Recent advances in annular pathobiology provide insights into rim-lesion mediated intervertebral disc degeneration and potential new approaches to annular repair strategies

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Abstract The objective of this study was to assess the impact of a landmark annular lesion model on our understanding of the etiopathogenesis of IVD degeneration and to appraise current IVD reparative strategies. A number of studies have utilised the Osti sheep model since its development in 1990. The experimental questions posed at that time are covered in this review, as are significant recent advances in annular repair strategies. The ovine model has provided important spatial and temporal insights into the longitudinal development of annular lesions and how they impact on other discal and paradiscal components such as the NP, cartilaginous end plates, zygapophyseal joints and vertebral bone and blood vessels. Important recent advances have been made in biomatrix design for IVD repair and in the oriented and dynamic culture of annular fibrochondrocytes into planar, spatially relevant, annular type structures. The development of hyaluronan hydrogels capable of rapid in situ gelation offer the possibility of supplementation of matrices with cells and other biomimetics and represent a significant advance in biopolymer design. New generation biological glues and self-curing acrylic formulations which may be augmented with slow delivery biomimetics in microcarriers may also find

application in the non-surgical repair of annular defects. Despite major advances, significant technical challenges still have to be overcome before the biological repair of this intractable connective tissue becomes a realistic alternative to conventional surgical intervention for the treatment of chronic degenerate IVDs.

Keywords Annular injury · Annular remodelling/repair · Intervertebral disc degeneration · Intervertebral disc repair · Rim lesions · Perlecan

Introduction

The intervertebral disc (IVD) is a tough, composite bio-material which acts as a viscoelastic weight-bearing cushion and is a major contributor to the spinal flexibility and mechanical stability properties of the spine [48]. The outer regions of the IVD, the annulus fibrosus (AF) consist of lamellae containing counter-gradients of types I and II collagen with the former predominating in the outer AF and the latter in the central region of the IVD and the nucleus pulposus (NP) [24]. Elastin fibrils are also present in interlamellar regions and in translamellar cross bridges [10, 106, 158] which interconnect and stabilise the annular lamellae [106, 131]. The type II collagen fibrillar network in the NP is relatively disorganised compared to the fibrillar arrangements of annular type I collagen. The interconnectivity provided by collagen and elastin fibres provides exceptional tensile strength to the AF and allows it to withstand very significant hoop stresses.

Aggrecan is the major space-filling proteoglycan of the NP where it forms massive macromolecular aggregates with hyaluronan which are entrapped within an amorphous

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type II collagen network [122]. These networks imbibe water into the IVD resulting in the generation of an internal hydrostatic pressure which equips the NP with the ability to act as a viscoelastic cushion to counter compressive axial spinal loading. Versican is another large CS-rich IVD proteoglycan found in the NP and in proteoglycan-rich regions between adjacent annular lamellae [74, 80] and in translamellar annular cross bridges [82] where it may have a role in the lubrication of collagen bundles. Furthermore, the G3 domain of versican is interactive with elastic networks and this may also facilitate its inter-lamellar localisation [56]. Members of the small leucine repeat proteoglycans (SLRPs), decorin, biglycan, fibromodulin, lumican and keratocan are also prominent disc proteoglycans. The SLRPs have important linking, shape determining and matrix organising roles and regulate cellular proliferation, matrix adhesion and response to growth factors and cytokines [27, 154, 155]. Direct evidence for the importance of the SLRPs in musculoskeletal tissues has been demonstrated using knockout mice. While functional overlap between SLRP members is evident, a major phenotype of biglycan, decorin, fibromodulin and lumican single or double knockout mice is age-dependent tendon laxity, ectopic calcification and arthritis [14, 18, 25, 121]. A recent study using the ovine annular lesion model has demonstrated fragmentation of fibromodulin and biglycan in areas of annular remodelling [83]. Isolation and characterisation of these fragments may demonstrate their utility as bio-markers for the assessment of annular remodelling processes.

Changes in the intervertebral disc during ageing and degeneration

The IVD undergoes profound age-related cellular and matrix changes leading to degeneration and a diminution in its weight-bearing capability. Discs of older spines exhibit a range of pathologies including abnormalities in the AF. These include the vertebral “rim lesion” (Fig. 1) originally described by Schmorl and Junghans [125] arising from discontinuities in the vertebral attachment to the AF and may arise from avulsion of the annular bony attachment in close proximity to the cartilaginous end-plate (CEP) (Fig. 1). At 30 years of age approximately 20% of L4L5 IVDs have rim lesions. This incidence rises to 90% at 80 years and the number of rim lesions per IVD also increases [146]. The anterior AF has 10% more rim lesions than the posterior annulus. Connections between rim lesions and radiating tears are rare before the age of 50 but are present in 50% of older spines [146]. Concentric (circumferential) annular tears are separations of the annular lamellae (de-lamellations), while radial annular defects result from clefts initiating within the NP. Despite vascular

ingrowth around annular rim lesions (see Fig. 1b, c) [79], evidence from human post-mortem studies indicates that these show a very limited ability to undergo spontaneous repair.

The ovine annular lesion model of experimental IVD degeneration

The ovine annular lesion model developed by Osti et al. [103] utilises controlled annular defects 5 mm deep by 5 mm wide [75, 77, 81] (Fig. 2). This model has provided some important insights into the temporal ECM changes that occur following induction of rim lesions. Compositional changes previously noted may contribute to the degenerative process, like loss of large high-buoyant density aggrecan type proteoglycans and an elevation in levels of the small leucine repeat proteoglycans (SLRPs) decorin and biglycan in the injured disc [76, 77]. Fragmentation of fibromodulin and biglycan further illustrate the ECM remodelling events in the AF-defect site. Transforming growth factor-beta (TGF- β) and basic fibroblast growth factor (bFGF) have been immunolocalised with blood vessel and nerve in-growth around experimental annular lesions [79, 81] and with cells expressing α -smooth muscle cell actin [81] (Figs. 3, 4). Six months after the creation of the lesion, penetration of blood vessels and the influx of cells into the inner AF were more advanced. However after 12 months, the blood vessels ceased to extend further into the defect and the number of associated cells had decreased [79, 81]. This increased cellularity in the mid and inner annular lesion may have resulted from increased proliferation of endogenous disc cell populations in response to TGF- β and FGF-2 which are known to stimulate cellular division and matrix synthesis [136].

Repair of the outer third of the experimentally injured AF is a universal finding but this repair process does not extend into the inner regions of the AF (Fig. 3a–d). Rim lesions also propagate in some cases along the track of the original defect as far as the contralateral AF or between adjacent annular lamellae leading to delamellations which may induce circumferential defects (Fig. 2d, e, g, h). Propagation of outer annular defects into the NP as radial defects are also frequently seen (Fig. 2i). All of these defects have previously been described in pathological human IVDs [104, 137] and severely impact on the biomechanical competence of the IVD [137]. Other changes which have been observed in the ovine annular lesion model of relevance to the etiology of human disc degeneration include changes in endplate vascularity adjacent to annular rim lesions [92], remodelling of vertebral bone [93], and osteoarthritic changes in the facet joints secondary to the development of experimental annular rim

Fig. 1 Morphology of rim lesions in H&E stained human IVDs. **a** Typical cleft type rim lesion (*arrow*), **b** repaired rim lesion (*asterisk*), **c** vasoproliferative rim lesion, **d** cystic rim lesion and **e** fragmented rim lesion

lesions [90]. Ovine IVDs containing experimental outer and circumferential annular lesions have compromised biomechanical properties [26]. Fixation failed to stabilise such lesion affected IVDs and did not prevent subsequent degenerative changes in the IVD and other spinal components [91].

Rim lesions and the etiopathogenesis of disc degeneration in man

There has been much debate over the years as to whether rim lesions result from degenerative changes in another component of the axial spinal motion segment or are

themselves causal agents leading to degeneration in discal and paradiscal components. There is no doubt with the longitudinal experimental design possible and the numerous well-controlled studies already conducted using the ovine annular lesion model on healthy ovine IVDs, that standardised, experimental, outer, annular lesions can set in motion a series of events which lead to (1) early spontaneous repair of outer annular defects; (2) time-dependant propagation of the non-repaired inner annular defect resulting in the development of delamellations, circumferential or radial tears and/or transverse tears through the NP to the contralateral AF resulting in severe distortion of annular architecture, loss of disc height and disc

Fig. 2 Schematic representation of the controlled rim lesions (top, arrow) used in the ovine model of experimental intervertebral disc degeneration and the defects which have been observed using this model (a–c). Histological evaluation of vertical disc sections stained with Masson-Trichrome (d–f) to assess collagenous reorganisation and toluidine blue–fast green to delineate the focal loss of anionic proteoglycan in the vicinity of the initial lesion site (g–i). Remodelling of the outer lesion consistent with a spontaneous repair response is evident in the outer lesion, however, experimental rim lesions are capable of propagating to form de-lamellations after 3 months (d, g) which may form circumferential concentric tears after 6 months (e, h) and radiating tears spanning from the original defect site to the contralateral annulus after 12 months (f, i)

degeneration. It is self evident that the biomechanical properties of such degenerate IVDs (and spinal motion segment) are severely, possibly irreversibly compromised; (iii) resultant changes also occur in the vascular supply underlying the endplate adjacent to the rim-lesion site and in vertebral subchondral bone density. These vascular changes further impact on the nutrition of the endogenous cell populations in the affected IVDs which rely heavily on diffusive processes exacerbating degenerative processes in the IVD; (4) consequential degenerative changes in the posterior zygapophyseal joints of the spinal motion segment affected by the rim lesion and in which disc degeneration has ensued. As to whether the above sequence of events (1–4) accurately reflect the initiating and perpetuating events underlying the etiopathogenesis of human IVD degeneration has yet to be definitively answered. Rim lesions certainly occur at similar frequencies in young,

otherwise structurally intact, healthy human IVDs and in older discs containing early degenerative changes. Rim lesions are therefore present in human IVDs devoid of any other obvious defect, apparently indicating that they could be an initiating event in the etiopathogenesis of human disc degeneration rather than occurring as a secondary feature in consequence to changes in some other spinal component. Similar longitudinal studies to those undertaken in sheep are not possible with human IVDs. Despite advances in high-resolution imaging techniques which can identify rim lesions, such longitudinal approaches have yet to be undertaken to follow systematically the impact of rim lesions on disc degeneration. The question of rim lesions and IVD degeneration causality, therefore, must remain open despite compelling evidence from experimental studies using the ovine annular lesion model implicating these as causative agents in IVD degeneration.

Fig. 3 Experimental ovine annular lesions demonstrating a spontaneous repair response in the outer annulus fibrosus (*asterisk*) after 3 months and lack of repair of the defect internally (**a–d**). The non-healed defect is visible towards the right hand side of each photosegment, an arrow at the left hand side marks where the defect was initially made. **a** Focal collagenous remodelling of outer AF spanning the original defect site (Masson Trichrome); **b** Focal loss of anionic proteoglycan along the plane of the experimental annular defect, (toluidine blue/fast green stain). **c** Re-organisation of collagen in the outer AF in response to the experimental annular defect (*asterisk*), (*picrosirius red* stain viewed under polarised light).

d Polarised light view of segment (**b**) depicting similar detail to segment (**c**). **e** Cellular infiltration into a defect site 6 months post-operatively, (H&E). (**f**) Cells in the vicinity of a defect site stain positively for α -smooth muscle actin 6 months post-operatively. A large proportion of the cells which migrate into inner annular repair sites express FGF-2 (**g–i**) and are associated with small blood vessels in regions of the AF undergoing remodelling consistent with an active repair response at least in the outer AF but not so in the inner AF; (**g**) 3 months; (**h**) 6 months; (**i**) 12 months post-operatively. The photosegments in **e–i** are typical higher power views from an area approximating to the boxed region in segment **b**

Immunolocalisation of FGF-2 and TGF- β in the remodelling AF: evidence of their potential in intervertebral disc repair processes

As already noted, FGF-2 has been localised to areas of the AF undergoing a reparative response in the ovine model (Fig. 2g–i). FGF-2 sustains the differentiated phenotype of cultured NP cells by maintaining their responsiveness to TGF- β [140]. FGF-2 also increases the numbers of inflammatory cells, including macrophages, lymphocytes, and fibroblasts in herniated disc and facilitates the resorption of material extruded into the epidural space [86]. Basic fibroblast growth factor also promotes the proliferation of chondrocytes in degenerated discs in an autocrine or paracrine manner [97]. IVD cells are also responsive to TGF- β in culture which stimulates MMP-2 and matrix production as well as cell proliferation [16, 105, 118].

TGF- β has been strongly localised to regions of annular lesions containing cells of a more rounded morphology than the typical elongated annular fibrochondrocyte (Fig. 4). The former cells also express α -smooth muscle cell actin suggesting a myofibroblastic phenotype. TGF- β exerts anabolic effects on articular chondrocytes and IVD cells [105], stimulates repair processes in cartilage defects [36] and enhances periosteal chondrogenesis [133]. A number of delivery systems have been developed for TGF- β to articular chondrocytes including hydrogels, periosteal grafts dipped in growth factor, liposomes and alginate beads [1, 43, 84]. Bone morphogenetic protein-7 (osteogenic protein-1, OP-1) is also a member of the TGF- β superfamily of growth factors which has been used in a number of applications to promote repair processes in articular cartilage [17, 57], IVD [7, 134], rotator cuff, ligament [9, 138] and genetically modified cells over expressing OP-1 have been developed to promote repair processes [42].

Perlecan and its roles in annulus development, remodelling and potential in therapeutic repair

An improved understanding of annular development and assembly may be insightful with regard to potential annular repair strategies. Our laboratory has embarked on a series of studies in the last few years to better understand the role of a multifunctional HS-proteoglycan, perlecan in FGF growth factor signalling in cartilaginous tissues and to assess how this regulates chondrogenic events relevant in annular development and remodelling [78]. Perlecan is a, large, modular, multifunctional proteoglycan with a 467-kDa protein core containing five distinct domains with homology to the laminin A chain, low density lipoprotein receptor, neural cell adhesion molecule and epidermal

growth factor [62, 94, 102]. In man, domain-I in perlecan is the main region of GAG substitution [19, 62, 94, 102] while domain V may also have an additional two HS attachment sites [54, 65]. When substituted with HS, perlecan domain-1 promotes binding to laminin-1 and collagen IV and can also act as a low affinity co-receptor for FGF-1, 2, 7, 9 and 18 while domains III and V act as a receptor for FGF-7 [63–65]. The low density lipoprotein (LDL) repeats in perlecan domain II bind connective tissue growth factor (CTGF) [2, 101], CTGF modulates bone morphogenetic protein (BMP) and transforming growth factor (TGF)- β signalling to co-ordinate morphogenesis, and chondrogenic and angiogenic events during skeletal development [2, 101]. Perlecan also interacts with a number of matrix stabilising molecules including fibrillin-1, nidogen-1 and 2, fibulin-2, fibronectin, proline/arginine-rich and leucine-rich repeat protein/prolargin (PRELP), von Willebrand factor protein-A related protein (WARP) and types XIII and XVIII collagen; and cell attachment proteins such as laminin, fibronectin and thrombospondin [6, 8, 45, 54, 78, 87, 141] and thus also has important roles to play in matrix organisation and stabilisation.

Immunolocalisation of perlecan in foetal human IVDs demonstrates its prominent pericellular distribution in the arcades of cells in the developing annulus and NP as well as the vertebral rudimentary cartilages (Fig. 5). Perlecan is particularly prominently expressed by the hypertrophic chondrocytes of the vertebral growth plates in the cartilaginous rudiments which is consistent with its known chondrogenic properties. It may be instructive to understand the pathobiology of perlecan in such transient developmental cartilages since it may be possible to harness some of these traits in annular remodelling processes in the mature IVD. Perlecan is also a prominent pericellular proteoglycan in the mature AF, although its levels are severely diminished with the age-dependant decline in cell numbers (Fig. 6e).

The pericellular matrix, cell–matrix interactions and annular mechanosensory processes

A number of studies have demonstrated that the pericellular matrix of AF cells display a complex morphology [12, 22, 23, 37, 60]. Extensive sinuous cellular processes interwoven through annular collagenous fibres are evident in cells of the outer AF as well as strings of 10–12 cells interconnected by type VI collagen which is a prominent pericellular AF component particularly in the mid and inner AF [23, 120]. This is consistent with type VI collagen's documented roles in the assembly of the basket-like chondron functional unit around cells of a chondrocytic background [46, 107]. The cells in the outer AF are

Fig. 4 Immunolocalisation of TGF- β in vertical sections of lesion affected ovine IVDs with a pan TGF- β Ab which detects all TGF- β isoforms. The areas shown are in the approximate area of the mid AF depicted by the boxed area in Fig. 2b. Negative control using equivalent concentration of mouse IgG in place of primary antibody (**a, b**); 3-month lesion (**c, d**); 6-month lesion (**e, f**); 12-month lesion (**g, h**); 26-month lesion (**i, j**). The photosegments depicted in the right hand column (**b, d, f, h, j**) are viewed with Nomarski DIC optics and are of the corresponding photosegments to their left (**a, c, e, g, i**), red blood cells are prominent in the DIC imaged blood vessels. The inner AF lesions depicted were incapable of undergoing repair despite the influx of cells and blood vessels to the lesion site and the demonstrated localisation of FGF-2 and TGF- β which are known to promote such processes

Fig. 5 Perlecan has important roles to play in matrix assembly and the development of the annulus fibrosus and other discal components. Low and medium power longitudinal mid sagittal sections through a 12-week-old human foetal spine stained with toluidine blue–fast green to depict the cartilaginous rudiments and developing intervertebral discs (**a–d**). The boxed area in (**a**) is presented at higher magnification in (**b**), as are the boxed areas in (**b**) in segments (**c** and **d**). Perlecan immunolocalisation in the boxed areas (**e**, **f**) is presented in segments (**e**, **f**), respectively by indirect fluorescent confocal microscopy using a rat anti perlecan primary mAb A7L6 to domain IV of perlecan and Alexa 594 conjugated donkey anti rat igG secondary Ab (red chromophore)

extensively interconnected through functional gap junctions forming a network through which the cells can communicate [23]. Loading of the IVD results in shearing forces between collagen fibrils in the annular lamellae and rotation of adjacent lamellae which absorbs the mechanical strains. The strings of mid and inner annular cells embedded in a dense pericellular matrix, located between adjacent lamellae, largely spared these tensile strains. However, the annular cells of the outer AF with their extensive interdigitating cellular processes woven into the collagenous architecture would be expected to be subjected

to tensional forces in situ and may be involved in annular mechanotransduction events [38]. The morphologic complexity of the annular cells with their interwoven ECM attachments to the annular collagen fibres indicates that the load transfer from ECM to cell membrane to cytoskeleton in all likelihood occurs by a complex pathway. The physical connections between focal cellular adhesions, the actin cytoskeleton and nuclear scaffold is thought to provide a pathway for mechanical signal transfer from the cell surface to the nucleus [50, 51]. Deformation of the cytoskeleton by intrinsic biomechanical stresses provides a

Fig. 6 Perlecan provides cell-cell and cell-ECM interconnections important for cellular attachment and possibly mechanosensory processes. Immunolocalisation of pericellular perlecan in short-term primary monolayer culture of ovine AF cells using a primary mAb A76 to perlecan domain-I and horse radish conjugated secondary antibodies and diaminobenzidine substrate for visualisation (*brown chromogen*), nuclei are counterstained with eosin (**a, b**). A negative control using equivalent concentration of mouse IgG in place of primary antibody is presented in (**c**). Localisation of the actin cytoskeleton layed down by the AF cells in monolayer culture using Phalloidin-oregon green, cell nuclei stained with DAPI (**d**). Immunolocalisation of perlecan in the pericellular matrix of AF cells in the native tissue (*brown chromogen*) using a mAb to perlecan domain IV (mAb A7L6) (**e**). Perlecan provides both cell-cell and cell-matrix connections which allow the monolayer cells to attach and spread out and also attaches the AF cells to the pericellular and interterritorial matrix in the native tissue

physical basis for transducing mechanical signals into transcriptional gene expression and protein synthesis through cell signalling [49, 52, 70].

Perlecan is a prominent pericellular matrix component of AF cells in vitro and in vivo. Furthermore, FGF-2, a major ligand for perlecan, has been suggested as a mechanotransducer molecule in articular cartilage and perlecan modulates FGF-2 bioactivity in growth plate cartilage [147–150]. Thus, the proposal of perlecan having some accessory role in

mechanosensory processes in the IVD is not without merit. We have examined the expression of perlecan in the pericellular matrix of AF cells in the native tissue (Figs. 5, 6e) and in short term monolayer culture (Fig. 6a–d). The AF cells are cultured in primary short-term (4 day) cultures to minimise cellular de-differentiation, in the absence of ascorbic acid, thus the intracellular procollagens are not hydroxylated or assembled into fibrillar collagen in the pericellular matrix. The triple helix of fibrillar collagens is

not thermally stable at 37°C without hydroxylation, and it is not secreted. Collagen VI also does not assemble into tetramers in the absence of ascorbate although the collagen VI monomers ($\alpha 1$, $\alpha 2$, $\alpha 3$) are secreted into the medium. Using this monolayer approach greatly simplifies observation of the early stages of the assembly of the annular pericellular matrix. Furthermore, without the surrounding ECM and the instructive properties it normally conveys [110], the AF cells synthesise a number of perlecan positive interconnections to adjacent cells and to the tissue culture plastic, and along with the actin cytoskeleton (visualised using phalloidin-oregon green, Fig. 6d) this facilitates the spreading out of these cells in culture. The fibrillar cellular extensions containing perlecan in the native AF are pericellular due to ECM constraining and intrinsic biomechanical forces. These monolayer experiments serve to demonstrate the propensity of the annular cells to assemble a pericellular matrix containing perlecan as an interactive component. This is consistent with perlecan's known multifunctional properties in a number of tensional and weight bearing connective tissues. Perlecan also has a pericellular localisation in the native AF and important interactive roles controlling matrix assembly and tissue homeostasis (Fig. 6e). Perlecan's chondrogenic, matrix organisational and stabilising roles and ability to sequester a range of anabolic growth factors in the pericellular matrix indicates that it has important attributes relevant to annular remodelling and repair processes, reinforcing its potential as an effector molecule worthy of further investigation in the context of repair biology. It will be very interesting in this regard to ascertain whether the therapeutic potential of 3D scaffolds of electrospun fibres of collagen and gelatin functionalised with recombinant domain-I of perlecan are fully realised [13].

Biological strategies for annular and intervertebral disc repair

Tissue engineering strategies for intervertebral disc repair have utilised a number of bioscaffolds [127]. Collagen sponge, collagen gel, agarose, alginate or fibrin gel cell carriers, alginate/chitosan hybrid fibre scaffolds [129], scaffolds assembled from electrospun collagen and gelatin functionalised with perlecan domain-I [13], chitosan salts cross-linked to genipen [95], a chitosan glycerophosphate hydrogel [114], a porous calcium polyphosphate carrier [128], elastic poly (1, 8-octanediol malate) 3D scaffolds [151], a composite demineralised bone matrix gelatin-(polycaprolactone triol malate)- poly(caprolactone triol malate) biphasic scaffold [152] and collagen/hyaluronan hybrid scaffolds [5] have all been examined in IVD and/or annular repair strategies. Exciting possibilities also exist for the use of replacement total IVDs using biocompatible and biomechanically competent PLGA and PLA scaffolds

seeded with AF and NP cells [88, 89]. Novel injectable hydrogels which can be seeded with a number of cell types have also been developed for NP replacement strategies [111, 145].

Therapeutic strategies focussed specifically on annular repair

Injectable scaffolds have potential application in the repair of annular defects since they allow easy filling of irregularly shaped defects and implantation of cells through minimally invasive surgical procedures. Due to the intricate structural organisation of the AF which is composed of highly oriented arcade-like, alternating stressed and relaxed lamellae in which fibrochondrocytes are aligned and containing counter-gradients of types II and I collagen in the inner and outer AF, respectively, this tissue has special requirements from an engineering perspective. AF cells have been grown on PDLA/45S5 Bioglass composite films [156] and composite foam scaffolds [40], an atelocollagen honeycomb-shaped scaffold with a membrane seal (ACHMS-scaffold) [124], and on micro-grooved membranes of polycaprolactone [61]. This provides control over the directional growth of the annular fibrochondrocytes in the constructs and recapitulates some of the spatial organisation of this tissue. Since engineering, a functional replacement for the AF is contingent upon recapitulation of AF structure, composition, and mechanical properties, some of the more recent advances in oriented electrospun scaffolds [58, 99], porous silk fibroin scaffolds [15] and microgrooved membranes [61] hold great promise in replacement strategies for the repair of annular defects. The interconnectivity and shape of tendon cells are important determinants which regulate production of type I collagen in vitro [68], a greater appreciation of the micromechanical control of cell and tissue development by biomatrices is emerging and newer approaches are attempting to guide cellular behaviour to facilitate correct tissue assembly and seamless integration with adjacent living tissues [31]. A novel 3D elastic scaffold which simulates the deformability of the AF and has good biocompatibility has also been developed using a malic acid based polyester poly (1,8-octanediol malate) (POM) scaffold [151]. This polymer supported the growth of AF cells, maintained their phenotype in culture and the cells produced proteoglycan and type II collagen in vitro. POM is metabolised through the tricarboxylic acid cycle, its degradative products are well tolerated, and POM consequently does not elicit a strong inflammatory response in situ [151]. Furthermore, POM has predictable degradative decay rates which allow the accurate determination of the culture period required for production of an engineered replacement AF.

A biphasic scaffold has been developed based on an outer demineralised bone matrix gelatin (BMG) scaffold which simulates the type I collagen rich outer AF and an inner biomaterial based on poly(polycaprolactone triol malate) (PPCLM) which has biomechanical properties more similar to the inner AF [152]. Incorporation of BMG into PPCLM resulted in a 50-fold increase in the tensile properties of the composite scaffold over PPCLM alone and produced a scaffold which could be prepared as sheets which could be subsequently assembled in concentric layers to mimic the lamellar structure present in the native AF. Both BGM and PPCLM demonstrated excellent biocompatibility and supported the growth of AF cells in culture. The phenotype of the cells was also maintained in culture with strong production of proteoglycan and type II collagen by AF cells on BMG-PPCLM and PPCLM matrix. Modification of porous silk fibroin scaffolds by functionalisation with RGD peptides transforms the properties of this scaffold material from that of the unmodified fibroin scaffold [15]. Coupling of RGD peptide influences AF cell phenotype when they are cultured on this matrix for 8 weeks with the cells synthesising higher levels of aggrecan and type II collagen than when grown on non-modified fibroin. The AF cells grown on the RGD functionalised fibroin displayed a phenotype similar to the cells of the inner AF while the AF cells grown on non-modified fibroin displayed a phenotype more similar to that of outer AF cells. This ability to differentially modulate gene expression profiles to simulate inner and outer AF cells in culture may prove useful in repair strategies aimed at recapitulating the spatial organisation of the AF.

An innovative development in scaffold design for annular repair is the use of natural polymer 3D scaffolds of electrospun collagen and gelatin functionalised with recombinant domain-I of the HS-proteoglycan, perlecan [13]. Recombinant perlecan domain-I substituted with HS has also been expressed in HEK-293 kidney cells, its FGF binding capability demonstrated and potential use as a scaffold additive to enhance FGF cell signalling by endogenous cell populations established [153]. Perlecan domain-I in native human perlecan contains up to three HS side chains. The rationale for the development of perlecan domain-I functionalised scaffolds for annular repair resides in their ability to bind FGF-2 although these HS chains can also bind FGF-1, 9, 18.

Integration of engineered annular constructs in repair environments

A major obstacle in IVD repair biology is how to effectively anchor the construct to facilitate its biointegration with the cartilaginous margins of a defect site which may be

somewhat devitalised [130]. Biological glues based on an adhesive protein extracted from the marine mussel *Mytilus edulis* [100] and a protein-based adhesive elastomer secreted by the Australian frog *Notaden bennetti* [34] have been used to glue porcine small intestinal submucosa [100] and may also be useful in annular repair strategies. “Frog-glue” offers a stronger alternative to fibrin glue, has been used for the reattachment of bucket handle tears in knee joint menisci and offers potential in the non-surgical sealing of annular defects as an integral part of a repair strategy [35]. Injectable, self-curing, acrylic formulations, also display potential in this regard and may be additionally supplemented with drugs and other biomimetics in microcarriers for their controlled release in a therapeutic setting to stimulate cellular responses and promote tissue repair [66]. Biphasic scaffolds have also been developed to mimic the known differences in the spatial organisation of the outer and inner AF and these should aid in the integration of constructs which recapitulate at least some aspects of the structure of the native AF. Multiphasic scaffolds have also been developed to produce biomaterials appropriate for replacement of osteochondral defects [71, 126]. Although designed specifically for the treatment of defects in articular cartilage a similar approach could be adopted for repair of IVD defects to effect direct integration of constructs into vertebral bone.

The search for cellular marker proteins and their use as phenotypic markers for the identification of AF and NP cells

A fundamental requirement to a proper understanding of disc degeneration and regeneration at the cellular level is a full appreciation of the cellular biology of the intervertebral disc. Currently, there are no definitive cellular biomarkers which can be used to define a normal AF or NP cell to differentiate these from one another or from an articular chondrocyte. Micro-array and RT-PCR approaches have been used to determine which disc cell markers are most appropriate as phenotypic markers in rat IVDs [28, 67]. In an initial study, CD-24 was suggested as an NP cell marker [28]. In a follow up study [67] while CD-24 was shown to be more highly expressed in NP cells than articular chondrocytes (AC), the discriminative criteria adopted (NP gene expression ≥ 5 -fold of AC cells) excluded CD-24 as a candidate NP marker. Sixty-three genes were expressed at a 5-fold greater level in NP compared to AF cells; 41 genes had ≥ 5 -fold greater expression in NP than AC cells. Real time RT-PCR confirmed that the expression of annexin A3, glypican-3 (*gpc-3*), keratin-19 (*k-19*), and pleiotropin were all significantly higher in NP cells than AF or AC cells, a finding confirmed using immunohistochemistry on rat disc tissues [67]. However, it remains to be established how useful these markers will be for the human intervertebral disc.

Several proteins have been investigated which convey functional adaptations to NP cells allowing them to survive under hostile low oxygen tension and high osmotic pressure environments. Tonicity enhancer binding protein (TonEBP) [139] and hypoxia inducible factor-1 alpha (HIF-1 α) [4, 115, 119] are two candidate proteins which allow NP cells to withstand hyperosmotic stress and an avascular hypoxic environment. TonEBP binds to the aggrecan promoter, regulates its transcriptional activity and the hydration status of the IVD. TonEBP is an interesting and functionally relevant marker for NP cells, however, it is also expressed by AF and kidney cells [11] and by costal chondrocytes but at considerably lower levels negating its suitability as an NP cell specific marker. HIF-1 α is produced by NP cells and is a key regulator of mammalian oxygen homeostasis [4, 109, 119]. HIF-1 α is tightly regulated by the oxygen tension of tissues, hypoxic conditions prevent ubiquitination and proteasomal degradation of HIF-1 α resulting in its accumulation and the trans-activation of downstream genes including those encoding glucose transporters and glycolytic enzymes capable of increasing anaerobic ATP synthesis [4, 109, 119]. Unlike other mammalian cells, HIF-1 α expression in NP cells is stabilised under normoxic conditions [119]. However, HIF-1 α is also expressed in a number of tumours [20, 21, 29] where it promotes the transcription of glycolytic enzymes which switch cells from aerobic to anaerobic glycolysis. HIF-1 α expression in the ovine and human IVD correlates with IVD pathology [39, 115, 119]. The normoxic stabilization of HIF-1 α in rat NP cells drives glycolytic processes which regulate aggrecan gene expression [4]. The glucose transporters GLUT-1, 3 and 9 are also targets for HIF-1 α in rat NP cells (but not AF cells) [115]. Thus, while HIF-1 α in isolation is not suitable as a specific NP marker, assessment of GLUT-1, 3 and 9 expression in combination with HIF-1 α and possibly other markers such as Sox 9, aggrecan and type II collagen in a mini array profile may facilitate the functional identification and differentiation of NP cells from related cell types. While in themselves each proposed marker may not be absolutely specific for NP cells, in combination and utilising thresholding techniques an identifiable NP signature may be identified. For example, the ratio of aggrecan to collagen in the IVD is 27:1 whereas in articular cartilage it is 2:1 [96]. Such assays will need to be validated in the adult human IVD which unlike rat IVDs lack notochordal cells and the regulatory factors they secrete.

Two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) is a powerful new technique whereby the proteome of several cell types can be compared simultaneously in one 2D gel format using in-gel enzymatic digestion, mass spectrometry and bioinformatics software to identify the full cellular proteome [69, 135]. 2D-DIGE has

been used to search for NP cell biomarkers to differentiate these from cells of a chondrocytic background [32]. Thirty differentially expressed proteins were identified in a preliminary study using DIGE, thus it may well be possible to identify useful NP and AF cell protein biomarkers using such technology in the not too distant future.

Cell-based therapies for the biologic treatment of disc degeneration

The IVD has a relatively sparse cell population available to effect repair processes and cell viabilities are also known to be significantly diminished with aging. The mean IVD cell density of 6,000 cells/mm³ compares with 14,000–15,000 cells/mm³ in human articular cartilage [47, 72]. The avascular nature of the IVD, poor nutrition and severe biomechanical demands placed on it are all refractory to repair processes and it consequently has an intrinsically poor healing capability. Of the cells to use for cell-based therapies for IVD degeneration, IVD cells themselves are the least likely candidates for use given the difficulty of harvesting sufficient numbers of viable autologous disc cells from symptomatic IVDs [112–114, 116, 117]. However, a number of cell types have provided promising results in therapeutic cell replacement strategies for disc repair, including costal and articular chondrocytes [30, 55, 73, 111], auricular chondrocytes [33] and mesenchymal stem cells (MSCs) [3, 41, 113, 114, 117, 123, 132]. A common pathological features of IVD degeneration is a chondroid metaplasia of the NP with clusters (chondrones) of chondrocyte-like cells within a dense hyaline-like matrix [59] which is different from the fibrocartilage of the normal NP. This is considered a degenerative feature, thus despite similarities in morphology between the chondrocyte and NP cells the rationale for the use of the latter cell type to effect disc “repair” must be questioned.

Nutritional constraints on cell-based therapeutic approaches for IVD repair

A number of factors affect disc cell metabolism apart from nutrient supply and removal of metabolic waste products. These include the presence of growth factors, changes in local biomechanical stresses and in the osmotic environment of the disc cell [44, 47, 72, 98, 143, 144, 157]. Stimulation of endogenous disc cell populations by administered growth factors and other biomimetics in slow delivery carriers within bioscaffolds aim to address some of these questions [53, 85].

The metabolism of disc cells is regulated by oxygen and nutrient levels in the tissue and also by the extracellular pH [44, 47, 143, 144]. The adult human IVD is virtually avascular apart from a sparse penetration of small vessels

in the outermost AF. The NP, inner AF and part of the outer AF is supplied by a network of small vessels that arise from the vertebral arteries and penetrates the subchondral plate terminating in capillary buds in the bone–cartilage end plate junction through which diffusive processes effect the nutrition of endogenous disc cell populations [44, 47, 143, 144]. The density of the capillary network feeding the disc diminishes with age and across the disc, with the highest density overlying the NP and lowest in the endplate adjacent to the outer AF. The density of the subchondral plate through which the capillaries pass also varies across the disc and its permeability decreases with age as the end plate becomes sclerotic. Such changes significantly diminish the nutritional status of the disc cell and undoubtedly contribute to cell death (apoptosis, senescence) with aging. This has to be taken into account in any prospective use of administered cells to replenish ECM components through cellular replicative and matrix biosynthetic processes. It may simply not be possible for a relatively large number of administered cells to undertake such tasks given that the degenerate IVD would be expected to have severely compromised routes of nutrition which have led to its demise in the first place. The use of stimulatory growth factors and/or slow release biomimetics in scaffolds similarly may be a futile gesture and may fail to stimulate replicative and matrix biosynthetic processes in endogenous disc cells unless the normal pathways to the disc cells for the supply of nutrients and removal of metabolic waste products have first been re-established.

Remodelling of the vertebral subchondral capillary bed in response to calcium agonists such as Nimodipine (marketed by Bayer as Nimotop®) has been observed in animal models of disc degeneration suggesting that the IVD nutritional status can be improved at least experimentally [142]. Nimodipine is a dihydropyridine calcium channel blocker originally developed for the treatment of high blood pressure, it has been used to increase blood flow to injured brain tissue and in the treatment of migraines. Oral administration of Nimodipine in 40 patients (30 mg QID for 5 days) significantly enhanced signal intensity of the subchondral bone and cartilaginous endplates in serial post contrast MRI studies using gadodiamide [108]. This indicated that the diffusive pathways to the human lumbar IVDs were significantly enhanced by Nimodipine treatment and suggests that this treatment may be beneficial in the nutrition of endogenous cell populations in age onset disc degeneration in man but has yet to be evaluated as part of a discal repair strategy.

Concluding remarks

The ovine annular lesion model [103] has provided useful spatial, temporal and longitudinal information on how rim lesions may impact on other discal components and has

provided important insights into annular remodelling and how it can influence the etiopathogenesis of disc degeneration in specific situations. There is a clear need to identify specific phenotypic marker proteins for AF and NP cells. This lack of cellular markers makes it difficult to evaluate the specific contributions of disc cell populations in cell-based IVD repair strategies. AF and NP cell markers would also be useful to assess how effectively an MSC or pluripotent progenitor cell population has been guided along a particular differentiation pathway by specific culture conditions to produce AF or NP cells for therapeutic applications. The nutrition of outer annulus is less critical than the inner annulus, however the nutrition of disc cells remains a centrally important issue in any repair strategy involving large numbers of administered reparative cells. Tissue engineering of the IVD has undergone impressive advances over the last few years particularly in the development of new biomatrices and the oriented and dynamic culture of annular fibrochondrocytes into planar, spatially relevant, annulus type structures. Improvements in our understanding of annular assembly has also provided insightful information applicable to new repair strategies. The multifunctional proteoglycan, perlecan, in particular has many attributes conducive to matrix assembly, remodelling and repair processes and is worthy of further consideration in annular and discal repair biology. Despite exciting advances in biological IVD repair, significant technical obstacles still have to be overcome before such approaches become realistic alternative therapeutic options to conventional surgical intervention procedures.

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