Mesenchymal stem cells as potential source for musculoskeletal diseases mainly for cartilage repair

(Ph.D. thesis)

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SUMMARY

Because of the lack of vascularity and paucity of cellularity, articular cartilage damaged by disease or trauma has a limited capacity for regeneration. Mesenchymal stem cells (MSCs) have the potential to differentiate into distinct mesenchymal tissues; including cartilage and bone hence they can be attractive cell source for cartilage tissue engineering approaches. Marrow samples were removed during bone surgery and adherent cell cultures were established. The cells were then passed into a newly developed **microaggregate culture system** in a medium containing transforming growth factor-B3, insulin, dexamethasone, and/or demineralized bone matrix. In vitro **chondrogenic activity** was measured as metabolic sulfate incorporation in pellet cultures. Cell aggregates were also analyzed by histology and by weight of newly synthesized cartilage. Our findings show that **DBM** possess all the necessary conductive features of a carrier, serving at the same time as a natural source of inductive chondrogenic factors.

One of our objectives here was to compare the in vitro chondrogenic potential of MSCs isolated from patients with rheumatoid arthritis (RA) and osteoarthritis (OA) with cells from normal donors. Culture-expanded **MSCs from RA and from OA** patients did not differ significantly from the normal population with respect to their chondrogenic potential *in vitro*, therefore these cells in these patients may serve a potential new prospect in the cartilage replacement therapy as well. **Galectin-1** stimulated the chondrogenic differentiation of mesenchymal cells in low concentration. We have evaluated the effect of recombinant human galectin-1 on the proliferation and survival of murine and human hematopoietic stem and progenitor cells. We show that low amount of galectin-1 increases the formation of granulocyte-macrophage and erythroid colonies. Gal-1 blocked BM progenitor cell migration induced by CY/G-CSF treatment, indicating a novel anti-inflammatory function of this lectin. We have found that low and moderate amount of galectin-1 stimulate the chondrogenic differentiation of MSCs. Therefore, recombinant galectin-1 might be used during cellular therapy of inflammatory joint and bone diseases as an anti-inflammatory agent.

INTRODUCTION

The **stem cells** are standing on the focus of interest in the last decade. Wide attention has been focused on adult stem cells as a reservoir and regeneration pool for various human tissues. The mesenchymal stem cells (MSCs) have the ability to proliferate into multiple mesenchymal lineages – as adipocyte, tenocyte, osteocyte, chondrocyte- and into other lineage as well under defined culture conditions.

Several pathological conditions associated with articular cartilage damage such as osteoarthritis (OA) and rheumatoid arthritis (RA) or trauma show limited capacity for tissue regeneration due to the lack of vascularity and paucity of cellularity. Thus, clinical needs for cartilage-generating therapies are diverse, prevalent, and increasing in concert with our aging population. Treatment of defects of articular surfaces is one of the most challenging problems of the daily orthopaedic practice.

The recently used orthopaedic techniques for cartilage repair are various, beside traditional **resurfacing procedures** (i.e. microfracture, drilling) several new techniques have been developed to promote a hyaline-like repair of the defected area. By the **bone marrow stimulation** breaking through the underlying bone (subchondral bone) one prepares minute canals for the mesenchymal stem cells to travel to the site of chondral lesion.

Widely known and accepted technique for focal chondral lesion and is often referred to as **„mosaic" plasty**. A drawback of the technique is that small and medium sized lesions might be treated only by it. The larger defect sizes may be repaired by **autologous chondrocyte transplantation**, but by these techniques could severe donor site morbidity occur, and two surgical interventions are necessary. Therefore the research is tending toward the treatments using stem cells (mesenchymal stem cell).

Transplantation cell therapy combined with appropriate growth factors is able to regenerate the cartilage matrix specifically in damaged areas hence it opens new perspectives in curing these diseases. The capacity of multipotent mesenchymal stem cells (MSCs) to differentiate into tissues of mesenchymal lineages, including bone, cartilage, fat, tendon and muscle according to culture conditions and specific growth factors make these cells ideal candidates for tissue engineering.

The new resurfacing techniques are contraindicated in the **autoimmune diseases**

because of the renewal of the defects by the continuosly standing inflammatory circumstances. The goal is to combine the in vitro chondrogenesis with relevant immunosuppression to avoid the endoprosthetic procedures in the future by the autoimmune patients. In this question we studied the potentially role of the galectin-1 on the evidence of the bibliography. **Galectin-1**, a member of the ancient beta-galactoside-binding lectin family, is a pleiotropic dimeric protein participating in a variety of normal and pathological processes, including cell adhesion, cell growth regulation, immunomodulation, inflammation, apoptosis, embryogenesis, and cancer progression. Galectin-1 (Gal-1) expressed by bone marrow stromal cells

AIMS

I. In vitro cartilage formation

- We had to developed a new culture system. This microculture system could significantly lower the cost and decrease the time required for the preparation of MSC aggregates compared to the original conical tube based culture system, while maintaining reproducible chondrogenic differentiation..
- One goal of our experiments was to examine the use of the demineralized bone matrix (DBM) in the in vitro chondrogenesis.
- We wished to analyze by histology the newly synthesized cartilage (comparison to the hyalincartilage)

II: The question is still opened whether MSCs isolated from RA and OA patients useful for tissue engineering purposes.

III. We want to test the ability of this microculture system for in vitro application: to study the effects of several chondroprotective and anti-inflammatory drugs, synovial fluid.

IV. We wanted to measure the effect of recombinant human Gal-1 on the in vitro chondrogenesis and to study his new anti-inflammatory effects.

MATERIALS AND METHODS

1., Isolation and cultures of human bone marrow-derived MSCs

To isolate human MSCs, bone marrow (BM) samples were taken from the femoral shaft of normal donors and patients underwent bone surgery or sternal punction after informed consent (patients: we studied 7 patients with RA, 7 patients with OA, and 9 control adult volunteers). Nucleated cells were isolated with a density gradient, all low-density cells were plated in 25-cm^2 flasks. After 2-4 days, nonadherent cells were discarded, and adherent cells were expanded in complete medium. When cells grew to 80% confluence, they were harvested with trypsin. These cells were further expanded, and the adherent cells between passage 3 and 7 were used as MSCs in all experiments.

2., In vitro cartilage formation

MSCs were placed in U-bottom tissue culture plates supplemented with dexamethasone, TGF-β3, and insulin, or with DBM alone, or with DBM plus growth factors. The 96-well tissue culture plates were then centrifuged and incubated. Cell aggregates were obtained at intervals of 14 days.

3., Histology and transmission electron microscopy

a., Cell aggregates (pellets) were harvested and stained with dimethyl-methylene blue.

b., Alternatively, pellets were stained with 1% toluidine blue, ultrathin sections were contrasted and examined with a electron microscope.

c., In vitro chondrogenic activity was measured as type II collagen expression in pellet cultures performed with quantitative RT-PCR. For measurement of newly synthesized proteoglycans and protein, incorporation of radioactivity (sulfate and leucine) was measured by liquid scintillation counting.

4. Demineralized bone matrix (DBM)

DBM was prepared as described by Gurevitch.

5. Pharmaceuticals

Chondroitine sulfate, glucosamine hydrocloride, chloroquine phosphate, aceclofenac, and niflumic acid were chosen as standard medications for OA or RA. We measured the effects of these drugs on chondrogenic differentiation of MSCs.

6., Galectin-1 Human recombinant galectin-1 was a gift from Dr Éva Monostori (Biological Research Center, Hungarian Academy of Sciences, Szeged).

7., Progenitor (colony-forming cell) assay

Quantification of the number of colony-forming units granulocyte-macrophage (CFU-GM) was performed using a semisolid methylcellulose CFC assay.

8., Gal-1-mediated effect of BM cell mobilization

Bone marrow HPCs (hematopoietic progenitor cells) were mobilized with CY/G-CSF or CY/G-CSF plus human recombinant Gal-1 in BDF1 mice. We measured the effect of Gal-1 treatment on the distribution of leukocyte subpopulations in blood and BM during HPC mobilization.

9., Statistical significance was assessed by a two-tailed unpaired Student's *t* test.

RESULTS

I. A new micromass culture system for chondrogenic differentiation of bone marrow-derived human MSCs

- In the present study, we have used a new micromass culture system, carried out in 96-well tissue culture plates. We have found that DBM is capable alone without growth factors for chondrogenic differentiation of human MSCs.
- After 14 days of chondrogenic culture, the extent of chondrogenesis was also assessed histologically using methylene blue and toluidine blue staining, as well as by transmission electron microscopy. Histological examination of pellets confirmed sulfate incorporation, showing an abundant cartilage-like, metachromatic-stained matrix and a dramatic increase in pellet size to untreated control cultures.

II. Chondrogenic differentiation of MSCs from RA and from OA donors

All MSC preparations from either normal donors or RA, as well as OA patients were able to synthesize the same amount of proteins and proteoglycans of culture in the presence of TGF-□3, insulin and DBM as determined by leucine and sulfate incorporation, respectively. Similar results were obtained when type II collagen mRNA levels measured by quantitative RT-PCR. Similar results were obtained comparing the size and the dry weight of pellets after 14 days of culture in chondrogenic medium.

III. Effects of chondroitin sulfate (CSA), glucosamine (GlcN), chloroquine and nonsteroidal anti-inflammatory drugs on cartilage formation of human MSCs

Chondroprotective drugs, CSA and GlcN induced protein synthesis during

chondrogenesis. Neither CSA nor GlcN had significant effect on normalized sulfate incorporation indicating that proteoglycan synthesis was not affected.

Chloroquine (CQ), markedly inhibited the de novo protein and proetoglycan synthesis on a dose-dependent manner.

The effect of two other nonsteroidal anti-inflammatory drugs, aceclofenac (Ac) and niflumic acid (Nac) on chondrogenic differentiation of MSCs was also investigated. Neither Ac nor Nac had any effect on leucine or sulfate incorporation at concentrations.

The inflammatory synovial fluid had a hypertophic effect for the newly syntetized cartilage.

IV. Galectin-1 effects

- **We have measured the effect of recombinant human Galectin-1 on murine and human HSC and progenitor cell proliferation and survival:**

Low amount of Gal-1 increases CFU-GM and BFU-E formation of bone marrow progenitor cells. In contrast, high amount of Gal-1 dramatically inhibit the growth of committed cells as well as that of much younger (stem/early progenitor) cells. The growth inhibition correlated with the apoptotic death of these cells. The effect of Gal-1, however, depended upon the differentiation states of the cells.

- **Dose- and time-dependence of Gal-1-mediated inhibition of BM cell mobilization:**

We analyzed the dose- and time-dependence of Gal-1-mediated inhibition of clonogenic myeloid progenitor cell mobilization in response to CY and G-CSF. We found that Gal-1 was a highly effective inhibitor of HPC mobilization in vivo in BDF1 mice.

- **Effect of recombinant galectin-1 on cartilage formation of normal, RA and OA MSCs**

Low concentration of Gal-1 promoted, contrarily high lectin concentration was unfavorable during induction of chondrogenic differentiation of MSCs.

Discussion

I.

In Hungary we established the research for cartilage from human MSCs. Mesenchymal stem cells provides a source of cells for the repair of musculoskeletal tissue. Using this cells large size chondral defect as well may be repaired in the future, replace the newly used matured cartilage for cartilage repair. MSCs with chondrogenic differentiation potential are obviously superior to the mature cells of these tissues.

This microculture system significantly lowered the cost and decreased the time required for the preparation of MSC aggregates compared to the original culture system, while maintaining reproducible chondrogenic differentiation.

The cells were passed into a microaggregate culture system in a medium containing transforming growth factor-β3, insulin, dexamethasone, and/or demineralized bone matrix. The demineralized bone matrix was capable without growth factors a good capacity for chondrogenesis and could be a good naturally source of growth factors for cartilage replacement therapy in the future.

II.

Culture-expanded MSCs from RA and from OA patients did not differ significantly from the normal population with respect to their chondrogenic potential *in vitro*. MSCs from RA and OA patients possess similar chondrogenic potential to that of MSCs isolated from healthy donors, therefore these cells may serve a potential new prospect in cartilage replacement therapy.

III.

With the micromass culture system newly synthesized tissues provide a new experimental paradigm to investigate cell functions and provide new and complementary information for animal experiments. In our study chondroitin sulfate and glucosamine enhanced, whereas chloroquine inhibited the NSAID agents did not altered the chondrogenesis in normal

donor or patient-derived MSC cultures.

IV.

Our findings enhanced the immunosuppressive and anti-inflammatory activity of galectin-1 has been well established in experimental studies. We determined a new anti-inflammatory effect of the lectin: inhibition of bone marrow cell mobilization.

Low concentration of Gal-1 promoted the chondrogenic differentiation of MSCs and that combined with the anti-inflammatory effect could be the galectin-1 a new potential agent for the cartilage repair by the inflammatory patients in the future.

LIST OF PUBLICATION

PUBLICATIONS PRESENTED IN THE THESIS:

1) Vas V, Monostori É, Fajka-Boja R, **Dudics V** and Uher F: *Biphasic effect of recombinant galectin-1 on the growth and death of early hematopoietic cells.* Stem Cells 2005;23:279-287. (***IF 2006: 7,924***)
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4) Kiss J, Urbán V, **Dudics V**, Vas V, Uher F: *A mesenchymalis őssejtek és az immunrendszer- immunszuppresszió gyógyszerek nélkül?* Orv Hetilap 2008, 140. évfolyam, 8. szám, 339-346.
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