Mesenchymal stem cells in skeletal muscle regeneration

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Chapter 8

General discussion

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In recent years, there has been considerable interest in rebuilding missing or damaged human tissues and organs through the applications of bioengineering principles and the use of stem cell and/or gene therapy supplementation technology.
Skeletal muscle is the most prevalent tissue in the body, representing roughly half the total body mass. It is responsible for the body's movements along with changes to the size and shape of internal organs. The frequent damage of skeletal muscle by means of trauma or acquired/genetic diseases makes skeletal muscle a suitable platform for the testing of new regenerative therapies.
Mesenchymal stem cells (MSCs) have attracted interest as candidate therapeutics for regenerative medicine owing to their ability to enhance hematopoietic cell engraftment and their contribution to tissue repair. Encouraging results after the infusion of MSCs has been reported in patients with osteogenesis imperfecta and in animal models of myocardial infarction. However, so far, their efficacy in myoregeneration *in vivo* has been limited.
The aim of the research presented in this thesis was to evaluate the participation of human MSCs in skeletal muscle regeneration *in vivo*. The outcome of this work added basic information improving the understanding of the myoregenerative aptitude of MSCs in a damaged skeletal muscle environment.

Models of myoregeneration

From among many skeletal muscle damage models described in the literature we have selected cardiotoxin (CTX)-induced damage, ischemia-reperfusion and minced muscle transplants to explore the participation of human MSCs in myoregeneration. All three models were established in immunodeficient mice, allowing the study of human cells. CTX-induced damage relates to muscular dystrophies in imitating a cycle of myofiber degeneration and regeneration which involves necrosis and loss of myofibers, cell infiltrates, proliferation of satellite cells and the differentiation of their progeny into skeletal muscle cells. By applying the agent to one specific muscle that can be easily harvested as a whole (i.e. the tibialis anterior muscle [TAM]) we could qualitatively and quantitatively determine the participation of MSCs in skeletal muscle regeneration over time (Chapter 2).
The ischemia-reperfusion of the dorsal skin fold was chosen as another injury model with a different pathogenesis. An important asset of this model is that exposure duration and thereby the degree of damage inflicted can be accurately controlled. Furthermore, the skin possesses multiple cell types derived from all three germ layers (i.e. ectoderm, mesoderm and endoderm) all

of which are subjected to the ischemic injury, so that introduction of MSCs into this environment allows one to study their multi-lineage differentiation capacity. The severity and the repair of pressure ulcers of the skin can be easily measured, allowing evaluation of the influence of the injected cells on ulcer formation and healing (Chapter 4). The lesion is also easy to dissect and to evaluate microscopically.
Both the TAMs and the pressure ulcers may be locally exposed to a high dose of external ionizing radiation without the risk of systemic effects due to damage of vital organs like bone marrow and the intestine. Such radiation may serve to selectively kill the endogenous satellite cells, thus excluding their participation in skeletal muscle regeneration (Chapter 4).
The model of minced muscle grafts was developed to allow studying the interaction of human MSCs with regenerating human muscle *in vivo* in the laboratory. It has been proposed that the cross interaction between human and murine cells may be suboptimal due to differences in the repertoire and structure of cell adhesion molecules and growth factor receptors they express at the cell surface as well as to differences in their response to signalling molecules. Such species differences - if of importance for the interaction between MSCs and regenerating skeletal muscle - would render the results of studies with human MSCs in mouse models impossible to translate to the clinic. Mincing destroys all myofibers, but not the satellite cells and the damage is therefore similar to that caused by CTX. In addition, the resulting small tissue fragments facilitate homogenous mixture with the MSCs. The results of this pilot study are reported in chapter 6. It was found for fresh mouse muscle pieces that the contribution of human MSCs to myoregeneration was much lower than that of mouse MSCs. Interestingly, when mixed with *cryopreserved* mouse muscle, the human MSCs performed as well as the mouse MSCs, which suggests that our CTX model, in which human MSCs are implanted into living mice skeletal muscle, may lead to underestimation of the therapeutic potential of these cells for human muscle diseases. Unfortunately, we have not been able to fully exploit this system due to the very limited supply of healthy human skeletal muscle tissue. However, we strongly feel that the current supply problems are surmountable and that these grafts can become a valuable tool for the study of cellular processes in normal and diseased human skeletal muscles.

MSCs in muscle regeneration

The role of MSCs in the regeneration of damaged tissues has been the subject of many studies. These have revealed several different mechanisms by which MSCs participate in tissue repair. In responds to stimuli in injured tissues, MSCs can differentiate into parenchymal cells and thus serve as a cellular

source for new tissue formation. By secreting bioactive factors, MSCs have also the potential to activate endogenous tissue remodelling.
Differentiation of MSCs along the adipogenic, condrogenic and osteogenic lineages has been unambiguously established. Their differentiation to cells of other tissues is still under debate. Nevertheless, in tissues composed of cells with physiological fusogenic properties like liver and muscle, MSCs can participate in tissue reconstruction through incorporation into the newly formed cellular units by heterotypic cell fusion and the adjustment of their gene expression program to that of the recipient cell.
One of the questions addressed in our studies concerns the mechanism(s) by which MSCs contribute to myoregeneration.
Overall, our results show that following implantation in an environment of skeletal muscle regeneration - independent of the damage model - MSCs participate in myofiber formation. Their contribution increased over time in CTX-injured muscles to reach 5% at 4 months after administration (Chapter 2). As mentioned before this level might turn out to be higher in human patients as a result of full species compatibility.
Morphological evaluation of injured skeletal muscles substantiates cell fusion to be the primary mechanism by which MSCs interact with regenerating endogenous myofibers. MSC nuclei incorporated in murine skeletal muscle fibers can undergo myogenic reprogramming as evident by the expression of human dystrophin and human β-spectrin in at least some of these syncytial structures.
The gradual increase over time of the frequency and size of clustered hybrid myofibers in damaged TAMs suggest that MSCs may acquire characteristics of myogenic precursor cells (Chapter 2).
However, our findings that MSCs do not contribute to any extent to myofiber formation in the absence of ongoing myoregeneration, i.e. in undamaged and irradiated skeletal muscle tissue and in skeletal muscle tissue deprived from satellite cells through cryopreservation) (Chapter 6) may rather imply that MSCs do not transdifferentiate into myogenic precursor cells and cannot substitute for satellite cells.
The observation that repair of muscle tissue supplemented with MSC does not differ from that of control muscles (TAM, Chapter 2 and PCM, Chapter 4) also preclude paracrine signaling of MSCs in these tissue.
The majority of the investigations described in this thesis were carried out with a stock of cultured human MSCs derived from bone marrow of a single donor to minimize variations due to different sources. The literature with which we had to compare our results, however, also contains important data obtained with MSCs derived from synovial membrane or adipose tissue. These studies suggest that some sources are better than others, but a comparison in a single study was not available. Therefore, separate experiments (Chapter 3) were

designed using MSCs collected from three different tissues of the same donor. No substantial differences were found in their myogenic fusion activity *in vitro* or participation in myoregeneration *in vivo*. However, the higher proliferative capacity and easy accessibility of adipose tissue-derived human MSCs make these cells a preferential source for clinical applications.
We extended our study of skeletal muscle regeneration to the panniculus carnosus muscle (PCM) for several reasons. Firstly, this skeletal muscle has an exceptional high rate of turnover/regeneration and secondly, the decubitus model allows the application of ischemia-reperfusion as a different kind of damage for inducing regeneration (Chapter 4). In this model, the MSCs injected into the dermis were found to transdifferentiate into various cell types (i.e. fibroblasts, adipocytes, endothelial cells and myoblasts) early after implantation. Surprisingly, these differentiated human cells were only transiently present in contrast to hybrid myofibers, which persisted for the whole observation period of 60 days. The hybrid myofibers were located only in areas where the MSCs were deposited close to the damaged PCM; there was no migration or spreading of the MSCs as in the CTX model.

Immunogenicity of MSCs

Following initial reports that allogeneic MSCs were not rejected, it is now generally accepted that these observations do not hold true. The sustained repair of tissue damage that requires *in situ* differentiation of MSCs into specialized cell types or their fusion with resident cells has been achieved only with autologous/syngeneic MSCs or in immunocompromised recipients. Similarly, successful use of MSCs as vehicles for the delivery of therapeutics depends on immunocompatible donor-recipient combinations.
In Chapter 7, we confirm the above by showing rejection of human MSCs in immunocompetent mice. This response was found to depend on major histocompatibility (MHC) class I expression on the surface of these MHC class II-negative cells as it could be alleviated by permanent downregulation of the MHC class I surface expression. This was achieved through the use of herpesviral immunoevasins (i.p. US11). *US11*-transduced human MSCs were, however, protected from rejection only in immunocompetent mice that had been depleted of NK cells. This is not unexpected as downregulation of MHC class I surface expression renders human MSCs vulnerable to NK cell recognition and cytolysis. Our findings imply that multiple viral immune evasion proteins are likely required to make human MSCs non-immunogenic and thereby universally transplantable.

Perspectives

Even though many questions still remain unanswered, this thesis provides several new insights into the prospects of MSCs for the treatment of skeletal muscle diseases.

In all three *in vivo* models of myoregeneration the contribution of the MSCs to skeletal muscle repair was at best modest.

Among the properties of MSCs that hold significant promise for their therapeutic use is their ability to multiply in culture for numerous passages allowing the collection and storage of many millions of cells under standard operation procedures. However, it is generally observed that following implantation into syngeneic and/or immune deficient recipients these cells gradually disappear at a much faster rate than can be accounted for by differentiation and incorporation into the repaired tissue. As a result, only a few percent of the transplanted cells remains in the treated tissue after 2-3 weeks. Improvement of post-transplant survival of MSCs has been demonstrated by the use of an extracellular matrix and it might be awarding to explore this approach further as a means into prolong and enhance their therapeutic impact. In addition, more research is needed to parameters and factors that influence the quality/regenerative capacity of MSCs, such as the age of the cell donor and the culture conditions of the cells as well as enhancing factors to be administered during/after implantation.

Our results with the minced muscle model provide preliminary evidence for the existence of a non-immune species incompatibility. The elucidation of this issue deserves high priority and it can be approached along the lines described here.

If the results for human MSCs in a human(-like) skeletal muscle environment are not much better than the ones obtained with human MSCs in the mouse models, focus on the application of other cell types like satellite cells, being the skeletal muscle-specific stem cells, and on pluripotent stem cells may be more rewarding. Some of these cells have been shown to possess very high repopulating capacity, but means have to be developed to improve their very limited systemic distribution before their therapeutic value in generalized muscular diseases can be investigated.

Whatever type of stem cells will eventually emerge as most suitable for enhancing the regeneration of skeletal muscle or for that matter of other tissues as well, it would be desirable to have such cells available in large quantities and to be non-immunogenic for off-the-shelf use. We have demonstrated that transduction with viral immunoevasin genes is a promising approach to the large scale production of universal donor cells.

Perfectioning of antigen downregulation along these lines may also further the clinical exploitation of the immunosuppressive and paracrine effects of MSCs by allowing their repeated administration.