

DOI: 10.21767/2471-8173.100035

The Role of Cell Clusters in Intervertebral Disc Degeneration and its Relevance behind Repair

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Abstract

Background context: The greatest risk factors for intervertebral disc degeneration are age, spinal level, excessive mechanical loading and genetic inheritance, a major cell-mediated change that occurs during degeneration is increased cell clustering. In fact, cell clusters formations are a histological hallmark behind intervertebral disc degeneration, yet a systematic review focusing on the cell clusters of the intervertebral disc is unknown. Therefore, the main purpose of this article is to discuss the role of cell cluster in intervertebral disc degeneration.

Methods: PubMed database was searched to identify studies about cells of the intervertebral discs from 1980 to 2016, and a total of 2824 published papers were identified. The inclusion criteria for this review was morphological, biochemical and molecular changes the cells of nucleus pulposus, inner and outer annulus regions undergo with advancement in disc degeneration. Large cell clusters of the intervertebral disc were the penultimate search. Exclusion criteria were whether the selected papers provided a firm relevance to the main purpose of this review.

Results: Recent and past studies confirm that cells clusters of the intervertebral disc are immunopositive to Ki-67 and proliferating cell nuclear antigen (PCNA) which are a marker for cellular proliferation. These proliferating clusters were of common occurrences in the nucleus and inner annulus regions increased around fissures and were especially abundant in Pfirmann grade IV and grade V disc which represents later stages of disc degeneration. Single cells and cell clusters were also reported to act as progenitor cells with immunopositive markers and gene expressions analysis, thus indicating that cell clustering phenomenon may be a repair response towards tissue damage.

Conclusion: Current evidence suggests that cluster formations are indicative of attempted repair response in intervertebral disc degeneration, and the innate capacity of these disc cells to act as progenitor cells signify that development of regenerative therapies is possible

Keywords: Intervertebral disc; Cell clusters; Chondrocyte-like cells; Progenitor cells; Regeneration

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Citation: Lama P, Kulkarni JP, Tamang BK (2017) The Role of Cell Clusters in Intervertebral Disc Degeneration and its Relevance behind Repair. Spine Res. Vol.3 No.3:15

Received: June 28, 2017; Accepted: October 18, 2017; Published: October 25, 2017

Introduction

Intervertebral discs (IVDs) are pads of white-fibro cartilage located between consecutive vertebral bodies. In normal adults, IVDs provide flexibility and facilitate movements of the vertebral column, allowing bending (flexion, extension) and torsion. They also act as minor shock absorbers and help in the even distribution of compressive forces on the vertebral bodies [1]. Individual

thoracolumbar IVDs are approximately 40 mm in diameter along the mid-sagittal plane, and their thickness increases from approximately 7-10 mm between thoracic and lumbar regions [2]. Each disc comprises of three distinct regions: a centrally located nucleus pulposus (np), a peripherally located annulus fibrosus (af), and cartilaginous endplates (cep) located above and below each individual disc [3,4]. Disc cells are present within the nucleus and annulus regions, and they undergo considerable

changes with age, excessive mechanical loading, and pathologies that weakens the disc tissue [5,6]. These changes are known to increase expression of matrix-degrading enzymes and other catabolic molecules that progressively contribute towards accelerated degradation of the tissue. Intervertebral disc cells attempt to restore the damaged matrix possibly by forming cell clusters [7] which may indicate repair attempts. Thus, the main purpose of this review is to evaluate the role of intervertebral disc cell clusters in degeneration and repair.

Methods

Micro-anatomy of IVDs

Nucleus pulposus (np) is the inner soft core of the intervertebral disc (**Figure 1**). It originates from the notochord and in the fetus and infants, it contains actively-dividing notochordal cells, which disappear at approximately eight years of age [8]. Interspersed at low density in the nucleus are chondrocyte-like nucleus pulposus cells [9], which may be present singly, or in pairs, and may frequently form small to large clusters, and occurrence of large clusters are considered to be a histological hallmark of intervertebral disc degeneration [10]. Along with the cells, the nucleus also contains type II collagen fibers which are randomly organized, and account for almost 20% of its dry weight [11]. Type III, V, VI, IX, and type XI collagen fibers are usually sparse and pericellular in location [12]. The collagen along with sparse elastin fibers normally hold the highly hydrated aggregating proteoglycan gel [13]. Proteoglycans are abundant in the nucleus pulposus, and the main proteoglycan aggrecan has a protein core to which up to 100 sulfated cationic glycosaminoglycans (GAG) chains are covalently attached [14]. The anionic charge carried on the surface by each GAG has a high affinity to attract ions and water making the water content of the nucleus approximately 80% [15]. Such organization of the nucleus tissue reinforces its hydrostatic pressure resisting capacities which is approximately 0.05 MPa in an unloaded cadaveric disc and up to 3 MPa in loaded discs [16].

Annulus fibrosus forms the outer part of the disc. Unlike the nucleus, the outer annulus contains a relatively dense population of fibroblast-like cells (**Figure 1**) which tend to be elongated and aligned parallel to the layers or lamellae of collagen fibers [9]. Towards the inner annulus region, the cells are more chondrocyte-like in appearances, and not necessarily aligned parallel to the collagen lamellae [17], these cells may appear in pairs or small clusters between the lamellae. Collagen types II, III and VI are present in the pericellular matrix of clustered and non-clustered inner annulus cells [12]. Each lamella consists of oblique and regularly arranged collagen fiber bundles, which are orientated at 30 degrees to the horizontal plane, with the direction of fibers alternating between adjacent lamellae [18]. Collagen forms 70% of the dry weight of the annulus [19], sparse elastin fibers are also present within and between the collagen fibers possibly helping the disc to return to its original arrangement following large deformations [20]. Elastin also binds the collagen lamellae together as these fibers pass radially from one lamella to the next, and exhibit a planar zigzag "crimp" pattern when viewed under

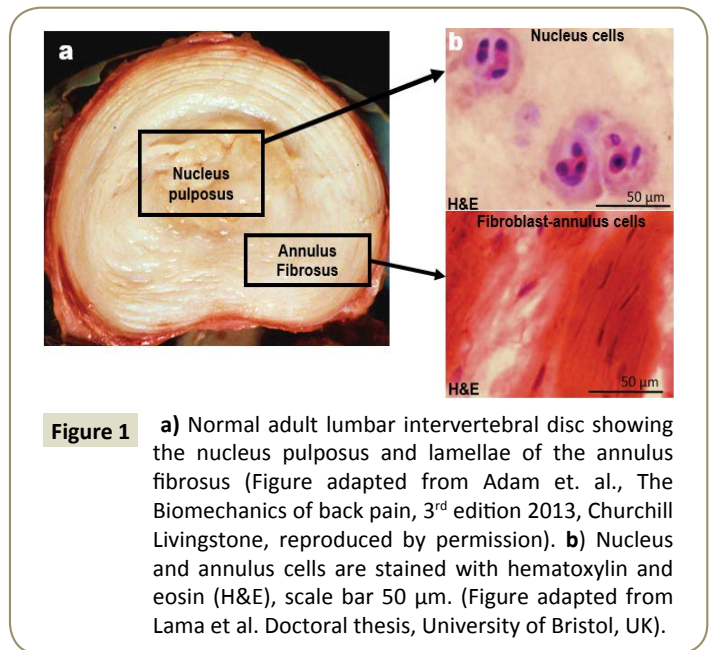


Figure 1 a) Normal adult lumbar intervertebral disc showing the nucleus pulposus and lamellae of the annulus fibrosus (Figure adapted from Adam et. al., *The Biomechanics of back pain*, 3rd edition 2013, Churchill Livingstone, reproduced by permission). b) Nucleus and annulus cells are stained with hematoxylin and eosin (H&E), scale bar 50 µm. (Figure adapted from Lama et al. Doctoral thesis, University of Bristol, UK).

a polarising microscope [21]. Glycosaminoglycans are suggested to be present between the collagen fibers and comprise 10% of dry weight of outer annulus and 30% of the inner annulus [14].

Cartilage end plates are the third morphologically distinct region of the disc. They usually are present in the form of a thin hyaline cartilage layer approximately 1 mm thick which is loosely bonded to the underlying vertebral endplate of perforated cortical bone [22]. The latter has abundant blood vessels and capillaries [23]. Cells of the cartilage endplate may also exist in pairs or clusters [24]. Function wise, cartilage endplate acts as a physical and chemical barrier helping to prevent nucleus herniating into the spongiosa of the vertebral bodies [25] and as a pressure distributor during loading [26] that consequently allows nutrition into the disc by the process of diffusion [27]. The cartilage endplate thus maintains internal pressurization of the discs by hindering expulsion of water and proteoglycan from the disc into the vertebrae [28,29].

Intervertebral disc cells

Two cell types normally populate the adult intervertebral discs: rounded chondrocyte-like cells of the nucleus pulposus and inner annulus fibrosus regions, and the flattened fibroblast-like cells of the outer annulus fibrosus [30]. Both cell types differ in their developmental origins. The rounded chondrocyte-like cells are suggested to be an embryological derivative of notochordal cells while the flattened fibroblast-like cells of the outer annulus region are mesodermal in origin [31].

Notochordal cells are the common cell type seen in the nucleus pulposus region in a fetus of 26-28 weeks. Notochordal cells are closely packed and have extensive intra-cytoplasmic glycogen storage capacities. Electron microscopic studies reveal that cell membranes of these closely packed cells are joined by a variety of cell-cell junctions [32]. In fetal tissue, the notochordal cells may also appear elongated and they lack a distinct pericellular

matrix [33]. After birth and with increasing age, notochordal cells separate, become rounded or irregular, develops a conspicuous pericellular matrix around it and the overall cell density begins to decline and totally disappear by the age of 8-10 years [34-36].

Cells of the nucleus pulposus embryologically originate from the notochord, and they completely transform into being more chondrocyte-like by 8-10 years of age [31], and appear in pairs or small, moderate and large-sized cell clusters. Viable and necrotic cells can both co-exist within a single cluster [34] but the mechanism of formation of heterogeneous “cell clusters” is still unclear and so is their significance. Presumably, cluster formation reflects the influence of the immediate pericellular matrix on the cells through continued accumulation of cell products, as the np region of the IVDs occurs in a relatively confined environment [25]. Cluster formation in the nucleus pulposus and inner annulus regions has been linked to gross structural changes that occur mainly during aging or injury [37].

Fibroblast-like cells of the annulus fibrosus are most numerous in the outer annulus regions. In IVDs they are usually elongated, thin and aligned parallel to type I collagen fibers [38]. Fibroblasts are often also termed “fixed cells” [39], and electron microscope studies show these cells can be spindle-shaped with a flattened nucleus and numerous branching processes [40]. The volume of cytoplasm and organelles inside the fibroblast depends on its activity and inactive fibroblast are known to have scanty cytoplasm with few organelles [41]. In contrast, activated fibroblasts increases its cytoplasmic activity especially when there is a need to lay down collagen fibers [42]. Fibroblasts divide to form more fibroblasts, but they cannot convert to other cell types and are prone to oxidative DNA damage and senescence [43]. Fibroblast-like cells of the IVDs aggregate in the outer annulus regions (**Figure 2**) especially during injuries, but they don't form clusters nor differentiate into cluster-like cells [30].

Cell clusters

In non-degenerate and young intervertebral discs, rounded cells of the nucleus and inner annulus often appear singly, in pairs or in small-sized clusters [44]. With age and degeneration, large clusters tend to appear in the nucleus pulposus and inner annulus regions [45]. Cell cluster can, therefore, be defined as three or more cells (**Figure 3**) in close proximity to each other within a large lacuna [46]. They have a distinct pericellular matrix rich in proteoglycans, fine fibrillar collagens, and non-collagenous molecules [47]. Clusters have been suggested to arise from cellular proliferation, increases its size, and is regarded as a histological hallmark of disc degeneration [10]. The principal mechanism behind cluster growth is not completely understood and they are suggested to arise due to cellular proliferation, as rabbit intervertebral discs in whole organ culture experiments show clusters being formed mainly by proliferation [35]. Similarly, in animal models of osteoarthritis, chondrocyte proliferation has been suggested to represent repair responses to tissue damage [48], and it has been demonstrated that compressive loading in rats promote clustering of the nucleus pulposus cells [49]. There is also evidence that disc cell proliferation can arise due

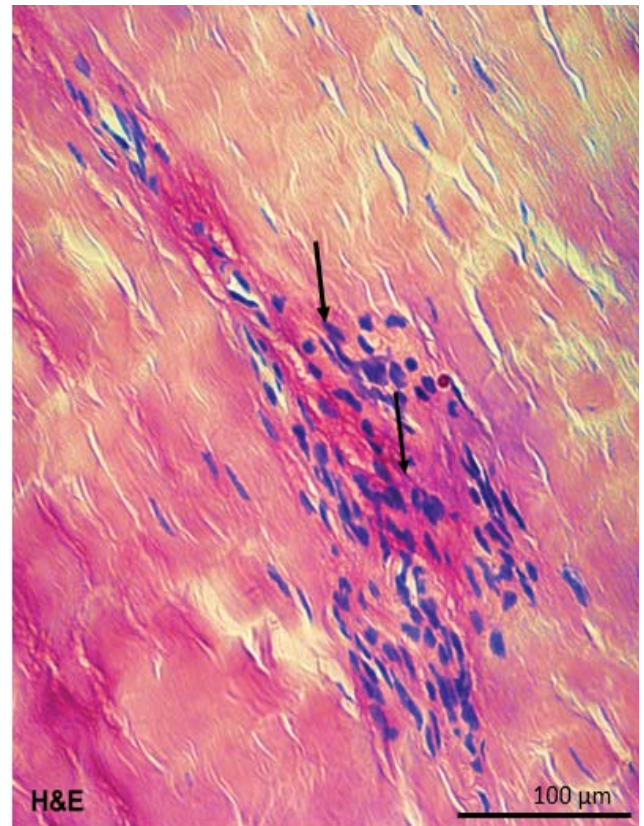


Figure 2 H&E stained fibroblasts-like cells of the annulus which are seen to exist in aggregation (arrows) in the outer annulus regions of a degenerated disc, Scale bar 100 μm (Figures adapted from Lama et. al.).

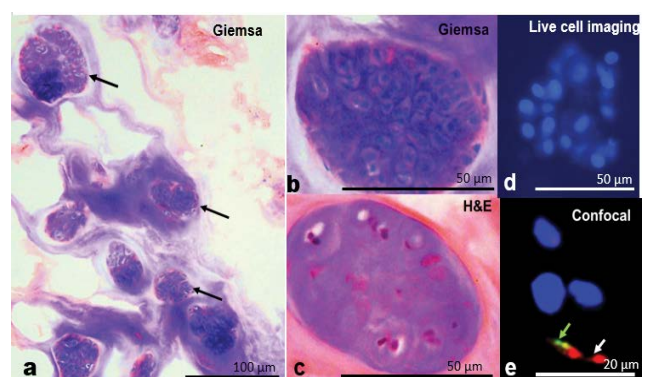


Figure 3 **a)** Groups of cell clusters around disorganised collagen lamellae in the inner annulus region of a degenerated disc with Giemsa stain. Scale 100 μm. **b and c)** H&E and Giemsa stained cell clusters under high magnification to show the cell number in each cluster, cell nuclei stained blue, scale bar 50 μm. **d)** DAPI stained cell nuclei with live cell imaging, scale 50 μm. **e)** Double immunofluorescence staining to co-localise DAPI-stained blue nuclei with integrin receptor $\alpha 5 \beta 1$ in red (white arrow) and focal adhesion kinase (green arrow) in green, scale bar 20 μm. (Figures adapted from Lama et al.).

to stimulation of chondrocytes by diffusible growth factors [50]. Similarly, cyclic mechanical compression has been shown to increase proteoglycans, collagen production and proliferation of nucleus pulposus cells [51]. However, as cell clusters are also most marked in tissues that are degenerate, clustering process can be suggested as failed tissue repair response [52], and it has also been hypothesized that increased density of cell clusters particularly those occurring at later stages of disc degeneration creates a nutrient-substrate deficit and interferes with the organization of the extracellular matrix [53].

Changes in disc cells with advancing degeneration

Disc degeneration has been suggested to be a phenomenon characterized by an imbalance between anabolism and catabolism of the matrix molecules [54]. Reduction in viable cell density has been associated with degeneration and aging. Recent research also suggests focal structural and cellular changes in the disc are often a consequence of radial fissures, disc prolapse, endplate fracture, internal or external collapse of the inner annulus [22]. Degenerative changes triggered by such intrinsic or extrinsic factors creates a stressful microenvironment for the disc cells and possibly reprograms it to increase synthesis of inflammatory cytokines [6,55] and may even allow it to enter into stages such as senescence [56,57]. The presence of senescence in the human disc cells has been shown by culturing them with B-galactosidase, and a recent study has shown that mean telomere length of the disc cells decreases as cellular expression of P16INK4A increases [58]. These studies show that cell senescence plays a direct role in disc aging and degeneration. There are two ways for the induction of cellular senescence: the first one is replicative senescence, caused by telomere shortening. Thus, replicative senescence may partly contribute to the higher percentage of senescent cells in clusters and this is also probably why senescence occurs along with the proliferating ones [59]. The second type of senescence is called stress-induced premature senescence, which results from oxidative stress [43,60] or overexpression of catabolic cytokines [61] leaving the cells unable to divide further even though they remain viable and metabolically active [62,63]. Both forms of senescence may make the disc cells function inappropriately in such a way that clusters of metabolically active senescent cells could effectively degrade a large surface area due to increased synthesis of matrix-degrading enzymes by these senescent cells and may widen the already existing fissure as cell clusters that produce degradative enzymes tend to aggregate around fissures. In general, matrix-degrading enzymes such as MMP-1, 2, 3, and 9 have been shown to increase with disc degeneration, and MMP's has been suggested to play a pivotal role in degrading the collagenous matrix of the intervertebral discs [30,64,65].

Cell clustering increases synthesis of degradative enzymes

With increasing grade of disc degeneration, cells of the nucleus and inner annulus increasingly form clusters, and clustering cells express elevated levels of matrix-degrading enzymes (**Figures 4a and 4b**) especially MMP-1, 2, 3, 7, 9, 10 and 13 [66,67]. Matrix-

degrading enzyme concentration and type (**Figure 5**) varies in the disc, with MMP-1, 3 and 13 being produced in greater quantities within the nucleus and inner annulus regions [68,69]. MMP's also tend to be localized mainly near clefts or tears (**Table 1**) in the inner annulus regions [65].

Elevated levels of matrix-degrading enzymes subsequently cleave collagen fibers, glycosylated, non-glycosylated protein and proteoglycan molecules into smaller fragments and may evoke rigorous inflammatory responses [70]. Loss of significant amount of extracellular matrix proteins and proteoglycans could additionally create a suitable environment for ingrowth of vessels and nerves.

Herniated and degenerated discs show cell clusters that tend to accumulate around fissures or proteoglycan-depleted regions (**Table 1**), and these cell cluster are usually greatest in tissues that are degenerated to Pfirrmann grade IV or V which indicates clusters may be a failed tissue repair process (10), thus depending upon whether the clusters appear in early, intermediate and

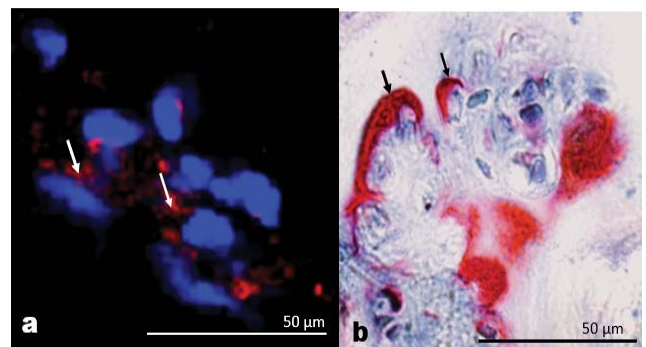


Figure 4 MMP-1 immunopositive cell clusters in its pro and active form in a herniated disc. **a)** MMP-1 positive cluster stained with Alexa red fluorophore 594 and visualised with confocal microscope, scale 50 µm **b)** Fast-red stained cell cluster, scale bar 50 µm. (Figures adapted from Lama et. al.).

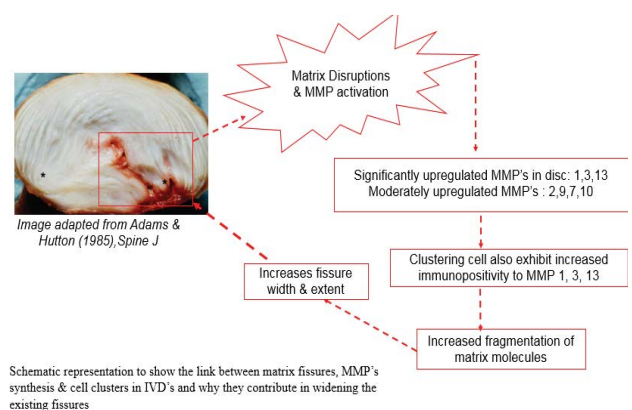


Figure 5 Schematic representation to show the link between matrix fissures, MMP's synthesis and cell clusters in IVD's and why they contribute in widening the existing fissures.

advanced stages of disc degeneration it may have an influence on the pathological state of the tissue.

Cell clusters behind attempted repair responses

Cell cluster formation mainly occurs in degenerated discs, so it has been suggested to be a distinctive feature of disc degeneration, but it can also be suggested as an attempted repair mechanism as cell clusters contain proliferating cells that are immunopositive to Ki-67 and proliferating cell nuclear antigen [10]. It has been shown that remodeling of matrix may occur by cluster formation, and this phenomenon usually involves swelling and distension of chondron allowing chondrocyte division and proliferation [71], which subsequently allow matrix synthesis. Clusters are also abundant in disc regions that show loss of fibrillar orientation [72] and are specifically located near lesions in experimental animal models [73]. Spatial association in human disc also indicates that disc cell clusters are around abundant near fissures (Figures 3a and 6a) and proteoglycan-depleted regions (Figure 6b), which is to perhaps replenish the lost proteoglycans and glycoproteins molecules. This shows that formation of clusters may occur as an intrinsic repair response to tissue damage, and it signify as a healing response that fails in the later stages of disc degeneration due to highly cross-linked collagen being beyond the repair capacities of the cells in clusters to completely remodel the matrix. Also, problems with metabolite transport, unavoidable daily loading stresses, age-related changes such as senescence, and continued exposure to environmental and genetic risk factors may progressively weaken the existing matrix molecules and attempted clustering response may not sufficiently allow adequate restoration of the matrix. In this cycle of attempted repair, especially at a later stage of disc degeneration clustering cells often abort its proliferative function by undergoing apoptosis, and may contribute towards enlarging existing fissure as the MMP's in discs do thus cell in clusters may act as a "double edged sword".

Progenitor capacities of disc cells

Recent evidence has shown that cells of the nucleus pulposus have the distinctive capacities to form colonies *ex-vivo*, and they

Table 1 Comparisons of tears/fissures, proteoglycan (PG) loss, cell clusters and MMP-1 immunopositive positive cells in nucleus pulposus (np), inner annulus (iaf) and outer annulus (oaf) regions in 'scoliotic disc' (SD), 'degenerated-in-situ' (DD) and 'herniated disc' (HD)^a.

| Variables | Scoliosis Disc (SD) | | | Degenerated Disc (DD) | | | Herniated Disc (HD) | | |
|-------------------------------------|---------------------|-----|-----|-----------------------|-----|-----|---------------------|-----|-----|
| | np | iaf | oaf | np | iaf | oaf | np | iaf | oaf |
| Tears/Fissures | + | + | + | ++ | ++ | *** | ++ | *** | *** |
| PG loss | + | ++ | + | ++ | ++ | *** | *** | *** | *** |
| Cell clusters | + | ++ | - | *** | *** | - | *** | *** | - |
| MMP-1 (cell count/mm ²) | + | ++ | + | *** | *** | *** | *** | *** | *** |

^amodified table from Lama et al. Absent: -; Rarely Present: +; Moderately Present: ++; Abundant: ***

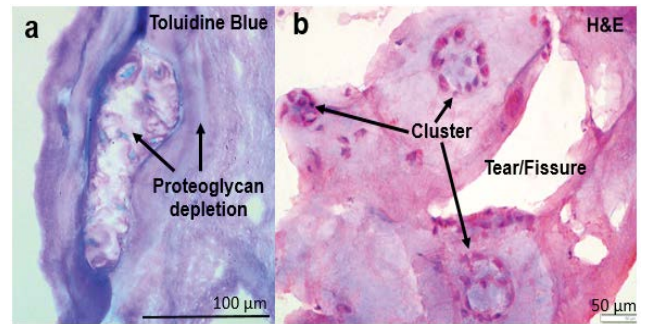


Figure 6 Cell clusters in the nucleus pulposus region of a degenerated disc to shows: **a)** diminished levels of proteoglycan staining with toluidine blue stain, scale bar 100 µm, and **b)** cell clusters localized around tears/fissures, scale bar 50 µm. (Figure adapted from Adams and Lama et al.).

Table 2 Intracellular regulators influencing disc cell cluster formations.

| Intracellular regulator | Activation | Cell clustering |
|-------------------------|---|---|
| SMADs | TGB-β on the cell surface activates SMADs protein complex during growth, differentiation, regeneration, and stress response | SMADs in the cell nucleus binds to DNA and control the activity of genes and regulate cell proliferation |
| SOX9 | Chondroprogenitor cells requires transcription factor Sox9 along with expression of Col2a1 for activation | SOX9 and Col2a1 directly regulates transcription and proliferation of chondrocytes |
| Notch-1 | Transmembrane ligand-activated protein that directly transduces extracellular signals on the cell surfaces | Notch-1 changes gene expression in nucleus that regulates interaction between physically adjacent cells |
| Integrin α5β1 | Receptor for fibronectin transduces extracellular signals by Focal Adhesion Kinases inside the cell | Regulates inflammatory cytokine production essential for human chondrocytes to enter various stages in cell cycle |

are immunopositive to stem as well as progenitor cell markers such as GD-2, a disialoganglioside that has been identified as a marker of bone marrow and umbilical cord mesenchymal stem cells [74]. Further, nucleus cells also have been shown to be immunopositive to Tie-2, a tyrosine kinase receptor that forms ligand with angiopoietin-1, that maintains the progenitor potential of the cells and protects them from apoptosis [75,76]. Progenitor populations of nucleus pulposus cells have been shown to be multipotent, and are capable of differentiating into adipocytes, osteocytes and chondrocytes [77] but these nucleus pulposus cells do not terminally differentiate as stem cell do, and maintain their phenotype as cartilage cells, a number of intercellular signalling mechanism is shown to be upregulated during proliferation and differentiation (Table 2), but understanding about these regulators and how they control accurate differentiation of the progenitor cells are still at a preliminary stage of investigation. However, with the current set

of evidences it can be rationalized that proliferating progenitor nucleus cells has the potential in restoring cell numbers and proteoglycan content which is essential for maintaining tissue homeostasis and for re-establishing biomechanical stability.

Discussion

Progenitor cell therapy

The clinical use of adult disc cells with proliferative and progenitor properties offers potential to regenerate tissue and this process requires correct identification and *ex-vivo* expansion of this progenitor cell line source in the disc. This is of importance to the present health-care environment as the use of allogeneic progenitor cells as a universal cell line could allow it to be of use in all suitable patients [78]. Cell therapy could replace damaged/apoptotic cells and restore cell numbers in injured tissues. Further, viable cells from non-disrupted disc regions can be extracted and used along with engineered constructs such as scaffolds to restore the fissured region, suitable scaffolds may temporarily resist the loss of proteoglycans from the disc and re-establish disc pressure to promote stability. Viable disc cells may also find a matrix to adhere and promote further restoration of the matrix. But, as the disc cell proliferate to form clusters they also

undergo senescence and produce mediators such as cytokines, MMPs and reactive oxygen species and these known changes has the potential to retard cell based therapies. Therefore, extended research on intervertebral disc cell and its matrix is important for successful development of less invasive cell-based therapies.

Conclusion

Cell clustering occurs in physically disrupted tissue that shows an elevated level of MMPs. Clusters may represent an attempt at repair that is frustrated by inadequate metabolite transport and is aborted by means of apoptosis. Clustering process may be repeated several times during tissue disruption to initiate a successful healing response, but these attempts fail. However, the finding of a distinct population of progenitor nucleus pulposus cells offers scope for enhancing the reparative capacities of disc tissue. Since the progenitor nucleus cells display high replicative potency strategies can be contemplated to allows these cells to successfully regenerate the matrix. Greater understanding on the intracellular mechanisms behind cell cluster formations and its regulation is of importance and in-depth knowledge behind it may even facilitate control over intrinsic healing responses that is initiated at early stages of disc degeneration.

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