Use of mesenchymal stem cells in musculoskeletal tissue engineering

– Theses –

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Introduction

Stem cell therapy is one of the greatest promises of biology in the new millennium. Regenerative medicine could certainly benefit from the healing potential of these cells. While embryonic and fetal stem cells hold great differentiation potential, they are also considered dangerous due to their tumorigenic potential and difficulties to regulate their differentiation.

Bone marrow transplantation routinely uses donor cells with high proliferative capacity to reconstitute the defected hematopoietic system of patients. These cells are termed hematopoietic stem cells. There are different, non-hematopoietic adult stem cells in the bone marrow and other mesenchymal tissues, the potential of which is just being realized in several areas of orthopaedic research. These MSCs are being evaluated for use in bone and cartilage regeneration, as well as for the reconstruction tendon, ligament, muscle, and other mesenchymal tissues.

In these studies we set out to investigate the possibility of using bone marrow derived MSCs, and to improve their differentiation into bone or cartilage.

In the first set of experiments we evaluated the putative osteogenic potential of statins (HMG-CoA reductase inhibitors) on human MSCs *in vitro*. They have been shown to increase bone mass in rats *in vivo* first in 1999.(Mundy *et al.*, 1999) Later, several studies suggested beneficial effect on OB cell lines.(Maeda *et al.*, 2004) At the same time, many other publications cautioned that statins may have detrimental effect on cell viability and cytoskeletal structure.(Ghosh *et al.*, 1997; Vamvakopoulos and Green, 2003) It would be preferred to use these simple organic compounds to differentiate MSCs into OBs compared to the expensive and potentially dangerous use of high concentration growth factors. So in these experiments we evaluated the effect of simvastatin and lovastatin on human MSCs, focusing on osteogenic markers and cell viability and morphology.

Another way to circumvent the use of recombinant growth factors is to use the cells themselves to produce them. This can be achieved by adenoviral transduction. This strategy was not effective in a sheep model.(Egermann *et al.*, 2006) In a second set of experiments we compared the reaction of sheep and human MSCs and OBs to AdBMP2 *in vitro*, to see whether the failure was due to a different reaction to the growth factor.

Finally, we investigated the potential of the transcription factor SOX9 for cartilage tissue engineering. SOX9 is a key regulator of chondrogenesis; its expression increases during the differentiation process, and decreases during hypertrophic (terminal) differentiation of chondrocytes.(Okubo and Reddi, 2003) Therefore is an ideal target for stable chondrogenic induction of MSCs. We optimized cell culture and the method of gene delivery, then conducted a series of experiments, where SOX9 over-expression was combined with mechanical load in an attempt to optimize and stabilize chondrogenic differentiation.

Methods

Human MSCs were isolated from bone marrow, expanded in culture flasks, and were used in the experiments afterwards. These were carried out on monolayer cells or MSCs suspended in a fibrin-PU scaffold. Gene delivery methods included virus-like particles, electroporation, cationic transfection and adenoviral transduction (lathofection). In the chondrogenic experiments the 3D scaffolds were mechanically stimulated in a custom-made bioreactor system.

Cell viability was assayed by calcein-ethidium combined staining, and lactate-dehydrogenase activity assay. Fluorescent Annexin-V-propidium-iodide combined staining was used to indicate possible apoptotic response. Cell number was determined by total DNA amount or MTT assay.

Gene expression data were obtained by real-time RT-PCR. This was used to follow both osteogenic and chondrogenic differentiation.

ALP activity, Ca-45 incorporation and von Kossa staining were used specifically to evaluate osteogenic differentiation. Chondrogenesis was assayed by S-35 incorporation (sulfated GAGs), and total GAG production was measured by DMMB colorimetry. OHP determination was used to measure total collagen production. Immunological methods included ELISA (TGF-β1), Western blot (SOX9) and immunohistochemical detection of aggrecan core protein.

Results

1. Lovastatin and simvastatin increased BMP-2 expression of MSCs. They also enhanced Ca-45 incorporation into their extracellular matrix above 1 μM concentration.
2. However, at these concentrations statins also severely decreased cell number and affected cell morphology, as well as caused apopototic staining of cells.
3. The cell morphology, and especially that of the extracellular calcium deposits suggest that the calcification was ectopic, and was a result of cell death, rather than physiological.
4. AdBMP-2 cell transduction was able to induce osteogenic differentiation in MSCs and to enhance OB function in both human and sheep cells. The recombinant factor was ineffective in both species at 50 ng/ml concentration.
5. ε-aminocaproic acid was shown to be a feasible fibrin degradation inhibitor for cartilage tissue engineering studies, because it did not interfere with the standard chondrogenic process.
6. Transfection methods (VLPs, electroporation and cationic transfection) proved to be less effective compared to adenoviral transduction on human MSCs. Only with enhanced adenoviral transduction (lanthofection) were we able to reach adequately high transduction efficiency.
7. SOX9 was able to initialize the chondrogenic differentiation of human MSCs in the absence of exogenous chondrogenic factors and dexamethasone *in vitro.*
8. Several chondrogenic genes were influenced by SOX9 in these cells, including well-known target genes (COL2A1, ACAN, COMP), but also lesser-known targets such as L-SOX5 and SOX6. We also pointed out the presence of putative SOX9 binding sites in the promoter region of these genes.
9. Mechanical stimulation was shown to enhance the expression of several other markers (PRG4, COMP, COL10A1)
10. The combination of the two treatments synergistically increased the production rate of sulfated GAGs.
11. This may be the result of increased TGF-β1 protein production, as it correlated positively with the GAG production rate, and other publications from our group

conclusively prove the major role of this factor in the mechanotransduction of MSCs.

Conclusions

In these studies we have investigated potential application of MSCs in tissue engineering of two closely related mesenchymal tissues: bone and cartilage. One of the great challenges in today's tissue engineering is the proper differentiation of the competent stem cells. This is not only difficult to achieve, but also, it should be done in a way that can later be translated into a clinical application.

Our investigations revealed that simvastatin and lovastatin both increased the expression of an osteogenic marker BMP-2, but at the same time, caused apoptosis. This suggests that the increased BMD observed in some clinical studies may be a result of decreased bone resorption rather than increased synthesis. Alternatively, the overall *in vivo* effects may be different to that seen *in vitro* due to the presence of many different cell types.

Differentiation factors can also be synthesized by the cells themselves. This is what we investigated in the next part of the study. We found that human and sheep cells reacted very similarly, both were irresponsive to 50 ng/ml rhBMP-2 and differentiated into OBs using the adenoviral transduction. This tells us that there are other causes behind the failure of gene therapy *in vivo*, for example strong immune response to the vector.

Finally, we tested SOX9 gene delivery in order to stimulate the chondrogenic differentiation of MSCs. The overall outcome suggests that this transcription factor alone is not able to fully differentiate MSCs into chondrocytes, and additional stimulation is necessary.

Overall, the gene delivery approach seems to be the most effective and least harmful way to direct MSC differentiation, however further refinement of its methods and *in vitro* cell culture environment may be necessary to utilize its full potential for cartilage tissue engineering.

Bibliography

Egermann M, Lill CA, Griesbeck K*, et al.* Effect of BMP-2 gene transfer on bone healing in sheep. Gene Ther 2006; 13 (17):1290-9.

Ghosh PM, Mott GE, Ghosh-Choudhury N*, et al.* Lovastatin induces apoptosis by inhibiting mitotic and post-mitotic events in cultured mesangial cells. Biochim Biophys Acta 1997; 1359 (1):13-24.

Maeda T, Matsunuma A, Kurahashi I*, et al.* Induction of osteoblast differentiation indices by statins in MC3T3-E1 cells. J Cell Biochem 2004; 92 (3):458-71.

Mundy G, Garrett R, Harris S*, et al.* Stimulation of bone formation in vitro and in rodents by statins. Science 1999; 286 (5446):1946-9.

Okubo Y, Reddi AH. Thyroxine downregulates Sox9 and promotes chondrocyte hypertrophy. Biochem Biophys Res Commun 2003; 306 (1):186-90.

Vamvakopoulos JE, Green C. HMG-CoA reductase inhibition aborts functional differentiation and triggers apoptosis in cultured primary human monocytes: a potential mechanism of statin-mediated vasculoprotection. BMC Cardiovasc Disord 2003; 3:6.

Publications

Grad S, Kupcsik L, Gorna K, Gogolewski S, Alini M. The use of biodegradable polyurethane scaffolds for cartilage tissue engineering: potential and limitations. Biomaterials 2003; 24 (28):5163-71.

Kupcsik L, Meury T, Flury M, Stoddart M, Alini M. Statin-induced calcification in human mesenchymal stem cells is cell-death related. Journal of Cellular and Molecular Medicine 2008. Epub doi:10.1111/j.1582-4934.2008.00545.x

Kupcsik L, Alini M, Stoddart MJ. Epsilon-Aminocaproic Acid Is a Useful Fibrin Degradation Inhibitor for Cartilage Tissue Engineering. Tissue Eng Part A 2008. Epub doi:10.1089/ten.tea.2008.0400

Li Z, Kupcsik L, Yao SJ, Alini M, Stoddart MJ. Chondrogenesis of Human Bone Marrow Mesenchymal Stem Cells in Fibrin-Polyurethane Composites. Tissue Eng Part A 2008. Epub doi:10.1089/ten.tea.2008.0247

Li Z, Kupcsik L, Yao SJ, Alini M, Stoddart MJ. Mechanical Load Modulates Chondrogenesis of Human Mesenchymal Stem Cells through the TGF-beta Pathway. J Cell Mol Med 2009. Epub doi:10.1111/j.1582-4934.2009.00780.x