An in vitro tissue model to study the effect of age on nucleus pulposus cells

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Abstract Differentiation between age (physiological) and disease-induced changes in the nucleus pulposus will facilitate our understanding of the mechanism(s) leading to the development of degenerative disc disease. The aim of this study was to develop an in vitro model that would allow the study of age-induced alterations of cell function in nucleus pulposus. Nucleus pulposus (NP) cells were isolated from intervertebral discs obtained from either calves (<9 months) or cows (>18 months). The cells were placed in culture and grown for 19 days. Although nucleus pulposus tissue was formed by the cells of the two different ages the more mature (older) cells formed less tissue as determined histologically by light microscopy. This was confirmed biochemically as the wet weight and proteoglycan content of the tissue formed by the older cells were significantly less than that of the younger tissue. The older cells accumulated less proteoglycans as determined by quantifying radioisotope incorporation. The older cells showed lower constitutive gene expression of collagen type II and aggrecan whereas collagen type I and link protein levels were similar to those of the younger cells. Metalloprotease (MMP) 13 gene and protein expression increased with age. There was no change in the levels of gene expression of MMP 2 and TIMP 1, 2, or 3 with age.

Cells obtained from NP tissue harvested from younger or mature animals showed both genotypic and phenotypic differences in vitro that resulted in the inability of the older cells to reconstitute their extracellular matrix to the same extent as the younger cells. This suggests that this in vitro NP tissue model will be suitable to determine the mechanism(s) regulating age-induced changes.

Keywords Nucleus pulposus · Aging · Tissue loss

Introduction

The intervertebral disc (IVD) consists of two interdependent tissues [31]; the nucleus pulposus which is surrounded radially by the concentric lamellae of the annulus fibrosus. Together, with the cartilage endplate, the components of the IVD anchor adjacent vertebral bodies and by doing so allow for spinal stabilization, load bearing, and movement. The nucleus pulposus is composed primarily of proteoglycans within a loose type II collagen framework [5, 23]. Aggrecan, which is the major proteoglycan, contributes to the gel-like nature of this tissue as it is highly negatively charged and is responsible for retaining the water, which enables the nucleus to resist compressive loads. Other proteoglycans such as versican and small leucine rich proteoglycans (SLRP) such as decorin and fibromodulin are also present, but their function in disc tissue is less well defined [5, 23]. As these proteins can bind growth factors they may be important for the maintenance of the nucleus pulposus. The collagen in the nucleus pulposus is believed to help resist swelling; however, this is controversial as a recent study suggested that it might not contribute to tensile properties as it does in the articular cartilage [27]. With age the IVD undergoes

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a variety of changes. The nucleus pulposus loses proteoglycans and thus water content and demonstrates an increase in proteoglycan degradation fragments, as well as changes in the amount of link protein and hyaluronate [23, 28]. Antoniou et al. demonstrated in a study of human lumbar spines that there was also decreased accumulation of newly synthesized aggrecan and type II collagen in the NP [1]. Changes in selected SLRPs such as fibromodulin have also been detected with aging in the NP [29]. Interestingly in rabbits, there is an increase in anabolic factors such as TGF β 1 and BMP 7 with age [17] suggesting that there may be resistance to growth factors perhaps as a result of down-regulation of the receptor protein as has been shown for IGF-1 [19]. In addition, increases in the levels of degradative enzymes have been detected with aging. Matrix metalloprotease (MMP)-2 expression has been shown to increase with age [32]. Other MMPs such as MMP-13 have been found in the non-degenerate disc at autopsy [22]. As well, lower levels of TIMP-3 protein, an inhibitor of MMP, has been observed in discs of adolescent rabbits as compared to 4-week-old animals [30]. It is likely that these alterations in the nucleus pulposus lead to abnormal biomechanics, and may contribute to the initiation of a cascade of events that result in disc degeneration. However, as many of the changes attributed to aging are also present in degeneration dissecting out which of these alterations are constitutive and related to age and which are associated with disease has been difficult [13]. For example, MMP-2 and MMP-13, although present in non-degenerate discs, are also believed to contribute to IVD degeneration. Delineation of age-related changes would allow study of the mechanisms regulating these processes and this could facilitate development of therapies to attenuate or prevent the development of these alterations.

As *ex vivo* nucleus pulposus tissue explant cultures cannot be used to study cell metabolism because of the tissue swelling that occurs when placed *in vitro* [4, 11], we developed the methodology to form nucleus pulposus tissue *in vitro* [25]. The tissue resembles the native nucleus pulposus and allows for the study of nucleus pulposus cells in a 3-dimensional tissue under controlled conditions. The purpose of this study was to determine if this *in vitro* model is appropriate to study age-related changes in NP tissue by examining whether nucleus pulposus cells show constitutive differences with maturation (age) in terms of matrix molecules and metalloproteases MMP-2 and MMP-13 and their inhibitors TIMP1, 2, and 3, molecules whose levels may be altered with age or disease. As the tissues formed by cells of different ages are maintained under similar conditions this study also provides insight into the effect of age on NP cells in the absence of impaired diffusion and nutrition.

Methods

In vitro nucleus pulposus tissue formation

To generate nucleus pulposus tissue *in vitro*, nucleus pulposus cells were isolated from bovine caudal spines obtained from animals of two different ages; calves up to 9 months (young) or cows between 18 and 24 months, at which time they are skeletally mature. All discs appeared normal and did not show any degenerative changes as determined by gross examination and histological examination of random tissues. The cells were isolated by sequential enzymatic digestion, as previously described [25]. Nucleus pulposus (NP) from multiple caudal discs from up to three animals were combined for each experiment to obtain sufficient numbers of cells for an experiment. Briefly, NP cells were seeded in Ham's F12 [25 mM HEPES, 10% fetal bovine serum (FBS)] on the upper surface of a porous cylindrical substrate (160,000 cells/mm²) encased in tygon tubing to prevent cell spillage over the edges. This substrate was composed of calcium polyphosphate (4 mm diameter $\times$ 2 mm height) synthesized as reported previously [20]. Cultures were transferred to Dulbecco's Modified Eagles Medium supplemented with 10% FBS 48 h later. On day 5, the FBS concentration was increased to 20%, and on day 7, ascorbic acid (100 μ g/ml) was added to the medium. These conditions were determined previously to result in nucleus pulposus tissue formation [25]. The tubing was removed at 14 days. Culture medium was changed every 2–3 days and fresh ascorbic acid was added with each change. The tissues were maintained for an additional 5 days and then harvested.

Evaluation of gene expression

Total RNA was isolated from each construct by Trizol[®] (Gibco BRL, Rockville, MD, USA) extraction following disruption of the tissue by mortar and pestle in liquid nitrogen. A total of 0.5 μ g RNA was reverse transcribed (Superscript First Strand Synthesis System, Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Relative gene expression was examined by semi-quantitative PCR using Taq Polymerase (Qiagen, Mississauga, ON, USA) and sequence-specific primers in reactions designed to amplify the template sequence of interest within the linear range. The primers for aggrecan core protein, type II collagen, type I collagen, MMP-2 and -13, and TIMP (tissue inhibitor of metalloproteases)-1, -2, and -3 have been reported previously. [26] PCR products were analyzed by electrophoresis on a 1.5% agarose/TBE gel containing ethidium bromide and visualized by UV

transillumination. The level of gene expression was semi-quantified by densitometry using LabWorks 4.0 Image analysis software (UVP BioImaging Systems, Upland, CA, USA) and corrected for 18S rRNA expression.

Proteoglycan synthesis

Twenty-four hours prior to harvest, the cultures were incubated with $^{35}\text{S}\text{-SO}_4$ (5uCi/well) to label newly synthesized proteoglycans. The tissues were harvested and digested by papain (Sigma, St Louis, MO, USA; 40 $\mu\text{g}/\text{ml}$ in 20 mM ammonium acetate, 1 mM EDTA, and 2 mM dithiothreitol) for 48 h at 65°C. The amount of radioactivity incorporated and retained in the matrix was determined using β -scintillation counter and normalized for DNA.

Proteoglycans were also precipitated from the media with 100% ethanol and resuspended in 4 M guanidinium hydrochloride. Total proteoglycan synthesis was determined by combining [^{35}S]SO₄ incorporation in both the NP tissue and culture medium.

Determination of DNA content

The tissue was removed from the substrate and lyophilized. The dry weight was measured using a Mettler balance. The tissue was then digested in papain and the DNA content determined using the Hoechst 33258 dye binding assay (Polysciences, Warrington, PA, USA) and fluorometry (emission wavelength 365 nm, excitation wavelength 458 nm), as previously described [25]. The standard curve was generated using calf thymus DNA (Sigma).

Determination of proteoglycan content

The proteoglycan content was determined by measuring the amount of sulfated glycosaminoglycans in the papain-digested tissues using the dimethylmethylene blue dye binding assay and spectrophotometry (wavelength: 525 nm) as previously described [25]. The standard curve was generated using bovine chondroitin sulfate (Sigma).

Processing for histological evaluation

The tissue was removed from the top of the substrates, fixed in 10% buffered formalin and processed for paraffin embedding. Five-micron sections were cut and stained with either toluidine blue or hematoxylin and eosin for examination by light microscopy.

Western blot analysis

The tissues were detached from the CPP, disrupted by Dounce homogenization in RIPA buffer (150 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% deoxycholate, 0.1% SDS, 2 mM EDTA) supplemented with protease inhibitor cocktail (Mini-Complete, Roche Molecular Biochemicals). Lysates were cleared by centrifugation for 15 min at 14,000g. The protein content was determined by the Pierce BCA protein assay, and equivalent amounts of protein (25 μg) were resolved by electrophoresis on 7.5% SDS-polyacrylamide gels, and transferred to nitrocellulose membranes (BioTrace NT; Pall Life Sciences, Mississauga, ON, USA) by electroblotting. Immunoblotting was performed using antibody reactive with MMP-13 and reactivity detected using HRP-conjugated goat anti-mouse IgG secondary antibodies (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), which was visualized using ECL Plus Western Blotting Detection System (Amersham Pharmacia Biotech, Buckinghamshire, UK) according to the manufacturer's instructions. The blots were stripped and re-probed for actin (1:10,000, AC-15, Sigma Aldrich, St Louis, MO, USA) to ensure equal loading of protein.

Statistical analysis

All experiments were done in triplicate and repeated at least thrice except the gene expression studies, which were done in duplicate. Student's *t* test was used to analyze the data and significance was assigned $P < 0.05$.

Results

Histological appearance of in vitro-formed nucleus pulposus tissue

The NP tissue formed in vitro after 19 days was evaluated histologically by light microscopy. The cells derived from both the young and mature animals formed a continuous layer of tissue (Fig. 1). The tissue consisted of NP cells surrounded by extracellular matrix that contained sulfated proteoglycans as indicated by toluidine blue staining. Occasional clusters of cells whose appearance, larger cells with clear vacuoles in the cytoplasm, was consistent with notochordal-like cells were seen in all tissues whether formed by the younger or older cells. The nucleus pulposus tissue formed by cells from the older cows appeared thinner with less extracellular matrix than that formed by the younger NP cells. This difference was confirmed by quantifying the weight and proteoglycan content of the two tissues. As shown in Table 1, there was significantly less

Fig. 1 Histological appearance of nucleus pulposus tissue formed in vitro. The nucleus pulposus tissues formed by younger (**a, b**) and older (mature) cells (**c, d**) were removed from the substrate at 19 days and processed for histological examination by light microscopy. Occasional clusters of cells with the appearance of notochordal-like cells (*arrow*) are present in both tissues. Asterisk indicates surface closest to the CPP substrate (toluidine blue)

tissue formed by the older cells ($P < 0.01$). As well the nucleus pulposus tissue formed by calf cells accumulated significantly more proteoglycans per cell than the NP tissue formed by cells from the older animals ($\mu\text{GAG}/\mu\text{gDNA}$: young = 99 ± 9.5 ; older = 27 ± 2.5 ; mean $\pm$ SEM, $n = 9$, $P < 0.01$).

Tissue cellularity

To determine if the difference in the amount of tissue was related to the cellularity of the tissue, the DNA content was determined. Although the tissue formed by the older cells had a slightly higher DNA content, the two tissues showed no significant difference in the amount of DNA present (Table 1) suggesting that differences in cellularity was not

the underlying mechanism leading to impaired tissue formation at this time.

Effect of age on proteoglycans synthesis and accumulation

To determine if there was a difference in the ability of the younger and older cells to produce and accumulate matrix molecules, the amount of proteoglycans synthesized and retained by the cells in the NP tissue was quantified. The younger cells synthesized significantly more proteoglycans ($P = 0.035$) than the older cells (Fig. 2a). Furthermore, more proteoglycans were retained in the tissue formed by the younger cells than that formed by the older NP cells ($P = 0.003$) suggesting that degradation of proteoglycans may also be occurring in the older tissue (Fig. 2b).

Table 1 Tissue physical properties

	Dry weight (μg)	DNA (μg)	Proteoglycan content ($\mu\text{g}/\mu\text{g DNA}$)
Young	1.4 ± 0.07	3.9 ± 0.68	99 ± 9.5
Mature	$1.0 \pm 0.07^*$	4.8 ± 0.60	$27 \pm 2.5^*$

All results expressed as mean $\pm$ SEM, $n = 9$

*Significantly different from young, $P < 0.01$

Expression of extracellular matrix genes

The gene expression of aggrecan, link protein and types I and II collagen was examined to determine whether there was differential expression with age (Fig. 3). The cells in the tissue formed by calf NP cells expressed higher levels of aggrecan core protein and collagen type II than the older

Fig. 2 Proteoglycan synthesis and retention. **a** The NP tissue was incubated with [³⁵S]SO₄ for 24 h prior to harvest. The media was collected and the proteoglycans released into the media were precipitated. The tissues were removed from the substrate and papain digested. The amount of radioactivity incorporated in the media and the tissue were quantified and combined to determine total proteoglycan synthesis by young and mature NP cells. **b** The percent retention of newly synthesized proteoglycans in NP tissue formed by the young and older NP cells was determined. The experiment was repeated thrice and the results combined and expressed as the mean ± SEM. **P* < 0.05 between the younger and older (mature) NP cells

cells. Not all matrix genes showed differential expression as there was no difference in the expression levels of type I collagen or link protein with age.

Expression of degradative enzymes and their inhibitors

As there was a difference in the percent retention of proteoglycans with age, the NP cells were examined for expression of MMP-2 and -13, enzymes that degrade extracellular matrix, as well as the inhibitors of metalloproteases TIMP-1, -2, and -3. The constitutive expression of MMP-13 was greater in the older cells than the younger NP cells (Fig. 4). The younger cells demonstrated variable

*significant difference from mature, *p* < 0.05

Fig. 3 Matrix gene expression in nucleus pulposus tissues. The nucleus pulposus tissue formed by young and older (mature) cells was removed from the substrate at 19 days and RNA extracted. Gene expression was determined by RT-PCR as described under the Sect. “Methods”. **a** Representative agarose gel from an experiment repeated thrice. **b** Gene expression was semi-quantified by densitometry and corrected for 18S rRNA expression. The data was combined and expressed as mean ± SE of three experiments

expression of these proteases but all tissues formed by older cells expressed these genes. There was no difference detected in the average constitutive expression of MMP-2 or TIMP-1, -2, or -3 between the younger and older cells.

To determine whether the increased gene expression of MMP-13 resulted in increased protein levels, protein expression was evaluated by western blot analysis. Tissues formed by the older cells had higher levels of both total and active MMP-13 (Fig. 5).

Discussion

This study demonstrated using nucleus pulposus tissue formed in vitro by NP cells obtained either from calves (6–9 months) or more mature cows (18–24 months), with no evidence of disc disease, that as the cells mature they develop constitutive changes in cell metabolism. The older cells formed less tissue than the younger cells, a result of synthesis of less matrix as well as decreased accumulation. The older cells expressed lower levels of aggrecan and type II collagen mRNA when compared to the younger cells. There was decreased synthesis of proteoglycans by the older cells. As well, there was decreased retention of newly synthesized proteoglycans in the extracellular matrix suggesting that there may be degradation occurring. The older

Fig. 4 Gene expression of matrix metalloproteases (MMP) and their inhibitors (TIMP) in nucleus pulposus tissues. The nucleus pulposus tissue formed by young and older (mature) cells was removed from the substrate at 19 days and RNA extracted. Gene expression was determined by RT-PCR as described under the Sect. “Methods”. **a** Representative agarose gel from an experiment repeated thrice. **b** Gene expression was semi-quantified by densitometry and corrected for 18S rRNA expression. The data was combined and expressed as mean $\pm$ SE of three separate experiments

cells were shown to express higher levels of MMP-13 at both the gene and protein levels. Many of these changes are similar to those detected in the aging human disc. For example in the older nucleus pulposus there is decreased accumulation of proteoglycans [1] and expression of degradative enzymes, such as MMP-13 [10]. The data

Fig. 5 Western blot analysis of MMP 13 protein expression. Total protein was extracted from nucleus pulposus tissue formed by young or older (mature) cells, separated by SDS-PAGE and immunoblotted for MMP-13 (**a**) or actin (**b**). The actin served to ensure there was equal loading of the samples. This is a representative blot from an experiment repeated thrice

suggest that this model is appropriate to study the factors regulating aging changes in NP.

The absence of a change in MMP-2 gene expression was unexpected as increased MMP-2 protein and gelatinase activity have been shown to occur in vivo with age in the human NP [22, 32]. However, the results of those other studies are not directly comparable to ours as they did not examine gene expression levels and there are other ways to increase gelatinase activity without altering gene expression. For example, MMP-2 gelatinase activity can be upregulated via ternary complex formation with MT1-MMP and TIMP-2 [8]. As we have shown in other experiments that TNF α induces gelatinase activity via this mechanism in NP cells (data not shown), further study is required to determine if changes in MMP-2 protein and/or activity levels occur with age in this in vitro model. TIMP-3 gene expression also did not change in our model, which differs from that described for aging rabbit discs [30]. However, the NP in rabbits is very different than bovine and human NP in that they are rich in notochordal cells. It may be that the TIMP changes detected in rabbits are species-specific and do not occur in human/bovine discs.

It is possible that the changes observed in this study were an artifact of culture, but this is considered less likely for several reasons. First, the NP tissue generated by calf cells in this culture system was characterized previously and shown to have selected biochemical and mechanical properties in keeping with the native nucleus pulposus from which the cells were obtained [25]. Second, there is maintenance of a small number of notochordal-like cells similar to that observed in the in vivo discs of the young and older cows [25]. Third, the NP cells in the in vitro-formed tissue can respond to pro-inflammatory stimuli similar to the in vivo cells as they produce proteases when exposed to TNF α . [26] Fourth, the younger and older NP cells were treated identically and yet behaved differently in vitro. Chelberg et al. also observed that disc cell phenotype can be maintained in vitro [3]. Others have also showed age-related changes can be detected in vitro. For example, Maeda and Kokubun observed age-dependant changes in proteoglycan synthesis. However this was a study of annulus fibrosus cells and not nucleus pulposus cells [14].

In our culture system the cells are maintained in a 3D tissue and not in monolayer culture and are already equilibrated to the tissue culture environment. It is well known that when the intact disc is placed in ex vivo culture it will swell approximately fivefold, and although this swelling can be minimized by culturing the disc under constraint by tubing or agarose there are still changes occurring when they are placed in vitro [4, 11]. This could alter nucleus pulposus cell biosynthesis and potentially obscure differences which is why in vitro-formed NP tissue might be better suited to study cell biosynthesis than ex vivo cultures

of native NP. However, the in vitro-formed NP tissue does differ from the native NP as it lacks the surrounding annulus fibrosus and is not maintained under hypoxic conditions. These may affect NP cell metabolism but it is not known how or in which way. Studies are currently under way to determine the effect of AF tissue on NP cells.

The constitutive changes we observed confirm that NP metabolism changes with age prior to the development of disease. It is not clear why the NP cell undergoes these changes in the production of matrix molecules and degradative enzymes with age but these alterations have been observed in other tissue types as they age [2, 6, 12, 16]. Alterations with age in potential regulatory processes such as signal transduction mechanisms, receptor levels, telomerase expression, and responsiveness to growth factors have been implicated [15, 18, 19, 24]. In the NP, it has been suggested that the changes that occur with age arise as a result of the decreasing vascularization of this tissue which may limit nutritional supply to the cells [7]. This is somewhat controversial as increased vascularization of the disc can occur with disease [9, 21]. Clearly the changes observed in the NP with age in our model were unrelated to nutrition as it was not limited in the tissue culture system used in these studies. This model should be able to delineate the mechanisms responsible for the changes that occur with age.

In summary, using a model system of in vitro-formed NP tissue age-related changes in constitutive levels of gene and protein expression of molecules such as proteoglycan, and MMP-13 were observed. This correlated with the formation of less tissue by the more mature (older) NP cells when compared to cells obtained from younger animals. This study confirms that the cells of different ages can retain genotypic and phenotypic differences in vitro under these culture conditions and that this approach may be appropriate to study the mechanisms regulating biosynthesis in young and older cells in the absence of nutritional and structural changes in order to identify ways to inhibit aging alterations and perhaps prevent the progression to degenerative disc disease.

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