Senescence in human intervertebral discs

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Abstract Intervertebral discs demonstrate degenerative changes relatively early in life. Disc degeneration, in turn, is associated with back pain and disc herniation, both of which cause considerable clinical problems in the western world. Cell senescence has been linked to degenerative diseases of other connective tissues such as osteoarthritis. Thus we investigated the degree of cell senescence in different regions of discs from patients with different disc disorders. Discs were obtained from 25 patients with disc herniations; from 27 patients undergoing anterior surgery for either back pain due to degenerative disc disease ($n = 25$) or spondylolisthesis ($n = 2$) and from six patients with scoliosis. In addition, four discs were obtained post-mortem. Samples were classified as annulus fibrosus or nucleus pulposus and tissue sections were assessed for the degree of cell senescence (using the marker senescence-associated- β -galactosidase (SA- β -Gal)) and the number of cells present in clusters. There were significantly more SA- β -Gal positive cells in herniated discs (8.5% of cells) than those with degenerative disc disease, spondylolisthesis, scoliosis, or cadaveric discs

(0.5% of cells; $P < 0.001$). There was more senescence of cells of the nucleus pulposus compared to those of the annulus fibrosus and in herniated discs a higher proportion of cells in cell clusters (defined as groups of three or more cells) were SA- β -Gal positive (25.5%) compared to cells not in clusters (4.2%, $P < 0.0001$). This study demonstrates an increased degree of cell senescence in herniated discs, particularly in the nucleus where cell clusters occur. These clusters have been shown previously to form via cell proliferation, which is likely to explain the increased senescence. These findings could have two important clinical implications: firstly, that since senescent cells are known to behave abnormally in other locations, they may lead to deleterious effects on the disc matrix and so contribute to the pathogenesis and secondly, cells from such tissue may not be ideal for cell therapy and repair via tissue engineering.

Keywords Herniation · Degeneration · Cell clusters · Stress-induced-premature-senescence (SIPS) · Catabolism

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Introduction

Normal human cells can undergo only a limited number of cell divisions in vitro. Beyond this they will not proliferate and are described as being senescent. Replicative senescence is a different process from programmed cell death and senescent cells remain viable, often for a long time, but with an altered morphology, phenotype and gene expression. For example, senescent cells can become less responsive to growth factors and other anabolic stimuli [14], produce

increased amounts of matrix metalloproteinase enzymes [17] and generally demonstrate a deterioration in cell function.

The accumulation of senescent cells in vivo with increasing age and with their altered pattern of gene expression, implicates cellular senescence in ageing and age-related pathologies [3]. Intervertebral discs demonstrate degenerative changes very early in life, with mild changes seen in the first decade of life and more significant changes from the second decade onwards [4]. Disc degeneration in turn is associated with back pain [10] and disc herniation [19], both of which are major clinical problems in their own right.

Although cell number, morphology and cell death have been investigated in the intervertebral disc, to our knowledge the phenomenon of cell senescence has not been studied systematically in human discs, particularly in relation to disease. Therefore in this study we undertook to use a biomarker of senescence, senescence associated β -galactosidase (SA- β -Gal) [5], to examine senescence in cells of discs from patients with either herniated discs or discogenic back pain. We hypothesised that intervertebral disc cells, like other cell types, would be liable to senesce and that senescence may be involved in the aetiopathogenesis of age-related disc degeneration.

Materials and methods

Discs were obtained from 25 patients with disc herniations, from 27 patients undergoing anterior surgery for either back pain due to degenerative disc disease or spondylolisthesis and from six patients undergoing corrective surgery for scoliosis (see Table 1 for details). Herniated discs (18 protrusions, four extrusions and three sequestrations) were differentiated into pieces of annulus fibrosus and nucleus pulposus where appropriate by the surgeon (and confirmed morphologically by microscopic examination); both regions were available for 21 samples and four consisted of nucleus pulposus only. Anterior surgery samples were more in tact, all of them incorporating outer and inner annulus with 15 also including some nucleus. In addition, discs were obtained at autopsy from four individuals, Articular cartilage was obtained from two patients; both aged 69 years, undergoing knee arthroplasty for osteoarthritis.

Senescence was assessed by the presence of SA- β -galactosidase; β -galactosidase is a lysosomal enzyme produced by all cells with optimal activity at pH 4.0 (L- β -Gal). Senescent cells produce greatly increased

Table 1 Patient details

Pathology	Age (years)		Levels
	Mean $\pm$ Standard deviation (<i>n</i>)	Range	
Disc herniation	41 $\pm$ 10 (25)	23–64	2 $\times$ L2-3; 9 $\times$ L4-5; 14 $\times$ L5-S1
Discogenic back pain	38 $\pm$ 8 (25)	22–60	11 $\times$ L4-5; 14 $\times$ L5-S1
Spondylolisthesis	28 and 36		2 $\times$ L5-S1
Scoliosis	24 $\pm$ 12 (6)	9–42	2 $\times$ T11-12; 1 $\times$ T12-11; 1 $\times$ L1-2; 1 $\times$ L2-3; 1 $\times$ L3-4
Post-mortem	46 $\pm$ 32 (4)	21–99	1 $\times$ T11-12; 2 $\times$ L4-5; 1 $\times$ L5-S1

amounts of the enzyme such that activity can be demonstrated at pH 6.0 [5]. At least 50 mg wet weight of tissue to be stained for SA- β -Gal was immersed for 24 h at 37°C in freshly prepared 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside (1 mg/ml 40 mM citric acid/sodium phosphate pH 6, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM MgCl₂; Sigma, UK), titrated to pH 6.0 [6]. Lysosomal (non-senescent) β -galactosidase activity was detected in adjacent tissue using the same solution but at pH 4.0. After incubation the tissue was briefly rinsed in ice-cold phosphate buffered saline (PBS), snap frozen and stored in liquid nitrogen. Cryosections (10 μ thick) were cut, fixed in 3% paraformaldehyde in PBS for 5 min at room temperature and counterstained with eosin. Osteoarthritic human articular cartilage was examined as a positive control [15]. By counting the number of positively stained (blue) cells in 200 cells in random fields across the section, the degree of cell senescence was assessed. The number of positive and negative cells present in cell clusters (defined as a group of three or more cells) was noted and from this the proportion of those, which were SA- β -Gal positive in clusters, was determined.

For statistical analyses, non-parametric statistical analyses (Mann–Whitney *U* test and Spearman rank correlations) were carried out using a software programme (Analyse-it Software Ltd, Leeds, UK). All values are quoted as means $\pm$ standard deviations.

Results

All samples of intervertebral disc and articular cartilage demonstrated non-senescent lysosomal β -Gal in

most cells (Fig. 1a, b). SA- β -Gal, in contrast, was present in far fewer cells. In osteoarthritic cartilage, for example, cells within cell clusters and at the fibrillated surface were SA- β -Gal positive (Fig. 1c). This is the same distribution as reported previously for osteoarthritic cartilage [15].

In the intervertebral disc the frequency of SA- β -Gal staining of cells was very variable, ranging from no cells to 39% ($n = 62$) of the cell population. There were significantly more SA- β -Gal positive cells in herniated discs (8.5%; $n = 25$) than those with degenerative disc disease, spondylolisthesis, scoliosis or cadaveric discs [$0.9\% \pm 2$ (range 0–16%; $n = 37$); $P < 0.001$; see Table 2]. In addition, there was more senescence in cells of the nucleus pulposus compared to those of the annulus fibrosus (for herniated discs: annulus 5%; $n = 21$, nucleus 13%; $n = 25$, $P = 0.009$; Fig. 2). Although numbers of post-mortem samples were very limited, they contained a higher percentage of senescent cells, the greater the age of the individual; the 21 year old had no senescent cells, the 28 year old had 0.5%, the 43 year old had 3% and the 92 year old had 2% SA- β -Gal positive cells. However, when the percentage of senescent cells was plotted against age for all the samples studied there was no significant correlation.

Cells in clusters (defined as groups of three or more cells) were more frequently SA- β -Gal positive ($11.8 \pm 26\%$; Fig. 3) than single or doublet cells ($2.1 \pm 4.1\%$). In herniated discs there was an even greater difference, with 25.5% (range 0–31%) of cells in clusters being SA- β -Gal positive compared to 4.2% (range 0–17%) of cells not in clusters ($P < 0.0001$). Clusters were mostly found in the nucleus pulposus, in 15 of 25 nucleus samples from herniated discs but only 8 of 27 were obtained via anterior surgery. SA- β -Gal positive cells were found in 14 of those 15 herniated

nucleus samples with clusters, but only one of the eight nucleus samples was obtained by anterior surgery.

No significant differences were found with type of prolapse (protrusion, extrusion or sequestration) or with time interval between onset of symptoms and surgery (which varied from 2 weeks to 10 years).

Discussion

The present study demonstrates for the first time that human intervertebral disc cells stain for the SA- β -Gal biomarker. This suggests that, like all other cell types investigated, they are subject to senescence. Replicative senescence is believed to be a protective mechanism for the individual against tumourigenesis [14]. However, because senescent cells have an altered, and often more catabolic metabolism, the trade off for this anti-tumour protection appears to be “ageing”. This has been suggested as one of the reasons why “Dolly”, the cloned sheep (using cells which have undergone many replications), and her siblings had premature arthritis and early ageing [18]. Replicative senescence can be clearly demonstrated in cells in vitro, with cell types having a typical number of cell divisions that they will undergo (alternatively known as the “Hayflick number”) [8] before ceasing replication and entering growth arrest in the transition phase G1–S of the cell cycle [14]. Human fibroblasts will undergo approximately 60 and chondrocytes 35 population doublings, respectively.

Martin and Buckwalter [12] have reported cell senescence in chondrocytes from articular cartilage; they found that the incidence of SA- β -Gal positivity in these cells in culture increased from $<0.1\%$ of cells from donors younger than 10 years old to $>0.4\%$ from those over 70 years of age. They conclude that

Fig. 1 Lysosomal- β -galactosidase was seen in most cells of all samples of intervertebral disc (a) and articular cartilage (b). Senescence associated- β -galactosidase (SA- β -Gal), in contrast, was limited to cells in clusters and near the fibrillated surface in

osteoarthritic cartilage (c). **a** Disc from 27-year-old disc herniation. **b,c** Articular cartilage from a 69 year old with osteoarthritis. Original magnification: **a** 10 $\times$, **b** 60 $\times$ and **c** 240 $\times$

Table 2 Frequency of SA- β -Gal staining in different patient groups

Pathology	% SA- β -Gal positive cells	Range
Disc herniation (both regions)	8.5 ± 8.5	0–39
Annulus	5 ± 9.8	0–39
Nucleus	13 ± 12.7	0–37
Discogenic back pain/spondylolisthesis (both regions)	0.9 ± 3.0	0–16
Annulus	0	
Nucleus	0.9 ± 3.0	0–16
Scoliosis	0	
Post-mortem	1.4 ± 1.4	0–3

chondrocyte senescence contributes to an increased susceptibility of osteoarthritis with age. There is a similar incidence in tissue sections of cadaveric discs in the present study with the younger individuals having the lowest incidence (0 and 0.5% senescent cells in discs of 21 and 28 year olds, respectively, and 2% in a 92 year old). The age dependence was not significant but the number of normal samples examined in this study was very limited. However, herniated discs have almost 10 $\times$ as many senescent cells as discs obtained at autopsy or anterior surgery. There was a much greater incidence of SA- β -Gal positive cells in cell clusters than in the population of single or doublet cells. Since

we have demonstrated previously that these cell clusters form by cell replication [9], this would seem to be a plausible explanation for this higher incidence on first inspection. However, when the incidence of clusters is examined in herniated discs in comparison, it becomes clear that other factors must also be involved. Other types of cells have been shown in some circumstances to become senescent prematurely. For example, high levels of oxygen or loading can lead to stress induced premature senescence (SIPS) of chondrocytes [11] [20] and fibroblasts [16]. The adult intervertebral disc, being virtually avascular, has lower oxygen content than most places elsewhere in the body. It would appear, however, that disc cells are well adapted to existing in such an environment, being capable of surviving at 0% oxygen [2]. When discs herniate, particularly if they become extruded or sequestered, the cells are likely to experience greatly increased access to oxygen, either via vascularisation within adjacent tissues or of the herniation itself (a common occurrence) [13]. In addition, the prolapsed disc is likely to experience a different loading pattern from normal and this in turn has been shown to lead to an increase in reactive oxygen species and hence to increased cell senescence in articular cartilage [11].

Within the anterior fusion samples one stood out as being very different from the rest, with 88% of cells within clusters being SA- β -Gal positive. On close

Fig. 2 Cells of the annulus fibrosus (**a**) were less often SA- β -Gal positive than those of the nucleus pulposus (**b**). Disc from 27 year old with disc herniation. Original magnification: 240 $\times$. Arrows negative cells

Fig. 3 Cells within clusters of herniated discs demonstrated the greatest frequency of SA- β -Gal positivity. Disc from a 47 year old with disc herniation (**a, b**). Original magnification: 240 $\times$

examination of the clinical and radiographic history of this patient it became obvious that, although surgery had been carried out for discogenic back pain, the magnetic resonance image interestingly demonstrated a small protrusion of this disc. This was not the case for any other such samples. Although it is dangerous to read too much into one sample, this does support the general results seen with other herniated discs in this study.

The relevance of the findings of this study is twofold. Firstly this demonstration of cellular senescence within disc cells suggest that they could, via their decreased anabolism and increased catabolism, be important in the pathogenesis of disc degeneration. Secondly, it could be very important to the current interest in biological therapies, both tissue engineering and genetic engineering. These techniques depend for their success on metabolically active cells. Hence if autologous intervertebral disc cells are used to implement such treatments, they may not function optimally, particularly if taken from herniated discs [7]. This reiterates the suggestion by Alini et al. [1] that these biological approaches are not currently appropriate to treat disc degeneration, but they may be in the future when we have a better understanding of disc cell biology.

Conclusion

Results from this study support our hypothesis that disc cells demonstrate senescence. However, the apparent increased degree of senescence seen in cells in herniated discs compared to non-herniated suggests that these cells have been induced to premature senescence by some environmental factor. The development of senescence in disc cells may be important not only in understanding the disease process, but also in providing guidance as to the appropriate optimal source of cells for cellular therapy in the future.

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