The differentiation of bone marrow mesenchymal stem cells into chondrocyte-like cells on poly-l-lactic acid (PLLA) scaffolds

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Abstract

While intervertebral disc (IVD) degeneration is associated with the majority of cases of low back pain, current treatments are symptomatic rather than curative. Tissue engineering offers a treatment that both cures the problem of disc degeneration and restores normal disc function. One of the major problems for any tissue engineering strategy, however, is ensuring that both the cells and matrices used are suitable for the target tissue. In this study, we have developed and studied a potential system for tissue engineering of the nucleus pulposus (NP) of the severely degenerate IVD. While cells from degenerate discs are not suitable for tissue engineering, bone-marrow-derived mesenchymal stem cells, which are capable of differentiating into chondrocyte-like cells such as those found within the NP of the disc, offer a potential source of cells. We have used transfection with adenoviral SOX-9, a transcription factor involved in differentiation of MSCs along the chondrogenic lineage, combined with culture in a specialised medium, to differentiate monolayer MSCs to NP-like (chondrocyte-like) cells, as shown by real-time quantitative polymerase chain reaction for NP-marker genes. We have also replicated these findings on porous, biodegradable three-dimensional (3D) poly-l-lactic acid scaffolds and shown expression and deposition of NP matrix markers such as type II collagen and aggrecan. We are therefore proposing predifferentiation of human MSCs and seeding on porous, biodegradable 3D synthetic polymer scaffolds as a realistic tissue engineering strategy for regeneration of the degenerate human IVD.

Keywords: Intervertebral disc; Mesenchymal stem cell; Polylactic acid; Confocal microscopy; PCR; TGF

1. Introduction

There has been much effort recently to identify a suitable method for tissue engineering of cartilaginous tissues, such as articular cartilage and intervertebral disc (IVD). This interest has arisen due to the lack of a satisfactory surgical interventions and the inability of symptomatic interventions to treat the root cause of the problem. While a number of different approaches have been published recently there are still two major issues that need to be tackled with any tissue engineering approach—the choice of a suitable scaffold and the choice of a suitable cell population.

The majority of work has concentrated on tissue engineering of articular cartilage using biodegradable scaffolds and hydrogels. These have included a variety of natural gels and hydrogels based on collagen, glycosaminoglycans, hyaluronic acid, agarose, gelatin and alginate [1–6]; as well as a variety of synthetic scaffolds which are based on polymers such as polylactic acid (PLA), polyglycolic acid (PGA), polyvinyl alcohol (PVA) and polyurethanes [7–13]. More recently investigators have proposed that calcium phosphate scaffolds may be useful...
for tissue engineering of the IVD [14] and polymer–alginate composites have been suggested as hybrid gel/scaffolds for tissue engineering of both cartilage and IVD [15,16].

The wide range of properties displayed by these different materials means that selection of the correct material is vital for a successful strategy. While culture in 3D gels such as alginate allows chondrocytes to retain their phenotype their lack of structural integrity make them unsuitable as a tissue engineering scaffold without being combined with a more rigid structure [15,16]. However, while a rigid scaffold structure is required to withstand the loads experienced within the articulating joint or the IVD (where loads can reach 4 MPa), not all polymer-based scaffolds are suitable for culture and differentiation of cells with a chondrocytic phenotype. For example, while PGA and PLGA polymers induce proteoglycan synthesis by chondrocytes [10], the co-polymer polyD,L-lactide-co-glycolide (PLGA) promotes osteoblastic cell attachment and differentiation over that seen on PLA scaffolds [17]. Poly-L-lactic acid (PLLA) has been found to offer the best compromise of mechanical stability and degradation rate and Rahman and Tsuchiya [8] demonstrated that chondrocyte redifferentiation was significantly improved in the presence of PLGA (as demonstrated by an increase in proteoglycans). Furthermore, long-term in vivo studies have shown that, unlike the rapidly degraded PGA and PLGA polymers, PLLA does not produce a significant inflammatory response and that in vivo degradation rates are similar to those shown in vitro [18].

The IVD is comprised of a central, gelatinous nucleus pulposus (NP), surrounded by a fibrous annulus fibrosus (AF), with cartilaginous end-plates on the superior and inferior surfaces [19]. As with articular cartilage, the mechanical properties of the IVD are dependant on the organisation of the extra-cellular matrix, which is controlled by phenotypically distinct populations of cells. Cells within the NP are chondrocyte-like, expressing a matrix of predominantly aggrecan and type II collagen molecules [20]. During degeneration there is an alteration in this extra-cellular matrix, with decreases in both aggrecan and type II collagen and an increase in type I collagen, leading to a more fibrous tissue and a reduction in hydration of the NP. Alongside these changes, fissures form in the NP, extending to the inner AF and this eventually leads to a loss of disc height and mechanical instability. In a large number of cases this instability is associated with neovascularisation and innervation of the tissue which leads to pain [21–25].

As with cartilage tissue engineering strategies a major problem for tissue engineering of the IVD is generating a large number of easily accessible cells with a normal phenotype that can be seeded onto scaffolds. Autologous cells would potentially be the most suitable choice, however both NP and AF tissues are relatively hypocellular (4000 and 9000 cells/mm², respectively, in normal tissue [26]) and therefore a large amount of tissue would need to be removed to recover a significant number of cells. However, since removal of tissue from degenerate discs has been shown to accelerate degeneration and removal of tissue from a normal disc is likely to induce degeneration [27], this option is not feasible. Furthermore, cells from degenerate IVD have an altered phenotype and increased senescence [20,28–31]. Thus expanding these cells in vitro to a number usable for tissue engineering would result in cells that could not be classified as ‘normal’. In addition, the use of allogenic cells raises issues of immunogenicity and disease transmission. As NP cells have a chondrocyte-like phenotype [20], we have explored the use of adult human bone marrow mesenchymal stromal cells (MSC) which are known to have the potential to differentiate along all connective tissue cell lineages [32] and thus could be used for autologous transplantation.

One of the key regulators of MSC differentiation to chondrocyte-like cells is the transcription factor SOX-9 [33–35]. Expression of SOX-9 regulates both maintenance of chondrocyte phenotype and expression of the type II collagen gene (Col2a1) [36,37] and studies have shown that overexpression of the SOX-9 gene can improve redifferentiation of osteoarthritic chondrocytes in both pellet and alginate cultures [38,39]. Recently Sive et al. [20] demonstrated its expression in NP cells of the adult IVD, showing a chondrocyte-like phenotype for these cells. This expression of SOX-9 in NP cells suggests that it may be possible to induce differentiation of MSCs to cells with an NP-like/ chondrocyte-like phenotype by upregulation of SOX-9 expression.

While MSCs have been shown to differentiate into chondrocyte-like cells in both alginate and pellet cultures [6,40], these cells are not easily retrieved from the matrix they synthesise. By combining optimised monolayer culture conditions and adenoviral transfection of SOX-9 to differentiate MSCs in vitro to NP-like (chondrocyte-like) cells we hope to develop a system which allows rapid differentiation and easy cell retrieval for seeding onto tissue engineering scaffolds such as PLLA scaffolds. Our aim was then to assess both the differentiation state and matrix production by scaffold-seeded cells to evaluate the feasibility of the strategy for tissue engineering of the IVD.

2. Materials and methods

2.1. Cell source and culture

To obtain human mesenchymal stromal stem cells, bone marrow was obtained from proximal femur samples removed during hip replacement surgery on a 62-year old male. The patient gave informed consent and local Ethical Committee approval was obtained for the use of the sample for research. Mononucleated cells were isolated using a Histopaque-1077 (Sigma) density gradient method [41]. These cells were cultured in a 75 cm² flask with minimum essential medium, α-modification (αMEM), supplemented with 10% heat-inactivated foetal calf serum (FCS), penicillin, streptomycin, amphotericin B, ascorbate and glucose at 37 °C in a humid atmosphere containing 5% CO₂. After 7 days, non-adherent cells were discarded and adherent cells were cultured to confluence, with medium changed every 3 days.
For differentiation studies, cells were cultured in either a standard medium (SM) or a chondrogenic medium (CM). SM consisted of Dulbecco modified Eagles medium, F12-modification, supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 250 ng/ml amphotericin B, 25 μg/ml ascorbate and 2 mM L-glutamine.

For cultures where CM was used, this contained Dulbecco modified Eagles medium, F12-modification, supplemented with 1.25 mg/ml bovine serum albumin (BSA), 100 U/ml penicillin, 100 μg/ml streptomycin, 250 ng/ml amphotericin B, 25 μg/ml ascorbate, 2 mM L-glutamine, 1 x 10^{-5} M dexamethasone, 10 ng/ml TGF-β1, 100 μg/ml sodium pyruvate, 40 μg/ml proline and ITS-X (10 μg/ml insulin, 6.7 μg/ml sodium selenite, 5.5 μg/ml transferrin and 2 ng/ml ethanamine; Gibco).

2.2. Adenoviral transfection of monolayer MSCs with SOX-9

At confluence MSCs from passage 3 were trypsinised and seeded into 25 cm² flasks in duplicate at a density of 5000 cells/cm². Following overnight culture, cells were washed with phosphate-buffered saline (PBS) and a pre-optimised multiplicity of infection (MOI) of 200 in 5 ml of SM per flask. For control flasks, 5 ml of SM without virus was added. Cells were cultured overnight, the medium was removed from each flask and replaced with either SM or CM. Cells were then cultured for 1, 7 and 14 days with medium replaced every 2 days.

2.3. Real-time PCR analysis of gene expression

At each time-point, cells were rinsed in PBS and cells lysed in TRizol (Invitrogen). Total RNA was extracted following manufacturers’ instructions. Briefly, chloroform was added to each sample and sample tubes centrifuged to enable phase separation. RNA was precipitated by addition of isopropanol to the aqueous phase, followed by centrifugation. Precipitated RNA pellets were washed in 75% ethanol and then resuspended in distilled RNAse-free water.

cDNA was prepared from RNA using Superscript II RT (Invitrogen). RNA (1 μg) was mixed with random prime hexamers (250 ng) and dNTPs (500 μm each dNTP) then incubated at 65 °C for 5 min. Tubes were cooled on ice, then 5 x 1 μl first strand buffer, 0.1 μl DTT, 0.4 U RNAseOUT RNase-inhibitor (Invitrogen) and 200 U reverse transcriptase were added, giving a final volume of 20 μl. Samples were then incubated at 42 °C for 90 min and finally heated to 70 °C for 15 min.

Gene expression was analysed by real-time PCR using an ABI Prism 7000 sequence detection system (Applied Biosystems). Housekeeping genes 18S and glyceraldehyde-6-phosphate dehydrogenase (GAPDH) primer/probe sets were predesigned and purchased from Applied Biosystems. Primers for SOX-9, type I collagen, type II collagen and aggregan were designed using the Applied Biosystems Primer Express software or derived from Martin et al. [42] (Table 1).

Reactions were carried out in duplicate in 96-well plates in a final volume of 25 μl. For the housekeeping genes the reaction mix contained 12.5 μl mastermix (Applied Biosystems), 9.25 μl RNAse-free water, 1.25 μl primer/probe mix and 2.5 μl cDNA. For target genes the reaction mix contained 12.5 μl SYBR green mastermix (Applied Biosystems), 5.5 μl water, 2.5 μl forward primer (900 nm), 2.5 μl reverse primer (900 nm) and 2 μl cDNA.

The PCR reaction consisted of an initial enzyme activation step at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. A cycle threshold value (Ct) value was obtained for each sample and duplicate sample values were averaged. The 2^{-ΔΔCt} method was then used to calculate relative expression of each target gene [43]. Briefly, mean Ct value of target genes in each sample was normalised to its averaged housekeeping gene Ct value (the average of the mean 18S and mean GAPDH values [44]) to give a ΔCt value. This was then normalised to control samples (ΔΔCt) and the 2^{-ΔΔCt} value obtained.

2.4. Seeding of PLLA scaffolds with MSCs

PLLA scaffolds were seeded in triplicate with untransfected or SOX-9 adenovirus transfected MSCs and cultured in either SM or CM. Cells were transfected in monolayer for 24-h before the start of the experiment, as described earlier. PLLA scaffolds were formed into 10 mm diameter x 3 mm thick discs by solvent casting and particulate leaching using NaCl particles sized 125–212 μm as the porogen [44–48]. The pore size distribution of the PLLA sponges was determined by mercury intrusion porosimetry using a Micromeritics Autopore IV 9500 porosimeter. The median pore diameter was 62.44 μm and the porosity was 90.4%. This scaffold porosity provided rapid access for the cells and media to all areas of the scaffolds, whilst maintaining structural integrity for the duration of the culture periods. Each scaffold was adhered to the well of a 24-well plate using silicone adhesive (NuSil) and prewetted with 2 ml SM for 1 h.

MSCs (untransfected and transfected) were trypsinised and resuspended at a concentration of 1 x 10^{4} cells/ml in SM. Medium was removed from the scaffolds and aliquots containing 5 x 10^{4} cells/ml were pipetted onto each scaffold. Scaffolds were then incubated at 37 °C for 1 h to allow cell attachment and then 2 ml of either SM or CM was added to the appropriate wells. Scaffolds were cultured for 1 and 4 weeks with medium changed every 2 days. At each time point samples were taken for real-time PCR analysis, western blotting and confocal microscopy.

Samples for real-time PCR were placed in 1 ml TRIzol and vortexed to disrupt the scaffold and lysed the cells, then frozen at −70 °C. RNA was extracted, reverse transcribed and used in real-time PCR for 18S, GAPDH, SOX-9, type I collagen, type II collagen and aggregan, as described earlier.

Table 1

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequences forward and reverse 5’→3’</th>
<th>Amplicon size (bp)</th>
<th>Genbank accession number</th>
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<tr>
<td>SOX-9</td>
<td>GACCTCCCGGCACTGGGAC CAGTACCTGCGCCCAAC</td>
<td>99</td>
<td>Z46629</td>
</tr>
<tr>
<td>Type I collagen (COL1A1)</td>
<td>CACGCGCTTCATCTACACG TTTTGATCCATCACGTTGC</td>
<td>83</td>
<td>*</td>
</tr>
<tr>
<td>Type II collagen (COL2A1)</td>
<td>GCTATGATCATGTCTACGTA CAGGAACTCGTGGCCGACCTT</td>
<td>79</td>
<td>*</td>
</tr>
<tr>
<td>Aggrecan</td>
<td>TCGAGGACAGCGAGGCC TCGGAGGTGTAGGCTAGA</td>
<td>85</td>
<td>*</td>
</tr>
</tbody>
</table>

Accession number given for primer designed in-house using Primer Express 2.0 software. Remaining primers (*) taken from Martin et al. [42].
2.5. Western blotting

Scaffolds were incubated with 1 ml 4M guanidine chloride (GuCl) at 4°C for 24 h. The GuCl extracted material was recovered by centrifugation and the scaffold removed to a fresh tube and washed twice with distilled water. Extract and washes were dialysed against 50 mM acetic acid for 24 h at 4°C and lyophilised. The lyophilised GuCl- and pepsin-extracted dialysates were resuspended in 100 and 200 μl of sample buffer, respectively, and analysed by electrophoresis on 7% (w/v) acrylamide SDS-PAGE gels; human type I and type II collagen standards were resolved alongside the samples. Resolved proteins were electroblotted onto PVDF membrane (Whatman) and blots were incubated with either a monoclonal antibody raised to the α1 chain of human type II collagen (AVT6E3) or a goat anti-bovine type I collagen, known to cross-react with human type I collagen (a generous gift from Professor Vic Duance). Subsequently, blots were incubated with horseradish peroxidase conjugated anti-species IgG antibodies and binding was detected on film using enhanced chemiluminescence (Amersham Pharmacia).

2.6. Immunohistochemistry and confocal microscopy

Scaffolds were stained immunohistochemically for collagen types I and II and aggrecan with an F-actin counterstain. Scaffolds were washed in PBS for 3 min, then stained with 3 μg/ml Hoechst 33342 (Molecular Probes) in PBS for 30 min at 37°C. Samples were then rinsed in PBS and fixed in 4% paraformaldehyde/2% sucrose for 15 min at 37°C. After a 5 min wash in PBS, scaffolds were permeabilised in 5% Triton-X-100/PBS for 5 min at 4°C, rinsed in PBS and then cut into four equal pieces to allow target staining. Staining was conducted in the dark wherever possible to maintain fluorescence intensity. Each sample was stained for 1 h at 37°C with the following antibodies: type I collagen (I-8H5, ICN; 1:100 dilution in 1% BSA in PBS), type II collagen (CHC1, Developmental Studies Hybridoma Bank; diluted 1:50) and aggrecan (HAG7E1, Abcam; diluted 1:100). Following incubation with primary antibody, samples were washed twice in 1% (v/v) Tween-20 solution for 3 min each, prior to addition of a rhodamine-conjugated goat ant-mouse secondary antibody (ICN; 1:20 dilution) for 1 h at 37°C. Samples were washed 2 × 3 min in 1% Tween-20 and for F-actin counterstaining a 1:40 dilution of Oregon green 488 phalloidin (Molecular Probes) was added and incubated for 30 min at 4°C. Slides were finally rinsed in PBS, air dried and analysed. Analysis was performed on a Zeiss 510 Laser scanning confocal microscope.

3. Results

3.1. Gene expression in monolayer MSCs

Real-time PCR was used to investigate the expression of SOX-9, collagen types I and II and aggrecan mRNAs by monolayer MSCs, either transfected with adenoviral SOX-9 and/or cultured in CM. Transfection of monolayer MSCs in SM raised SOX-9 mRNA levels almost 3000-fold after 24 h, while transfected MSCs in CM showed a 3500-fold increase. By day 14 levels of SOX-9 mRNA in SM-cultured cells were 300-fold higher than controls, but expression in CM-cultured cells was maintained at around 3200-fold higher. Monolayer MSCs cultured in CM alone showed only slight changes in SOX-9 mRNA expression over controls (Fig. 1A).

Expression of type II collagen mRNA by SOX-9 transfected MSCs increased over the 14 days to around...
23-fold higher than controls, while culture of transfected MSCs in CM caused a 2000-fold increase over controls. Cells cultured in CM alone showed only slight changes from controls (Fig. 1B).

When SOX-9 transfected MSCs were cultured in either SM or CM for 1, 7 or 14 days aggrecan mRNA showed less than a 10-fold change, however when untransfected MSCs were cultured in CM aggrecan mRNA expression decreased almost 100-fold by day 14, compared to controls (Fig. 1C).

Type I collagen mRNA expression showed only minor changes from controls, with the largest increase seen at 14 days in cells cultured in CM (7.4-fold). Cells transfected with SOX-9 and cultured in either SM or CM showed initial decreases in type I collagen mRNA expression, followed by an increase to slightly above control levels (Fig. 1D).

3.2. Gene expression in MSCs seeded on PLLA scaffolds

Real-time PCR showed that in SOX-9 transfected samples, SOX-9 mRNA expression was increased over controls almost 350-fold, while the addition of CM to SOX-9 transfected cells caused an increase of almost 3700-fold over controls. By week 4, expression in SOX-9 transfected samples was around 60 times higher than controls, but over 21,000 times higher where SOX-9 transfected cells were supplemented with CM. Culture in CM alone, however, only caused a 23-fold increase in SOX-9 mRNA expression over controls by week 4 (Fig. 2A).

By week 4, type II collagen (COL2A1) mRNA expression was increased by 130-fold in samples cultured in CM and 300-fold in SOX-9 transfected samples cultured in CM, however SOX-9 transfection alone caused only a four-fold increase by week 4 (Fig. 2B).

After 4 weeks, PLLA-scaffold seeded MSCs transfected with SOX-9 showed a 43-fold increase in aggrecan mRNA expression over controls, while additional supplementation caused a 725-fold. Interestingly by week 4, culture in CM had caused a 100-fold increase in aggrecan mRNA levels, compared to nearly a 10-fold decrease at 1 week (Fig. 2C).

Type I collagen (COL1A1) mRNA expression appeared to be increased by culture in CM, as by week 4 levels were 10- and 17-fold higher in CM samples and SOX-9+CM samples, respectively, than SM controls. Less than a one-fold change was observed between SOX-9 transfected samples in SM and untransfected controls in SM, suggesting that SOX-9 is not responsible for control of type I collagen mRNA expression (Fig. 2D).

3.3. Western blotting

Western blotting was used to detect collagen types I and II in both control and SOX-9+CM samples at weeks 1 and 4. While type I collagen was present predominantly in the pepsinised extract, type II collagen was present only in the GuCl sample (Fig. 3). Untransfected MSCs cultured on

![Fig. 2. Real-time PCR analysis of gene expression by MSCs cultured on PLLA scaffolds. Untransfected MSCs were either cultured in SM (used for normalisation) or CM (MSC+CM), or SOX-9-transfected and cultured in SM (MSC+SOX-9+SM) or CM (MSC+SOX-9+CM), then mRNA expression levels for SOX-9 (A), type II collagen (B), aggrecan (C) and type I collagen (D) were measured. Expression levels were normalised to housekeeping genes and untransfected controls using the 2^(-ΔΔCt) method.](image-url)
PLLA scaffolds in SM expressed type I collagen protein at both 1 and 4 weeks, with α, β and γ bands being present. In the type II collagen gels, neither the untrasfected MSCs cultured in SM nor the SOX-9 transfected cells cultured in CM showed expression of type II collagen. However by week 4, type II procollagen was detectable in both samples. In the SOX-9+CM samples at week 4, a partially processed pN-intermediate molecule was detectable, although fully processed α-chains, or β-chains were not detectable. The ease of extraction of type II collagen into GuCl and the requirement for pepsin digestion in order to solubilise type I collagen are consistent with the forms of these molecules detected by western blotting.

3.4. Confocal microscopy

Confocal microscopy was used to investigate distribution of collagen types I and II and aggrecan within PLLA scaffolds seeded with MSCs cultured in SM and MSCs transfected with SOX-9 and cultured in CM over 1 and 4 weeks. Type II collagen was virtually undetectable in samples where untransfected MSCs were cultured in SM at 1 week (Fig. 4A), while in samples where MSCs were SOX-9 transfected and cultured in CM expression could be seen in areas of high cell density (Fig. 4B). By week 4 there was some deposition of type II collagen in MSC+SM samples (Fig. 4C), but much more intense and widespread staining was seen in the samples where SOX-9 transfected cells were cultured in CM (Fig. 4D). Again this staining appeared to be predominantly in areas of high cell density, where more rounded cells were present.

Type I collagen showed extensive staining after 1 week and staining intensity did not seem to be affected by culture conditions (Fig. 4E and F). Expression was most prominent around cells closely associated with the PLLA scaffold and type I collagen appeared to be distributed around pores in the scaffold.

While aggrecan was present in control samples, expression was much more widespread in the SOX-9+CM samples after both 1 week (Fig. 4G and H) and 4 weeks (data not shown) and appeared diffusely throughout the matrix.

4. Discussion

The matrix of both the normal NP of the IVD and articular cartilage are comprised of an interwoven network of proteoglycans, predominantly aggrecan, and collagen (particularly type II collagen), with embedded chondrocytic cells. In both osteoarthritis and IVD degeneration there is a change in cell and matrix biology, a disruption of the matrix and a loss of function. In the case of the IVD, degeneration causes dehydration and loss of the hydrophilic properties of the NP matrix (which normally attracts water and gives the disc a high swelling pressure that provides the discs height and functionality), leading to a loss of disc height and eventually to disability and pain.

While a number of novel treatments for repair of OA cartilage have been proposed, current treatments for IVD degeneration are generally symptomatic and are designed to relieve the pain and restore some function to the disc, but without actually treating the problem or repairing the...
disc itself. By using a combination of cells and synthetic or natural biomaterials, tissue engineering offers a technology that will both regenerate the matrix and fully restore normal function to either the IVD or the articulating joint. To do this the scaffold is required to immediately restore function, while the cells seeded onto the scaffold replace the matrix. The scaffold must therefore also allow maintenance of the cells’ phenotype and be fully biodegradable over the time-period required for restoration of the matrix.

A wide range of synthetic polymer-based materials are available, however many have no practical application in the continuously loaded environment of the spine or the articulating joint. As demonstrated by Rahman and Tsuchiya [8], PLLA offers an important combination of mechanical stability and retention of cellular phenotype, in particular improved proteoglycan production by chondrocytes over other polymer scaffolds.

While removal of tissue from the site of injury and subsequent reimplantation of expanded cells may be seen as the method of choice, a number of practical issues make these cells unsuitable. In particular, evidence suggests that the changes that occur in the degenerate disc make the use of degenerate NP cells unfeasible and since the removal of NP tissue from a normal disc is thought to induce degeneration, we have investigated the use of human MSCs derived from bone marrow. While it is known that human MSCs are capable of differentiating into chondrocytic cells, one of the biggest challenges for any tissue engineering strategy is achieving this in an in vitro environment. However, although both alginate bead and pellet cultures have been shown to allow MSC differentiation, neither of these methods lends itself to tissue engineering strategies. Therefore we have studied both the predifferentiation of MSCs in monolayer and seeded onto PLLA scaffolds to investigate the feasibility of such methodologies to repair the degenerate IVD.

4.1. Predifferentiation of monolayer MSCs

In this study, we have shown that adherent monolayer MSCs, which would normally have an undifferentiated, fibroblastic phenotype, can be differentiated into chondrocytic cells through adenoviral transfection with SOX-9 and culture in a CM. Monolayer transfection with SOX-9 and culture in SM caused an upregulation of SOX-9 and type II collagen mRNA expression over a 14-day time-course, but showed very little change in aggrecan and type I collagen mRNA levels. When SOX-9 transfected MSCs were cultured in CM, expression of SOX-9 was maintained at a higher level over the 14 days than by transfected cells cultured in standard medium. Similarly type II collagen mRNA levels were higher by 14 days, but there was again little change in either type I collagen or aggrecan gene expression. This suggests that in monolayer, the combination of SOX-9 transfection and culture in CM is sufficient to initiate differentiation to a chondrocyte-like phenotype, and that a combination of the two treatments induces more of a chondrogenic change than either treatment alone. This method has advantages over current 3D-culture systems, such as pellet or alginate bead culture, in that it allows cells to be used in subsequent tissue engineering applications. Furthermore, overexpression of SOX-9 causes more rapid
differentiation of MSCs than 3D-cultures systems and while addition of growth factors, such as TGF-β, improves this differentiation it is not sufficient alone to induce differentiation. Also, since transfection of dividing, monolayer MSCs with adenoviral SOX-9 is transient, careful control over the level of virus used for the transfection should allow the initiation of differentiation to be performed ex vivo with the viral vector being lost before reimplantation of cells into the body; this would circumvent safety and regulatory issues.

4.2. Predifferentiation of MSCs seeded onto PLLA scaffolds

For MSC differentiation to chondrocyte-like NP cells using SOX-9 transfection and culture in CM to be applicable to a tissue engineering strategy, it was important to ensure that this phenotype was maintained when MSCs were seeded onto PLLA scaffolds. When the same combination of treatments was applied to MSCs seeded on PLLA scaffolds a similar differentiation pattern was observed, with increases in SOX-9 and type II collagen over both controls and either SOX-9 transfected or CM-cultured, untransfected cells.

Importantly, unlike the monolayer culture, aggrecan levels were raised following all three treatments, with the greatest increase again being seen in the combined SOX-9 transfection + CM treated cells by week 4. As aggrecan levels were increased 100-fold in untransfected, CM-cultured cells, this suggests that its expression is regulated by cell morphology, or the presence of a 3D matrix, rather than simply by the over-expression of SOX-9. The increase in aggrecan expression could also be caused by the PLLA scaffolds, which have previously been shown to induce aggrecan synthesis in chondrocytes [8] and could cause similar increases in expression in MSCs, particularly if these cells have been partially differentiated by the over-expression of SOX-9 and culture in CM.

Unlike the other genes examined, type I collagen gene expression levels remained relatively constant throughout. The presence of type I collagen bands on the pepsinised western blot gels and staining throughout the matrix in confocal microscopy samples suggests the deposition of a stable, cross-linked type I collagen matrix, which is not affected by these culture conditions, or over-expression of SOX-9. Importantly, however, the western blotting and confocal microscopy data do show that type II collagen protein is also being synthesised and deposited within the matrix when cells are SOX-9 transfected and cultured in the presence of CM. Furthermore, widespread expression of aggrecan throughout the matrix after 1 week on the scaffold, following transfection with SOX-9 and culture in CM, combined with the increased expression and deposition suggest that it would be possible to form a suitable matrix within a PLLA scaffold with some further manipulation. This increased synthesis and deposition of aggrecan is vital, as any functional tissue engineered construct must mimic the proteoglycan-rich, hydrophilic nature of the normal tissue, which has a proteoglycan:collagen ratio of 27:1 in the case of normal NP and a ratio of 2:1 in the case of native articular cartilage [49].

4.3. Clinical application

For this method to have a viable clinical application as a tissue engineering treatment to repair either osteoarthritic cartilage or the degenerate IVD it is imperative that the correct matrix is deposited within the scaffold. This will require tighter regulation of cell phenotype to prevent deposition of type I collagen and enhance the expression of both type II collagen and proteoglycans, in particular aggrecan. This matrix production may be achieved by addition of alternate growth factors, such as IGF-1 [50] and CDMP [51] that stimulate proteoglycan synthesis. It is also possible that cell–cell interactions, once the scaffolds have been implanted, would ensure that a suitable matrix is produced and this would again improve the repair process. Furthermore, modification of the PLLA surface chemistry or physical characteristics may produce a scaffold that allows predifferentiated MSCs to deposit a matrix which is identical to that seen in normal tissue. If growth or/and transcription factors can be incorporated into scaffolds and released slowly over time it is possible that MSC differentiation and matrix production could be achieved without the use of viral transfection and this would circumvent any potential regulatory issues regarding residual virus following implantation into the body.

5. Conclusions

In conclusion we have shown that using SOX-9 transfection and culture in a specialised medium it is feasible to differentiate human MSCs into chondrocytic cells in vitro and that when these cells are seeded onto PLLA scaffolds they retain their phenotype. Furthermore, when seeded on PLLA scaffolds these cells synthesise and deposit the same matrix molecules as seen in vivo. We have therefore shown, for the first time, that tissue engineering using predifferentiated human MSCs seeded onto biodegradable scaffolds is a feasible clinical possibility.

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