ELSEVIER

The Spine Journal 9 (2009) 210–215

THE SPINE JOURNAL

Increased cell senescence is associated with decreased cell proliferation in vivo in the degenerating human annulus

Helen E. Gruber, PhD*, Jane A. Ingram, BS, Daniel E. Davis, MS, Edward N. Hanley, Jr., MD

Department of Orthopaedic Surgery, Carolinas Medical Center, PO Box 32861, Cannon Research., Room 304, Charlotte, NC 28232, USA

Received 10 December 2007; accepted 30 January 2008

Abstract

BACKGROUND CONTEXT: During disc degeneration, there is a well-recognized loss of cells. This puts the remaining cell population at high risk for any further decrease in cell function or cell numbers. Cell senescence has recently been shown to be present in the aging/degenerating human disc. Senescent cell are viable, metabolically active, persist, and accumulate over time, but cannot divide. Little is known about the relationship between renewal of the disc cell population via cell proliferation and disc cell senescence.

PURPOSE: To determine the percentage of senescent cells and proliferating cells in the human annulus in vivo.

STUDY DESIGN/SETTING: Human annulus specimens were obtained from surgical subjects and control donors in a study approved by the authors' Human Subjects Institutional Review Board.

PATIENT SAMPLE: One Thompson Grade I disc, 4 Grade II discs, 9 Grade III discs, and 12 Grade IV discs were studied.

OUTCOME MEASURES: The percentages of senescent cells and the percentage of proliferating cells.

METHODS: Immunohistochemistry was used to detect senescent cells using an antisenescence-associated beta-galactosidase antibody, and an antiproliferation antibody (Ki67). An average of 410 cells/specimens was counted to determine the percent senescence, and an average of 229 cells was counted to determine the percent proliferation.

RESULTS: Cell proliferation was low in both surgical and control normal donor annulus tissue (4.09%+1.77 (26), mean+SD (n)). There was no significant difference in the percentage of proliferating cells for more degenerate discs versus healthier discs (4.7%+1.6 (21) for Grades III and IV vs. 5.3%+1.9 (5) for Grades I and II). More degenerated Grades III and IV discs contained significantly greater percentages of senescent annulus cells than did the healthier Grades I and II discs (44.4%+20.0 (21) vs. 18.8%+11.0 (5), respectively; $p=.011$). A significant negative correlation was present between the percentage of senescent cells versus the percentage of proliferating cells, $r=-0.013$, $p=.013$. No correlation was present between age and the percentage of senescent cells or age and the percentage of proliferating cells.

CONCLUSIONS: Because senescent cells cannot divide, senescence may reduce the disc's ability to generate new cells to replace cells lost to necrosis or apoptosis. Senescent cells also accumulate in the disc over time, such that their metabolic patterns may contribute to the pathologic changes seen in degenerating discs. Novel data presented here show a significant negative correlation between the percentage of senescent cells and the percentage of proliferating cells during disc degeneration. Molecular work is underway in our lab to help us determine whether senescent cells in the disc secrete factors that can result in decreased proliferation in neighboring cells.

Keywords: Disc degeneration; Cell proliferation; Cell senescence; Immunohistochemistry

FDA approval status: not applicable.

The authors do not have a financial relationship that creates, or may be perceived as creating, a conflict related to this article.

Support in whole or in part was received from the North American Spine Society, a nonprofit foundation.

* Corresponding author. Department of Orthopaedic Research Biology, Carolinas Medical Center, PO Box 32861, Cannon Research, Room 304, Charlotte, NC 28232, USA. Tel.: (704) 355-5665; fax: (704) 355-5620.

E-mail address: helen.gruber@carolinashealthcare.org (H.E. Gruber)

1529-9430/09/$ – see front matter
doi:10.1016/j.spinee.2008.01.012

Introduction

Cell senescence (also termed replicative senescence) occurs when normal cells stop dividing. This phenomenon was initially described more than 40 years ago during studies of cultured human fibroblasts [1]. Senescent cells are viable, but exhibit alterations in phenotype and altered gene expression patterns [2–5]. Senescent cells may have altered responsiveness to external stimuli and may secrete factors, which can influence neighboring cells or their nearby extracellular matrix. There is currently a great deal of interest in the manner in which cell senescence may contribute to age-associated loss of function or age-related pathology in vivo, and molecular studies are directed toward elucidating mechanisms and pathways which activate the senescence program in cells [6].

The current views of cell senescence not only recognize that it is a condition in which cells can no longer respond to mitogenic signals and thus cannot proliferate, but also point out that senescence is associated with alterations in nuclear structure, protein processing, gene expression, and cell metabolism. The senescent state is a response to specific trigger(s) or multiple signaling pathways, including telomere uncapping, oxidative stress, DNA damage, and oncogene activation [3,7,8]. Senescence represents a general cellular response mechanism which, when activated, results in numerous morphologic and functional changes [2].

Our laboratory and two other groups have recently published papers pointing to the importance of senescence in the disc. Roberts et al. have provided evidence that there was a greater proportion of senescent cells in herniated than non-herniated discs, and more senescent cells in the nucleus pulposus compared with the annulus [9]. Our laboratory showed that that the proportion of senescent cells increased significantly with increasing stages of disc degeneration ($p<.0001$) [10]. The third paper on disc cell senescence came from Le Maitre et al. and determined that the senescent disc cell phenotype is associated with increased catabolism involving metalloproteinase 13 and aggrecanase [11]. This finding is important because it links senescence with matrix degradation, one of the major problems in disc degeneration. This study also showed that disc cells exhibit accelerated senescence with decreased telomere length. (Shortening of telomeres [the nucleoprotein complexes at the ends of chromosomes] has been suggested as one of the hypotheses to explain senescence.) An additional publication from our group provided information on senescence using laser capture microdissection and microarray analysis [12]. We identified two senescence-related genes, which were significantly up regulated in more degenerated discs compared with healthier discs: growth arrest-specific 1 gene (GAS) (which inhibits DNA synthesis, inhibits cell cycle progression in vitro, and is expressed in senescent fibroblasts [13,14]), and lysyl oxidase-like 2, which is expressed in senescent human fibroblasts [15].

EVIDENCE & METHODS

Context
Because of the significance of disc degeneration and the loss of disc cells as the degenerative process progresses, it is important to understand the mechanism by which the cells are lost. This is particularly important in the biological treatments and strategies toward disc regeneration or prevention of degeneration in which growth factor or gene transfer strategies are used to target disc cells, or if strategies need to introduce new cells into the disc.

Contribution
The results of this study show that overall there is low cell proliferation in all annulus tissues in control and degenerative discs, showing that this is a very difficult tissue to proliferate and renew itself. There was no significant difference for the percent proliferating cells for the more degenerative discs versus the healthier discs, however, the greater grades of degeneration contain greater percentages of the senescent annulus cells than the healthier grades, respectively. It also appears that as the percent senescent cells goes up, the percent proliferating cells goes down.

Implications
Cell senescence and the factors controlling these metabolic events may be primary factors in disc degeneration. The disc environment is austere and this article allows insight into potential mechanisms where the disc cells are lost and no longer renewing themselves.

– The Editors

In the present study, we examine the percentage of cells in human annulus specimens and investigate the relationship of proliferation to cell senescence.

Methods

Source of disc tissue

Experimental study of disc specimens was approved prospectively by the authors' Human Subjects Institutional Review Board. Patient specimens were derived from surgical discectomy procedures. Surgical specimens were transported to the laboratory (less than 30 minutes after surgical removal) in sterile tissue culture medium and placed in

10% neutral buffered formalin. Care was taken to remove all granulation tissue and to sample only disc tissue. Two normal donor specimens were also used in this study, obtained via the National Cancer Institute Cooperative Human Tissue Network; it was shipped overnight to the laboratory in sterile tissue culture medium and placed in 10% neutral buffered formalin.

Histologic specimen preparation

Specimens were fixed in 10% neutral buffered formalin (Allegiance, McGaw Park, IL) overnight and then transferred to 70% ethyl alcohol (AAPER, Shelbyville, KY) and held for paraffin processing using a Shandon Pathcentre Automated Tissue Processor (ThermoShandon, Pittsburgh, PA). Specimens were embedded in Paraplast Plus (ThermoShandon) paraffin and 4 μm serial sections were cut with a Leica (Nussloch, Germany) RM2025 microtome. Sections (serial or adjacent) were mounted on Superfrost-Plus microscope slides (Allegiance) for immunohistochemistry as described below.

Immunocytochemical localization of senescence-associated ß-galactosidase

Slides were deparaffinized in xylene and hydrated through graded alcohols to distilled water. The remainder of the procedure was performed using the Dako AutostainerPlus (Dakocytomation, Carpenteria, CA). Endogenous peroxidase was blocked using 3%H_20_2 (Sigma, St Louis, MO) in methanol (Allegiance, McGaw Park, IL). Slides were incubated for 1 hour with the anti-β-galactosidase antibody (Promega Corporation, Madison, WI) at a 1:25 dilution. The secondary antibody used was LSAB2 Link Antibody (DakoCytomation) for 10 minutes followed by peroxidase-conjugated streptavidin (Dakocytomation) for 10 minutes and DAB (diaminobenzidine) for 5 minutes. Slides were removed from the stainer, rinsed in water, counterstained with light green, dehydrated, cleared, and mounted with resinous mounting media. Negative control slides were processed as described above but with the absence of the primary antibody.

Immunocytochemical localization of proliferating cells

Localization of proliferating cells using anti-Ki67 antibody was carried out as described above using the Ki67 antibody (DakoCytomation) at a dilution of 1:50; negative controls used anti-mouse IgG (DakoCytomation) at 1:50.

Cell counts

The percentage of cells positive for localization of ß-galactosidase or Ki67 was determined using the OsteoMeasure software of OsteoMetrics, Inc. (Decatur, GA) at a magnification of 200×. The overall mean number of cells scored to obtain the percentage of senescent cells was 410+180 (26) (mean+SD (n)), and for the percentage of proliferating cells was 229+99(26). The proliferation counts scored a lower total number of cells because only nuclei showed positive immunolocalization.

Table 1
Demographic features for specimens studied for immunocytochemical localization of senescence-associated ß-galactosidase and Ki67[a]

Subject #	Site	Thompson score	Gender	Age	Other information
1	L	I	M	23	Surgical
2	L	II	F	29	Surgical
3	L	II	F	18	Surgical
4	L	II	M	31	Surgical
5	L	II	F	16	Surgical
6	L	III	F	47	Surgical
7	L	III	F	68	Control donor; cause of death: PE
8	L	III	F	33	Control donor; cause of death: stroke
9	L	III	F	40	Surgical
10	L	III	M	37	Surgical
11	L	III	M	44	Surgical
12	L	III	M	37	Surgical
13	L	III	M	57	Surgical
14	L	III	F	26	Surgical
15	L	IV	F	45	Surgical
16	C	IV	M	58	Surgical
8	L	IV	F	33	Control donor
17	C	IV	M	42	Surgical
18	L	IV	F	56	Surgical
19	L	IV	M	59	Surgical
20	L	IV	M	55	Surgical
21	L	IV	F	34	Surgical
22	L	IV	M	56	Surgical
23	L	IV	F	59	Surgical
23	L	IV	F	59	Surgical
24	L	IV	F	46	Surgical

[a] Twenty-six discs were studied from 24 subjects.
L, lumbar; C, cervical; M, male; F, female; PE, pulmonary embolism.

Statistical analyses

Statistical analysis used standard methods using GraphPad InStat, version 3.06 (San Diego, CA). A p value of .05 was considered statistically significant. Analyses performed included t tests and regression analysis.

Results

Twenty-six discs from 24 subjects were evaluated in this study. Table 1 summarizes the demographic data for the study population. Mean subject age was 42.3 years+14.3 (mean+SD) with a range from 16 to 68 years. The Thompson grading system was used to score disc degeneration over the spectrum of stages from Thompson Grade I (a healthy disc) to discs with advanced degeneration (Thompson Grade V) [16]. The study group included 1 Thompson Grade I disc, 4 Grade II discs, 9 Grade III discs, and 12 Grade IV discs.

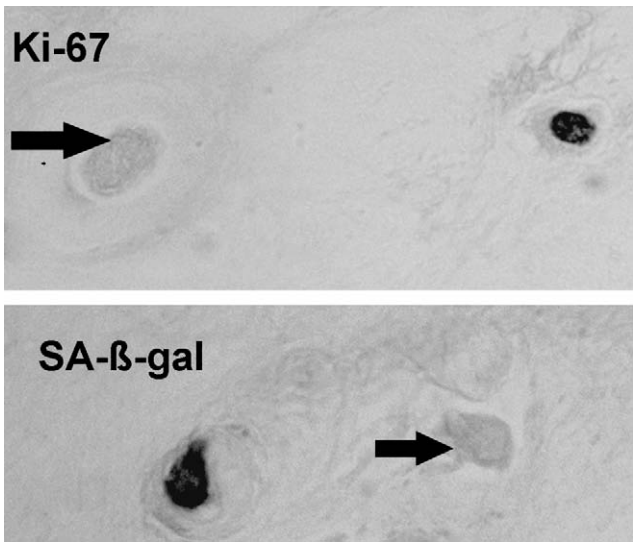

Fig. 1. Immunocytochemical localization of (top) proliferating disc cells using Ki67 and of (bottom) senescent disc cells using senescence-associated-ß-galactosidase. Dark localization product marks positive cells; note the presence of negative cells that lie nearby (arrows). (SA-ß-gal, senescence-associated-beta-galactosidase.)

Cell proliferation findings

Fig. 1 (top) presents a representative image of immunolocalization of the Ki67 antibody in a cell of the human annulus in vivo. Note the presence of a nearby cell, which is not dividing and thus shows no localization product. The percentage of proliferating cells was low in the study population: 4.09%+1.76 (26) with a range from 1.81% to 9.33%. The three normal donor discs also showed a low proportion of dividing cells (3.8%, 3.7%, and 1.8%). No correlation was present between the percentage of proliferating cells and subject age (p=.48). There was no significant difference in the percentage of dividing cells in healthier Grade I/II discs compared with Grade III/IV discs (Fig. 2).

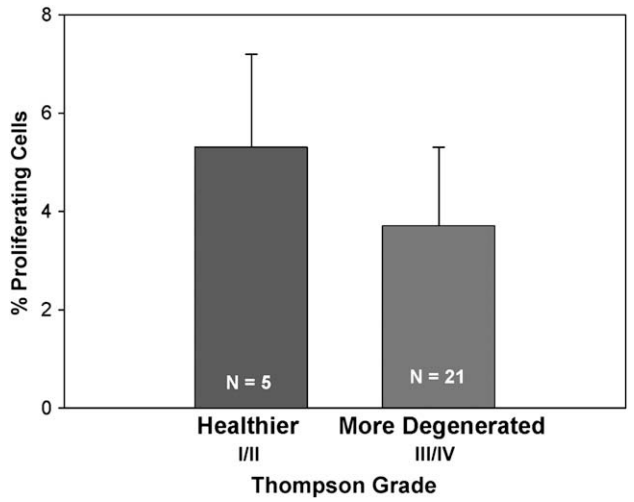

Fig. 2. The proportion of proliferating cells in healthier Thompson Grade I/II discs (dark bar, left) did not significantly differ from that present in more degenerated Grade III/IV discs (lighter bar, right). Bars show means+SD; n values are shown within the respective bars.

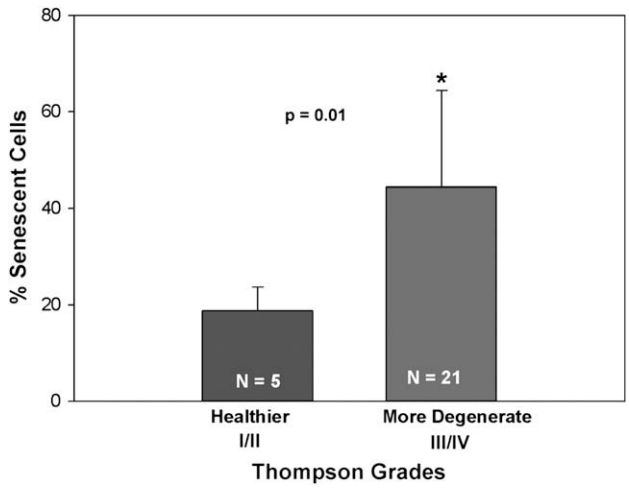

Fig. 3. The proportion of senescent cells was significantly greater in more degenerate Thompson Grade III/IV discs (lighter bar, left) compared with that present in healthier Grade I/II discs (dark bar, right), *p=.01. Bars show means+SD; n values are shown within the respective bars.

Cell senescence findings

The study population contained specimens from 12 herniated discs and 16 nonherniated specimens. Statistical analysis showed that there was no significant difference in the percentage of senescent cells in the herniated versus nonherniated groups (herniated: 35.16%+12.5 (mean+SD) versus nonherniated: 38.87+20.66).

Cytoplasmic localization of antisenescence-associated-ß-galactosidase antibody is shown in Fig. 1 (bottom). Note the presence of nearby healthy (non-senescent) cells, which show no localization product. The overall percentage of senescent cells in the study population was 39.55%+21.1% (26) with a range from 0.002% to 76.4%. As shown in Fig. 3, there were significantly more senescent

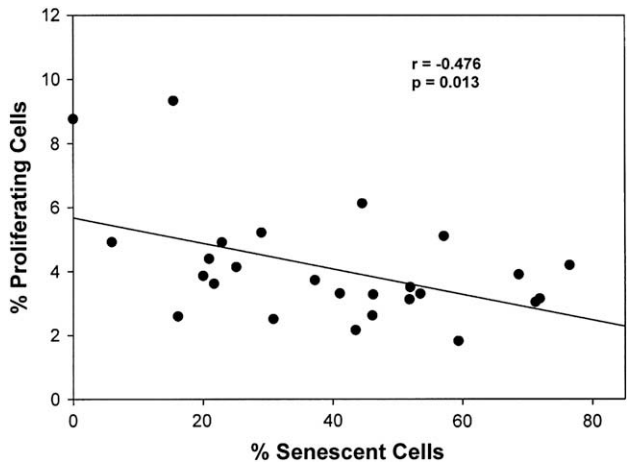

Fig. 4. A significant negative correlation was present between the percent senescent cells and the percent proliferating cells (r=−.476, p=.013).

cells in the Thompson Grade III/IV discs compared with the healthier Grade I/II discs (p=.01). No relationship was present between the percentage of senescent cells and subject age (p=.28).

A significant negative relationship was present between the percentage of senescent ells and the percentage of proliferating cells as shown in Fig. 4 ($r^2=-0.227$; p=.013).

Discussion

In the present work, we examined tissue from the annulus fibrosus. Ultimately, it is the extracellular matrix of the annulus fibrosus, which fails in disc degeneration; dehydration and matrix fraying culminate in the formation of tears within the annulus during biomechanical loading and torsion. This is believed by many to be the major source of incapacitating low back pain conditions. Additionally, nucleus and annulus disc material may rupture through these tears, causing radicular pain. Long-standing disc degeneration and mechanical insufficiency may result in adaptive hypertrophic changes in the adjacent osseous and soft-tissue structures, resulting in spinal stenosis syndromes in later life.

It has long been appreciated that the healthy human intervertebral disc contains a small cell population; with aging and degeneration, this population decreases even further [17]. This puts the remaining cell population at high risk for any further decrease in cell function or cell numbers. Cell senescence has recently been shown to be present in the aging/degenerating human disc [9,10,18]. Senescent cell are viable, metabolically active, persist and accumulate over time, but cannot divide. Little is known about the relationship between renewal of the disc cell population via cell proliferation and disc cell senescence.

The work presented here showed a low percentage of proliferating cells in vivo in the annulus. The antibody used to localize proliferating cells was Ki67, a reliable, commonly-used marker, which is detected only in proliferating cells; we chose this marker over proliferating cell nuclear antigen because proliferating cell nuclear antigen may also localize in cells which have recently undergone arrest [19]. It should also be noted that there were two cervical specimens in the study population with percent senescence levels of 41.08% and 71.2%, and percent proliferation values of 3.3% and 3.0%.

Previous work by other investigators has also found a low proliferation rate in the disc. Paajanen et al. studied lumbar prolapsed disc tissue with immunolocalization of Ki67 and reported a percent proliferation ranging from 0.2% to 2.6% [20]. Ford et al. also used immunolocalization of Ki67 in a study of five prolapsed discs, and reported that few cells showed localization but did not quantify the fraction of proliferating cells [21].

Peng et al. reported that 6.24% of the cells in a study of 16 discs showed proliferation, and a 9.18% level in nongranulation tissue in discs from patients with low back pain [22]. These investigators also reported a higher proliferation rate present in the disc granulation zone of their study subjects who reported pain (40.85%); the authors suggested that this higher level may possibly be in response to growth factors present in these regions.

One other report quantified the proportion of proliferating cells in the human disc. Johnson et al. have shown proliferation in disc cells present in clusters [19]. Cells that were not found in clusters showed a low proportion involved in proliferation (1–7%). This study reported that proliferation in the inner annulus of degenerating discs was higher than the outer annulus. In addition, the proportion of proliferating cells in the inner annulus and in the nucleus correlated with the stages of disc degeneration of the specimens.

Our present work appears to be the first to investigate the relationship between cell proliferation and cell senescence in the same disc specimens. Our work showed that there was a significant, negative correlation between cell senescence and cell proliferation (Fig. 4, p=.013) such that discs with greater proportions of senescent cells contained lower proportions of proliferating cells. This correlation model showed that 22% of the change in proliferation could be explained by the presence of cell senescence. Correlations, however, do not establish a cause and effect relationship. Thus, further research is essential to further identify factors influencing the low cell proliferation rate in the disc, and to determine if, in fact, senescent cells can actually reduce or prevent normal disc cells from proliferating, and to determine the actual effect of the accumulations of senescent cells over time.

Is there any molecular evidence of why such generally low proliferation rates are present in the degenerating disc? A previous study from our lab used laser capture microdissection to harvest cells from disc tissue and then examine gene expression patterns of these cells with microarray analysis [12]. GAS, which inhibits DNA synthesis, inhibits cell cycle progression in vitro, and is expressed in senescent fibroblasts [13,14,23], was found to be significantly up regulated in more degenerated discs compared with healthier discs. In the developing mouse embryo, GAS was seen by Lee et al. to only be expressed by chondrocytes after cartilage started to differentiate [24]. It is interesting that GAS1 expression has been found to positively correlate with inhibition of endothelial cell apoptosis in endothelial cells [25]; this potential role of GAS1 in disc cells is unexplored to date.

Clearly, the cell factors and microenvironmental factors, which control disc cell proliferation, an important component of the disc's reparative response, are poorly understood. Current research is underway in our laboratory to investigate whether GAS is expressed by both senescent and nonsenescent cells, and to determine if senescent disc cells may have secretory products, which reduce proliferation rates in nearby cells.

Acknowledgments

We gratefully acknowledge funding for this research by the North American Spine Society, and also thank The Brooks Center for Back Pain Research for general support. We also thank Mrs. Natalia Zinchenko for expert technical help.

References

[1] Hayflick L. The limited in vitro lifetime of human diploid cell strains. Exp Cell Res 1965;37:614–36.
[2] Ben-Porath I, Weinberg RA. The signals and pathways activating cellular senescence. Int J Biochem Cell Biol 2005;37:961–76.
[3] Ben-Porath I, Weinberg RA. When cells get stressed: an integrative view of cellular senescence. J Clin Invest 2004;113:8–13.
[4] Campisi J. Senescent cells, tumor suppression, and organismal aging: good citizens, bad neighbors. Cell 2005;120:513–22.
[5] Campisi J. The biology of replicative senescence. Eur J Cancer 1997;33:703–9.
[6] Chen J, Goligorsky MS. Premature senescence of endothelial cells: Methusaleh's dilemma. Am J Physiol Heart Circ Physiol 2006;290: H1729–39.
[7] Ehrenreiter K, Piazzolla D, Velamoor V, et al. Raf-1 regulates Rho signaling and cell migration. J Cell Biol 2005;168:955–64.
[8] Oshimura M, Barrett JC. Multiple pathways to cellular senescence: role of telomerase repressors. Eur J Cancer 1997;33:710–5.
[9] Roberts S, Evans EH, Kletas D, Jaffray DC, Eisenstein SM. Senescence in human intervertebral discs. Eur Spine J 2006;15(Suppl 3): S312–6.
[10] Gruber HE, Ingram JA, Norton HJ, Hanley EN Jr. Senescence of cells in the aging and degenerating intervertebral disc. Immunolocalization of senescence-associated β-galactosidase in human and sand rat discs. Spine 2007;32:321–7.
[11] Le Maitre CL, Freemont AJ, Hoyland JA. Accelerated cellular senescence in degenerate intervertebral discs: a possible role in the pathogenesis of intervertebral disc degeneration. Arthritis Res Ther 2007;9:R45.
[12] Gruber HE, Mougeot J-L, Hoelscher GL, Ingram JA, Hanley EN Jr. Microarray analysis of laser capture microdissected-anulus cells from the human intervertebral disc. Spine 2007;32:1181–7.
[13] DelSal G, Ruaro ME, Philipson L, Schneider C. The growth arrest-specific gene, gas1, is involved in growth suppression. Cell 1992;70: 595–607.
[14] Gonos ES. Expression of the growth arrest specific genes in rat embryonic fibroblasts undergoing senescence. Ann N Y Acad Sci 1998;851: 466–9.
[15] Pascal T, Debacq-Chainiaux F, Chrétien A, et al. Comparison of replicative senescence and stress-induced premature senescence combining differential display and low-density DNA arrays. FEBS Lett 2005;579:3651–9.
[16] Thompson JP, Pearce RH, Schechter MT, Adams ME, Tsang IKY, Bishop PB. Preliminary evaluation of a scheme for grading the gross morphology of the human intervertebral disc. Spine 1990;15: 411–5.
[17] Buckwalter JA. Aging and degeneration of the human intervertebral disc. Spine 1995;20:1307–14.
[18] LeMaitre CL, Freemont AJ, Hoyland JA. Accelerated cellular senescence in human intervertebral disc degeneration. Transactions of 52nd Annual Meeting, Orthopaedic Research Society, Abstract #411. 2006. Ref Type: Abstract.
[19] Johnson WEB, Eisenstein SM, Roberts S. Cell cluster formation in degenerate lumbar intervertebral discs is associated with increased disc cell proliferation. Connect Tiss Res 2001;42:197–207.
[20] Paajanen H, Haapasalo H, Kotilainen E, Anapuu M, Kettunen J. Proliferation potential of human lumbar disc after herniation. J Spinal Disord 1999;12:57–60.
[21] Ford JL, Jones P, Downes S. Cellularity of human annulus tissue: an investigation into the cellularity of tissue of different pathologies. Histopathology 2002;41:531–7.
[22] Peng B, Hao JH, Hou S, et al. Possible pathogenesis of painful intervertebral disc degeneration. Spine 2006;31:560–6.
[23] Evdokiou A, Cowled PA. Growth-regulatory activity of the growth arrest-specific gene, GAS!, in NIH3T3 fibroblasts. Exp Cell Res 1998;240:359–67.
[24] Lee KKH, Leung AKC, Tang MK, et al. Functions of the growth arrest specific 1 gene in the development of the mouse embryo. Dev Biol 2001;234:188–203.
[25] Spagnuolo R, Corada M, Orsenigo F, et al. Gas1 is induced by VE-cadherin and vascular endothelial growth factor and inhibits endothelial cell apoptosis. Blood 2004;103:3005–12.