined using Student's t test ($p < 0.5$). The results are shown as mean ± standard deviation.

Results

1. Characterization of mesenchymal stem cell populations by surface markers and differentiation capacity

We prepared stromal cultures from the bone marrow or from the abdominal and inguinal adipose of adult C57Bl/6 mice. After eighth passages the bone marrow (BM-MSC) and adipose tissue-derived (Ad-MSC) mesenchymal stem cell cultures both consisted of adherent cells with fibroblast-like morphology. According to FACS analysis, these cell populations were positive for Sca-1, CD44 and CD73 surface markers but only Ad-MSC expressed CD90.2. The cultures were uniformly negative for the tested hematopoietic markers. The cells of both MSC populations were differentiated into osteoblasts or adipocytes in the presence of appropriate inductor media. Thus, based on their plastic adherence, morphology, cell surface markers and differentiation potential both bone marrow- and adipose tissue-derived cultures can be considered as mesenchymal stem cells. Similarly, MSC populations corresponding to all of the above mentioned criteria could be obtained from the bone marrow, aorta, spleen and thymus of young (14 days old) C57BL/6 mice as well.

2. Inhibition of mitogen- and alloantigen-induced T-cell proliferation in vitro

Bone marrow, adipose tissue and spleen-derived MSCs had the ability to inhibit (about 65-70% extent) mitogen (ConA)-induced T cell proliferation. The aorta-derived MSCs had more moderate inhibitory activity (approx. 40%) while the thymus-derived cells did not affect T-cell proliferation. Identical results were obtained in mixed lymphocyte reactions. Thus, MSC populations derived from different sources may vary significantly from each other in their immunosuppressive activity *in vitro.*

3. Immunosuppressive activity of MSCs in the presence of certain enzyme inhibitors

We tried to explore the mechanism of immunosuppression by inhibiting enzymes responsible for the synthesis of different soluble mediators released by MSCs in the presence of activated T cells. In the presence of indomethacin (inhibitor of PGE2 synthesis) or L-NMA (nitric oxide synthase inhibitor) T cell proliferation is partially but not completely restored, thus we could conclude that prostaglandin(s) and NO play a role in the immunosuppressive activity of MSCs.

The indolamine-2,3-dioxygenase (IDO) inhibitor methyl-tryptophan, however, did not modify the T cell proliferation blocking ability of MSCs, so at least in case of mouse MSCs IDO does not seem to contribute to the immunosuppressive activity.

4. PGE2 production of MSCs in the presence of activated T cells

After consideration of the above mentioned results, we focused on the examination of PGE2 production by MSCs. The basic PGE2 production of BM-MSCs was 1200 pg/ml, this value increased up to 4000 pg/ml in the presence of naive T cells. In case of ConA-stimulated lymphocytes + MSC cultures, we measured 16,000 pg/ml PGE2 in the supernatants. If the T-cells and bone marrow MSCs were spatially separated, PGE2 secretion of the stem cells did not rise above 1200 pg/ml. Thus the activated T lymphocytes are capable of increasing the PGE2 synthesis of mesenchymal stem cells, but direct cell-cell interactions are obligatory for their effect.

5. The impact of the inflammatory environment on the prostaglandin E2 production of mesenchymal stem cells

To model inflammatory environment, recombinant cytokines were added to BM- and Ad-MSCs *in vitro*. High concentration (50 ng/ml)

of TNF-α significantly increased the PGE2 production of MSCs whereas lower concentration (10 ng/ml) was found to be ineffective. When IFN-γ was added to MSCs no significant change was observed in any of the examined concentrations (100 and 20 ng/ml). However, if the two cytokines were used together even in low concentrations (10 ng/ml TNF-α and 20 ng/ml IFN-γ) we got significant increase in the amount of secreted PGE2. Thus, we can conclude that the inflammatory environment enhances the immunosuppressive mediator production of MSCs. The effect of the tested mediators is strongly synergistic as IFN-γ significantly enhances the TNF-α-induced PGE2 production of mesenchymal stem cells.

The addition of 10 μM indomethacin to BM-MSC cultures could completely inhibit not only spontaneous but also inflammatory mediator-stimulated PGE2 secretion. PGE2 production induced by the simultaneous presence of TNF-α and IFN-γ could be inhibited with L-NMA, though only to a 60-70% extent. In contrast, the NOS inhibitor did not have significant effect on the PGE2 secretion stimulated by 50 ng/ml of TNF-α.

6. The immunosuppressive activity of MSCs in experimental autoimmune encephalomyelitis

As the presented *in vitro* results and literature data suggest that the immunosuppressive activity of MSCs can be used for therapeutic purposes, we examined the effect of our cells on experimental autoimmune encephalomyelitis (EAE), a preclinical animal model of human multiple sclerosis.

In the first phase of the disease mice treated on the 7th day with the intraperitoneal administration of $2x10^6$ bone marrow-derived mesenchymal stem cells temporarily exhibited more serious symptoms compared to the untreated control group. During the most severe stage of the disease (day 15-18th) there is absolutely no

difference between the stem cells treated or untreated animals in the extent of paralysis. Subsequently (after day 19th), however, mice undergone cell therapy improved significantly faster than untreated control counterparts. Therefore in our model MSC treatment does not inhibit the induction and/or progression of EAE but rather promotes disease remission. As in case of EAE the main effector cells of the ongoing inflammatory processes of the brain and spinal cord are microglial cells, we focused on the analysis of the impact of MSCs on microglia *in vitro*.

7. The effect of MSCs on microglial cells

We prepared mixed glial cell cultures from the bain of newborn CD1 mice then microglial cells were selectively isolated by mild two-step trypsinisation. The obtained microglia can be characterised by a multi-branched, resting cell shape. In the presence of MSCs the morphology of microglia considerably changed: they became spread, activated and gained an amoeboid phenotype even if we spatially separated the two cell types from each other with semi-permeable membrane.

Then we examined the effect of MSCs on microglial cells, in functional tests. First we studied the changes in the phagocytic activity of microglia which can be quantified as incorporated yeast particles. During the 60-minute incubation microglia alone can engulf 15 ± 1 yeast cells, whereas in the presence of mesenchymal stem cells the intensity of phagocytosis increased to 24 ± 2, which proved to be significant. Thus, microglia cells became activated in the presence of mesenchymal stem cells, which can be proven by changes in the morphology, cell size and increase of phagocytosis as well.

8. The cytokine production of microglial cells in the presence MSCs

Next, we wanted to know the type of activation microglia gain resulting from their interaction with MSCs, therefore, we compared the effects of MSCs and LPS on the cytokine production of microglia. In the presence of endotoxin (LPS) microglial cells increased their production of TNF-α, while we could not observe changes in the secretion of IL-10 and PGE2, i.e. microglial cells undergone classical activation and gained an M1, inflammatory phenotype. However, high levels of IL-10 and PGE2 were detected in the supernatants of microglia-MSC co-cultures indicating the shift of the cytokine milieu toward an immunosuppressive direction: an M2, alternative activation of microglia occurred in these cultures. If the two cell types were separated spatially in transwell cultures the concentrations of inflammatory mediators increased, i.e. in this case the immunosuppressive effect of MSCs requires cell-to-cell contact.

Next, we wanted to find out which cell type produce the pro- and anti-inflammatory cytokines mentioned above in the microglia-MSC co-cultures. The results of two-stage transwell experiments and gene expression studies revealed that the majority of TNF-α and IL-10 are produced by microglial cells, whereas mesenchymal stem cells secreted only PGE2 in significant amounts.

9. The effect of MSCs on the antigen presenting capacity of microglial cells

The antigen-presenting ability of microglial cells was examined in the presence of ovalbumin (OVA)-specific T cells and different concentrations of OVA. We found that microglial cells efficiently presented ovalbumin which was further enhanced in the simultaneous presence of mesenchymal stem cells. This is supported by our observation that the expression of cell surface molecules playing a key role in the antigen-presenting ability of microglial cells (MHC-II and CD86) is also increased in the presence of MSCs. Accordingly we

hypothesise that due to mesenchymal stem cells a type of regulatory, alternatively activated microglial cell is formed that can be characterised by the production of anti-inflammatory mediators, amoeboid cell morphology, intensive phagocytosis, and increased antigen-presenting activity. This result is very important in the therapeutic aspect, as regulatory microglial cells formed in the presence of MSCs may be effective in the treatment of autoimmune or neurodegenerative disorders.

10. The role of inflammasomes in the microglia-MSC interaction

We investigated more thoroughly the interaction between mesenchymal cells and microglial cells by examining the gene expression of several different mediators (IL-1α, IL-1β, IL-6, Arg-1 and MCP1). Among these IL-1β stood out: microglial cells significantly reduced the expression of this mediator in the presence of MSCs. This raised the possibility that MSCs (also) exert their immunosuppressive activity through inflammasomes.

In support of this idea we examined the expression of NLRP3 of microglial cells which was reduced by half in the presence MSCs. Thus, MSCs can effectively inhibit the expression of the NLRP3 gene by microglia and simultaneously they can reduce the secretion of IL-1β as well.

Conclusions

1. We managed to isolate adherent cell populations showing fibroblast-like morphology from the bone marrow, adipose tissue, thymus, spleen and the aorta as well. The cells can be considered mesenchymal stem cells based on their surface markers and differentiation potential toward adipocytes and osteoblasts.
2. These MSCs – except for cells from the thymus – can inhibit mitogen- and alloantigen-induced proliferation of T lymphocytes *in vitro*, therefore they have similar immunosuppressive activity.
3. Bone marrow and adipose tissue-derived MSCs are capable of producing large amounts of prostaglandin E2, which is considered as a potential immunosuppressive mediator, in the presence of activated T cells or inflammatory cytokines.
4. The most important inflammatory cytokines - TNF-α and IFN-γ - enhance the production of PGE2 by mesenchymal stem cells synergistically.
5. The PGE2 production of MSCs is not regulated by one special mediator or non-redundant molecular mechanisms, but rather by several signaling pathways.
6. Mesenchymal stem cells can successfully treat experimental autoimmune encephalomyelitis.
7. The yeast phagocytosis, cytokine production, morphology and antigen presenting capability of microglial cells are significantly altered in the presence of MSCs. This suggests that mesenchymal stem cell induce the alternative (type M2) activation of microglial cells.

8. The presence of MSCs does not completely inhibit, rather only modulates the process of classical activation which is triggered by antigens from pathogens such as fungal cells or endotoxins.
9. PGE2 produced by MSCs might be one of the most important soluble mediators that acts on the microglia cells and can modify their inflammatory and anti-inflammatory cytokine production. Blocking the action of inflammasomes also plays a significant role in the MSC-microglial interaction.

List of publications

Publications related to the dissertation:

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