Eur Spine J (2006) 15 (Suppl. 3):S406–S413
DOI 10.1007/s00586-006-0183-z

REVIEW

Regeneration of intervertebral disc by mesenchymal stem cells: potentials, limitations, and future direction

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Received: 21 May 2006 / Revised: 16 June 2006 / Accepted: 21 June 2006 / Published online: 15 July 2006

Abstract Over the past few years, substantial progress has been made in the field of stem cell regeneration of the intervertebral disc. Autogenic mesenchymal stem cells in animal models can arrest intervertebral disc degeneration or even partially regenerate it and the effect is suggested to be dependent on the severity of degeneration. Mesenchymal stem cells (MSCs) are able to escape alloantigen recognition which is an advantage for allogenic transplantation. A number of injectable scaffolds have been described and various methods to pre-modulate MSCs' activity have been tested. In future, work will need to address the use of mesenchymal stem cells in large animal models and the fate of the implanted mesenchymal stem cells, particularly in the long term, in animals. This review examines the state-of-the-art in the field of stem cell regeneration of the intervertebral disc, and critically discusses, with scientific support, the issues involved, before stem cells could be used in human subjects.

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Keywords Mesenchymal stem cells · Intervertebral disc degeneration · Intervertebral disc regeneration · Tissue engineering

Stem cells in orthopaedics

Stem cells are defined as unspecialized cells capable of long-term self-renewal and differentiation into specialized cells. Properties and functions of stem cells have been extensively studied in the development of organisms [33], cancer [56], wound healing [62, 69], and regenerative medicine. In the latter, it has been investigated for tackling complex pathogenic conditions such as neurodegenerative diseases [38], hematopoietic impairment [59], and musculoskeletal degeneration [79, 81]. In the development of organism, the single totipotent cell after fertilization divides and specializes into pluripotent cells, such as embryonic stem cells that are necessary for fetal development. The pluripotent cells then further specialize into multipotent cells that commit into lineages with tissue-specific functions. Cells have been successfully identified in or isolated from embryonic [70], fetal [34, 73], or adult tissues [45] and demonstrated to have stem cell-like properties in vitro and in vivo. The maintenance, survival and activity of these stem cells is suggested to be dependent on the special micro-environmental niche [45], such that uncommitted pluripotent stem cells can be induced to differentiate to form a particular cell type by the nature of the environment. Additionally, the pluripotency of stem cells depends on the source, the method of isolation, and conditions of ex-vivo cell processing.

Adult stem cells

Adult stem cells are the undifferentiated cells in adult tissues that retain differentiation potential to become cell types of their origin. Their use is far less controversial than fetal or embryonic stem cells, as the derivation does not involve cloning. And because of their limited differentiation potential (multipotent), they are less likely to form tumors, although some are thought to be related to certain tumors [56].

Adult stem cells normally have a role in replacing cell loss during normal tissue turnover. Well known examples include haematopoietic stem cells in the bone marrow that replace blood cells, and dermal stem cells in the skin that replace shed skin cells [45]. Adult stem cells are also thought to be important in wound repair [62]. They appear to be 'plastic' as the stem cells derived from one tissue have been demonstrated to trans-differentiate into tissue of another type in vitro or in vivo [29]. There has been increasing sources of multipotent adult stem cells identified ex vivo or in vivo such as skin [78], liver [73], bone marrow [73], brain [21, 85], peripheral blood [59], skeletal muscle [76], cardiac muscle [37] and synovial membranes [14, 15]. In orthopedics, considerable attention has been paid to the use of stem cells in bone marrow, and more recently, adipose tissues [16] for cell-based regenerative medicine.

Mesenchymal stem cell source and property

Bone marrow has been the primary source for two stem cell populations: the hematopoietic stem cells (HSCs) and the stromal mesenchymal stem cells (MSCs). Recent studies suggest that adipose aspirate is also a rich source of MSCs [19], but their property and application in vivo remains to be fully investigated. MSCs are a heterogeneous cell population that has been shown to differentiate into bone, cartilage, fat, and fibrous tissues. In ex vivo culturing conditions, differentiation of MSCs into specific cell types can be guided by applying appropriate growth factors or chemicals [54]. Recent in vivo evidence has demonstrated the therapeutic effect of implanted MSCs in tissue repair [43, 44]. MSCs are able to populate injured tissues through either local implantation or via systemic delivery. For example, MSCs directly transplanted into damaged heart muscles, can generate new muscle cells, while intravenous introduction of MSCs can induce angiogenesis in the heart and enhance the proliferation of existing cardiac vasculature. MSCs can escape the normal process of alloantigen recognition [63], which is considered an advantage in clinical applications that involve allogenic transplantation.

Tissue repair in orthopedics by MSCs

In combination with biological or chemically engineered scaffolds and/or growth factors, MSCs have been used in treating articular cartilage defects in animal models as well as bony defects in both animal models and human trials with satisfactory results [27, 55]. MSCs' injection into the knee joint of a goat model of osteoarthritis has been shown to induce regeneration of the meniscus and arrest the progression of articular destruction [46]. In view of similar characteristics to osteoarthritis [17], the feasibility of using MSCs in arresting or even reversing the degeneration of intervertebral disc has recently been investigated by a number of research groups.

Intervertebral disc degeneration and potential therapeutic approaches

Intervertebral disc degeneration

In general, the intervertebral disc (IVD) is populated by chondrocytes in the end-plates (EP), vacuolated notochordal cells and/or chondrocyte-like cells in the nucleus pulposus (NP), and fibroblast-like cells in the annulus fibrosus (AF). The NP and AF contain abundant extracellular matrix, particularly collagens and proteoglycans, which are present in different proportions in the two structures [82]. In humans, the NP is dominated by chondrocyte-like cells as the IVD matures. It has been postulated that NP cells in adults arise from an invasion of chondrocytes from EP that may have caused the notochordal NP to become fibrocartilagenous NP [32]. The change of NP phenotype is also thought to relate to the diminishing notochondal cells as a result of apoptosis [31]. The mature NP of the IVD becomes similar to hyaline cartilage, expressing markers of chondrocytes such as collagen II, collagen IX, aggrecan, and SOX9 [23, 61, 74, 91].

There is a lack of consensus concerning the cause of degeneration. Whether the degeneration is the result of a loss of mechanical integrity in the disc or the mechanical loading itself being a precipitating factor of the degeneration, is still unresolved [1, 82]. Recent evidence suggests that genetic factors are also important [5, 11, 28, 68, 80]. Nonetheless, degeneration of IVDs has been characterized extensively indicating that degeneration involves changes in disc morphology, composition of ECM, as well as loss of disc cells, proteoglycan and water content. Such changes have been suggested to be the consequence of an up-regulation of inflammatory mediators and metalloproteinases (MMPs)

in the degenerated IVD [9, 22, 86]. The process of degeneration is thought to begin in the NP with a loss of proteoglycans and water; the consequential loss of shock-absorbing capacity thus resulting in the structural failure of the IVD, leading to microfracture, osteophyte formation, disc herniation and possibly pain.

Emerging biological approaches in treating IVD degeneration

The majority of current biological therapies aim to restore proteoglycan level or synthesis within the degenerated IVD, mainly because of the observation that the loss of proteoglycan in the NP is the first event in IVD degeneration. For instance, the use of *SOX9*, *TGFbeta1*, *TIMP1*, and *BMP2* has been shown to preserve the architecture of disc tissue and/or increase collagen and proteoglycan synthesis through adenovirus-mediated gene therapy [48, 53, 83]. On the other hand, treating disc cells or direct intradiscal injection with growth factors OP-1(BMP-7), GDF-5, or intradiscal delivery of adenovirus coupled *LMP-1* expression vector can also elevate the proteoglycan level [3, 30, 40, 77, 89]. Cell therapy is an alternative approach, and the regenerative effects of transplantation of autologous cells, such as mesenchymal stem cells [12, 65, 66, 90], nucleus pulposus cells [50, 52, 84], and cartilaginous chondrocytes [18, 20] into the IVD, have been demonstrated.

MSCs in regeneration of IVD: current pre-clinical studies

Use of autogenic MSCs in retarding disc degeneration

Current MSC studies in adolescent rabbits and rats focus at the fate of the stem cells in normal discs or the consequence of induced disc degeneration. Sakai et al. described the use of MSCs to regenerate the IVD. Using a rabbit model of nucleus aspiration to induce degeneration, MSCs were injected embedded in an atelocollagen matrix. The cells survived over a 4-week period and proteoglycan content was enhanced in the implanted discs [66]. In later studies, implantation of autogenic GFP-tagged MSCs also resulted in preservation of annular structure, re-establishing a disc nucleus positive for glycosaminoglycan and keratin sulfate proteoglycans, as well as partial restoration of disc height and disc hydration [64, 65]. In addition, the authors suggested that the MSCs in the re-established nucleus had differentiated into a chondrocyte-like/nucleus pulposus cell phenotype expressing collagen II, keratin sulfate, and chondroitin-4-sulfate [65]. In conclusion, although autogenic MSC implantation could not fully regenerate the disc, it could indeed overcome and counter the degeneration process to some extent. To enhance the full potential of MSC therapy, perhaps other factors such as mechanical stimulus, efficient removal of degeneration by-products, or inactivation of the degeneration precipitating factors need to be considered. Most importantly, the enhancement may perhaps simply require MSCs to have appropriately differentiated into ''true'' disc cells, or be efficient enough to act as helper to induce endogenous disc cell proliferation and differentiation, which has not been sufficiently evaluated in animal models to date.

Potential of allogenic MSCs in IVD regeneration

Extending the concept of stem cell therapy further, investigators have exploited the use of allogenic stem cells. This has the added advantage of off-the-shelf availability. Moreover, as the cause of disc degeneration is thought to be multifactorial, the use of allogenic stem cells could eliminate potential autogenic precipitating factors such as genetic predisposition [2, 7, 28, 51, 71], or the diminished potency of stem cells due to natural aging [72]. In fact, IVD is suggested to be immune-privileged due to its avascular nature. A study, showing allogenic nucleus pulposus cell transplantation did not elicit lymphocyte infiltration, is consistent with this notion [50]. The problem of immune rejection is likely to be even less for allogenic MSCs, since MSCs are capable of escaping alloantigen recognition [41, 63].

Allogenic MSC transplantation has been attempted in normal rabbit lumber IVD, with LacZ-tagged MSCs surviving in the nucleus pulposus for 6 months producing proteoglycan and collagen II, suggesting that allogenic MSCs have similar regeneration potentials as autogenic cells [90]. Allogenic transplantation has also been investigated in normal coccygeal IVD of adult rats [13]. When transplanted in a hyaluronan gel scaffold, bone marrow MSCs survived in the nucleus pulposus over a 4-week period [13]. Thus the potential of allogenic stem cells is worthy of further investigations using longer time points and larger animal models.

Pre-modulated MSCs for IVD regeneration

Cells in the nucleus pulposus (NP) are heterogeneous, comprising of notochordal cells and chondrocyte-like nucleus pulposus cells. The origin, function and characteristics of NP cells, or the signals required for NP

cell differentiation are still not clear. It has been proposed that notochordal cells in the nucleus pulposus act as 'organizers' or behave as stem cells for IVD maintenance and repair [26]. Thus, there is a need to investigate potential notochordal or NP cell signals for IVD regeneration. However, a reliable source of notochordal or NP cells is still elusive.

The pre-loading of biological signals to MSCs prior to implantation, to ''direct'' MSCs or host cell differentiation has been tested to enhance stem cell therapy [43, 49, 88]. Studies have attempted to stimulate MSCs to differentiate into chondrocyte-like or NP-like cells using cytokines or genes prior to implantation. For instance, pre-conditioned human MSCs carrying a *SOX9*-expressing adenovirus seeded into synthetic PLLA (poly-lactic acid) polymer scaffold resulted in strong and sustained induction of aggrecan and collagen II expression over a 4-week culture period [57]. In another study, MSCs cultured in alginate when stimulated with TGFβ1 under hypoxic conditions showed upregulation of SOX9, collagen II, integrins and proteoglycans. The hypoxia responsive gene, *HIF-1a*, was also upregulated, suggesting MSCs may have differentiated into a phenotype consistent to that of NP cells [60]. The importance of TGFb signaling was again highlighted when human MSCs' spheroid cultures were stimulated with TGFβ3 [75]. Microarray analyses of the gene expression profiles were more consistent with annulus fibrosus cells of the IVD than a chondrocyte profile [75].

Besides cytokines, it is known that co-culturing with differentiated cells can determine the fate of MSCs. Adipose-derived rabbit MSCs can activate chondrocytic markers such as collagen II and aggrecan by NP tissues through soluble mediators [39]. Also, the activation of MSCs' differentiation by IVD cell stimulation through direct cell-cell contact in co-cultures of human MSCs and NP cells can better induce upregulation of chondrocyte-like markers, and is dependent on the ratio of the two cell types [58]. Conversely, MSCs can also stimulate proliferation and proteoglycan synthesis of NP cells through cell-cell contact [87]. Thus, the priming of MSCs with disc cells in co-cultures, or in the use of cytokines, morphogens and transcription factors, have the potential to enhance the efficacy of IVD regeneration using MSCs.

Stage of degeneration and regenerative potential

It has always been assumed that regenerative therapies, be it cell-based or molecular-based is most effective at the early stages of degeneration, when the anatomical derangements are relatively minor. However, this assumption has not been investigated scientifically, and recent preliminary evidence in a rabbit model suggests that MSC therapy may be more effective in the later stages of degeneration [12]. While the reason for this is not known, this finding is worthy of further investigation, as this has significant implications for future clinical trials and the choice of patients for therapy.

Choice of scaffold

Like tissue engineering, the choice of scaffolds for stem cell therapy is also thought to be important for cell engraftment. Scaffolds provide a provisional matrix in a three-dimensional microenvironment, to localize cells for cellular and molecular interactions appropriate for cell survival and differentiation. The importance of scaffold is highlighted in a comparison of implantation using intact NP and isolated NP cells, with findings indicating that the inclusion of extracellular matrix of the nucleus is more critical than the number of cells to be implanted for arresting IVD degeneration, using a rabbit model [50]. Therefore, a well-chosen scaffold may be important for effective regenerative cell therapy.

To date, the choice of a scaffold, either natural or synthetic, is numerous with a trend towards designing nano-scale dimension scaffolds. The natures and properties of various scaffolds for orthopaedics has been reviewed [4, 35] and will not be discussed here, in detail. In brief, to choose a scaffold for cell implantation into the IVD, a number of parameters should be considered: (1) immunogenicity, (2) architectural and mechanical properties, (3) biocompatibility and biodegradability, and (4) method of graft delivery.

Given that IVD degeneration may be related to inflammatory response such as osteoarthritis, the choice and design of scaffold should aim at minimizing immunogenicity to avoid further degenerative damages. It should not be toxic to the disc cells such as extreme pH that could induce necrotic or apoptotic responses. An appropriate microscopic architecture of the scaffold is vital, allowing cell attachment and migration for promoting graft integration into host tissue. In particular, when considering the confined avascular nature of the IVD, the geometry and porosity need to be considered for appropriate diffusion of solutes such as nutrients and signaling molecules. On the other hand, they should be biodegradable, allowing replacement with an endogenous matrix as the disc regenerates. Finally, IVD is a highly-compressed structure, sensitive to physical disturbance and may result in degeneration even subject

to the slightest damage. Thus, injectable scaffolds are preferable for IVD engineering, allowing needle-guided graft delivery to minimize trauma of the disc to be regenerated.

Several scaffolds have been tested and shown to be effective as supports for cell implantation into IVD. Fibrin gel has been used as a scaffold [6] that simulates the coagulation cascade to form fibrin matrix through fibrinogen and thrombin [42]. Other studies have used collagen gels [64, 66, 67], hyaluronan gel [13] and genipin cross-linked chitosan [47]. The established MSC-based regeneration models are expected to provide a useful tool for further investigation of the compatibility of MSCs with these and other types of scaffolds in vivo.

Discussion and perspectives

MSCs applications have been highlighted as a potential therapeutic option in regenerative medicine. Current findings are encouraging showing the potential of autogenic and allogenic MSCs to arrest IVD degeneration or even partial regeneration in various animal models. However, it is clear that this technology is still in its infancy and further developments are required while many issues await clarification.

Firstly, a clear understanding of the etiology of disc degeneration is of utmost importance, since the presence of endogenous disposing factors may render any therapy ineffective or only temporary. Secondly, at present, not a lot is known about the cellular and molecular changes in the IVD with aging and degeneration. Thus aiming to fully regenerate the disc in a middle-aged individual, may not be physiologically appropriate, as the majority within the ''normal'' population may have some age-related degeneration and molecular changes at this stage. Thirdly, while current therapeutic attempts are aiming to replace or rejuvenate NP cells, the precise phenotype or characteristics of a NP cell is not known. Although they are chondrocyte-like, they do not behave in the same way as chondrocytes. As there are currently no specific markers for NP cells, it is not known whether MSCs can indeed differentiate into NP cells. Finally, current evidence for efficacy comes from animal degeneration models in which the degeneration is traumatically induced by needle puncture. The relevance of such models of disc degeneration in human subjects is not known, and the issue of choice of animal model is thus of importance.

Many of the small animal models such as rabbits and rats currently used are physiologically different from human IVD both in terms of mechanical loading and cellular composition. They appear to have a larger number of notochordal cells that persist through adult life, while in humans, notochordal cells rapidly disappear after birth. Larger animal models such as goats or primates could be considered, as their NP structure and mechanical loading are more similar to humans. However, these models are expensive and limited molecular tools are available for scientific investigations. Thus, the authors propose that small animal models can serve as the driving force for the initial studies, with a wealth of biological molecular reagents and probes to study cell fate. The availability of high resolution in vivo imaging of rodents such as high magnetic field MRI and bioluminescence imaging are emerging, that provide non-invasive and continuous assessment of the regenerative process [10, 25, 36, 40]. Before human clinical trials, there should be an intermediate step in a large animal model to determine efficacy. Thus the choice of animal models is dependent on the the biological question being asked.

It is also worth noting that the survival of cells in the nucleus pulposus has been suggested to be governed by the diffusion of metabolites and hence the distance of NP cells from the endplates [8, 24]. Therefore, it is possible that different diffusion distances between large and small animal models may result in different outcomes of the cell therapy. In particular, nutritional supply may be a particular problem in large animals' and humans' IVDs due to the larger diffusion distances.

Based on the potential and possible limitations of using MSCs in IVD regeneration, future work shall be focused on the ways of optimizing the efficacy as well as delineating the biological process involved. For instance, the optimal cell number and choice of scaffold can be investigated by systematic comparison. Determining the window of degenerative stage for the best efficacy is also important for defining the subjects in future human trials. It is also worth investigating whether allogenic transplantation is able to rejuvenate the degenerated discs to an extent similar to autogenic transplantation, as allogenic application is more clinically applicable because of easy shelf availability. In addition, previous studies have been carried out on small animals, the efficacy of regeneration awaits to be tested on larger animal models. On the other hand, understanding the biological process underlying the regenerative effect is vital to the design the future strategies for efficacy improvement. The fate of MSCs should be carefully tracked after implantation, with special attention paid to the cell phenotype, induced functions, and long-term survival (> 1 year) of MSCs.

Yet to investigate whether MSCs have themselves differentiated or have induced the differentiation of other cells into true disc cells, the authentic phenotype and molecular signatures of disc cells need to be unambiguously defined.

In conclusion, although considerable progress has been made in the field of stem cell therapy for intervertebral disc regeneration, there are still many questions and challenges that need to be addressed, before stem cell therapy can be recommended as a treatment option in IVD degeneration.

Acknowledgments The work described in this paper was partially supported by a grant from the University Grants Committee of the Hong Kong Special Administrative Region of China (HKU7496/05M).

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