LABORATORY INVESTIGATION

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Disparity between MR Imaging and Histochemical Grading in Human Intervertebral Disc Degeneration

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Objective : In order to establish the index of degeneration, the authors performed a histochemical study with Safranin-O staining and investigated the occurrence of apoptosis in the human intervertebral disc.

Methods : Eighteen intervertebral disc specimens surgically extracted from the patients and two additional specimens from the autopsied cases were stained with Safranin-O for proteoglycan according to a standard protocol. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate- biotin nick end labeling(TUNEL) was used to detect the fragmented DNA known to be associated with apoptotic cell death and classification scheme was formulated for categorization of the degree of Safranin-O staining (normal, moderate reduction, faint) by modification of Makin's histological-histochemical grading. The Kruskal-Wallis H test and Chi-square test were used for statistical analysis.

Results : The statistical results showed a significant difference in the mean age between "normal" Safranin-O staining group and the others (19.3 versus 55, 43.4, p=0.021). However, there was no statistically significant correlation between Safranin-O staining and MR grading of disc degeneration. Only six of eighteen surgical specimens and none in autopsies showed positive apoptotic cells in TUNEL staining.

Conclusion : The determination of the degree of degeneration in surgically obtained disc tissue *per se* by histochemical staining or by the degree of apoptosis that corresponds to its morphologic change was not feasible.

KEY WORDS : Disc Degeneration · Histochemical grading · Safranin O · Apoptosis.

Introduction

Human intervertebral discs undergo age-related degenerative changes that contribute to some of the most common causes of impairment and disability for middle aged and older persons : back stiffness or pain and neck pain. Various radiologic, biochemical, and structural alterations take place during the process of aging and degeneration of the intervertebral disc and these changes have been used as a scale for the grading system of disc degeneration [5,23]

The degree of degeneration has widely been documented by the grading systems based on its morphologic change, whether by its gross look or its appearance in the magnetic resonance imaging(MRI), in the evaluation of intervertebral disc degeneration. However, a microscopic or histochemical grading system of disc degeneration that is suitable to match these morphologic changes has not been established yet. As an alternative, the degree of apoptosis has previously been proposed as a scale of disc degeneration by many investigators.

Since Gruber et al. first reported in 1998 apoptotic cell death in the lumbar disc annulus, many authors have used apoptosis as a scale of disc degeneration[9]. However, an apoptosis as a natural phenomenon, its incidence in the normal undiseased discs, or its degree in the various stages of disc degeneration has not been adequately clarified. In the current study, the authors performed a histochemical study with Safranin O staining and investigated the occurrence of apoptosis to establish the index of degeneration in the human intervertebral disc.

Materials and Methods

Eighteen specimens of intervertebral disc were extracted from the individuals with a herniated disc, spondylolisthesis,

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or spinal stenosis after the performance of each surgical procedure. Two additional specimens extracted from autopsied cases were also included. Among the 18 patients, the male to female ratio was 1:1 and the mean age of the patients was 46.7 years (range; 17 to 71 years). The specimens from autopsied cases were from a six year-old male and a 30 year-old female. Of the 20 specimens, 10 were obtained from L4-5, 8 from L5-S1, 2 from L3-4 (Table 1).

For the proper preparation of surgical specimens, the authors carefully tried to avoid from mixing the cartilaginous endplate or outer annulus fibrosus with surgically obtained disc tissues. Specimens were fixed in 4% glutaraldehyde and were also cryofrozen in Tissue-Tek O.C.T. Compound (Miles, Elkhart, IN) and kept in a freezer at -70°C until sectioning. Samples were sectioned (10㎛ thick), and stained with Safranin-O for proteoglycan according to a standard protocol. In brief, hydrated sections were sequentially immersed for 5 minutes in hematoxylin, 5 minutes in running tap water, 4 minutes in 0.02% fast green, 10 seconds in 1% acetic acid, and then 6 minutes in 0.1% Safranin-O. For the routine light microscopic examination, specimens embedded in paraffin were stained with Toluidineblue (Fig. 1).

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) was used to detect the fragmented DNA known to be associated with apoptotic cell death (Fig. 2). End labeling was performed by use of the Apoptag Plus in situ apoptosis detection kit (ONCOR, Gaithersburg, MD). Counter-staining was performed with Propidium iodide.

The degree of disc degeneration by morphology was evaluated according to the criteria of Pfirrmann et al (Table 2)[19] for MRI classification of disc degeneration. The classification scheme was formulated for the categorization of the degree of Safranin-O staining (normal, moderate reduction, faint) by modification of Mankin's histological-histochemical grading[16] (Fig. 3).

The Kruskal-Wallis H test and Chi- square test were used for statistical analysis and P-value less than 0.05 was accepted as statistically significant.

Fig. 1. A photograph of microscopic finding after ×200 (A) and ×400 (B) magnification of disc specimen stained with Toluidine blue after having embedded in paraffin.

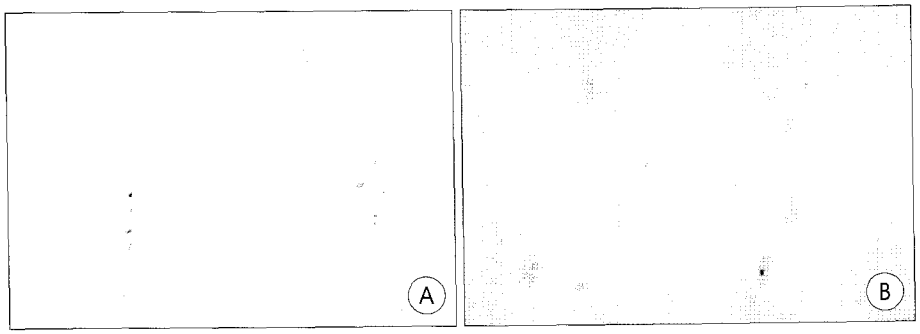

Fig. 2. Photographs of the disc specimen stained with TUNEL that was used to detect the fragmented DNA known to be associated with apoptotic cell death (A, B). End labeling was performed by use of the Apoptag Plus in situ apoptosis detection kit and counter-staining was performed with Propidium iodide.

Results

The results are summarized in Table 3 to 5. Three specimens were classified as "normal" for Safranin-O staining. Seven were classified as "moderate reduction" and 10 as "faint". The Kruskal - Wallis test results showed a significant difference

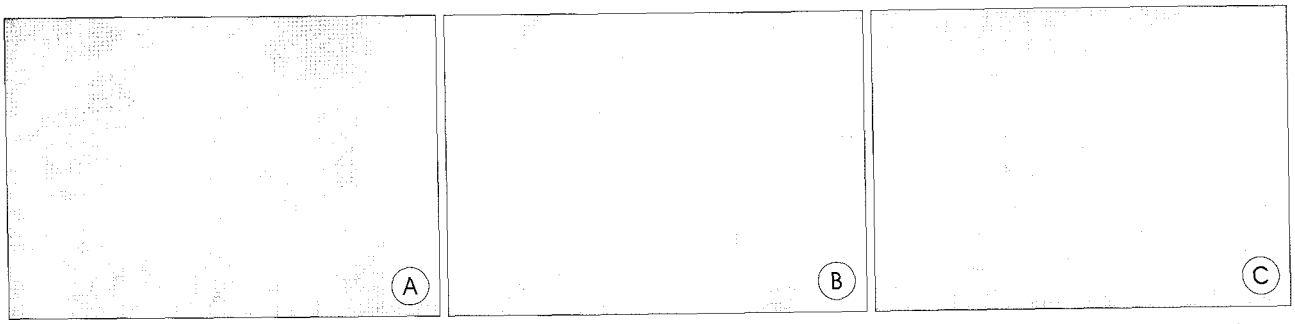

Fig. 3. Sample photographs of each class of the disc specimens stained with Safranin O after×200 magnification. The classification scheme was formulated by categorizing the degree of Safranin-O staining into 'normal' (A), 'moderate reduction' (B), and 'faint' (C) after modifying the Mankin's histological-histochemical grading.

Table 1. Demographic data of surgical specimens

No.	Age	Sex	Level	Diagnosis*	MR grading	Safranin O staining	TUNEL staining†
1	22	F	L5S1	HIVD	3	faint	6/17(35%)
2	17	M	L45	HIVD	3	faint	8/19(42%)
3	51	M	L5S1	HIVD	4	faint	0/14
4	48	M	L45	HIVD	4	faint	12/26(46%)
5	56	M	L5S1	Spondylolisthesis	5	moderate	0/21
6	55	M	L45	HIVD	4	moderate	0/4
7	68	F	L45	HIVD	5	faint	4/6(67%)
8	49	F	L34	HIVD	4	moderate	0/20
9	71	M	L5S1	Spinal stenosis	4	moderate	0/33
10	49	F	L45	HIVD	4	moderate	0/29
11	57	F	L45	Spondylolisthesis	5	faint	6/32(19%)
12	57	M	L45	HIVD	3	moderate	0/16
13	31	F	L5S1	HIVD	4	faint	0/23
14	22	M	L5S1	HIVD	3	normal	0/18
15	51	M	L5S1	HIVD	3	faint	0/12
16	48	F	L45	Spondylolisthesis	4	moderate	0/11
17	41	F	L34	HIVD	4	faint	8/15(53%)
18	48	F	L5S1	HIVD	5	faint	0/11
19	30	F	L45	Autopsy	-	normal	0/28
20	6	M	L45	Autopsy	-	normal	0/20

*HIVD : herniated intervertebral disc, †TUNEL staining : Number of positive cells/Number of total cells in 30 high power fields

Table 2. Classification of disc degeneration on MRI[19]

Grade	Structure	Distinction of Nucleus and Anulus	Signal Intensity	Height of Disc
I	Homogeneous, bright white	Clear	Hyperintense, isointense to CSF	Normal
II	Inhomogeneous with or without horizontal bands	Clear	Hyperintense, isointense to CSF	Normal
III	Inhomogeneous, gray	Unclear	Intermediate	Slightly decreased
IV	Inhomogeneous, gray to black	Lost	Intermediate to hypointense	Moderately decreased
V	Inhomogeneous, black	Lost	Hypointense	Collapsed

Table 3. Comparison of mean age among three different Safranin O staining groups

Safranin-O staining	N	Mean age	Standard deviation
Normal	3	19.3	12.2
Moderate reduction	7	55.0	8.0
Faint	10	43.4	15.9
Total	20	43.9	17.2

The Kruskal-Wallis test results showed a significant difference in the mean age between "normal" safranin O staining group and the others (p=0.021)

in the category of the mean age between "normal" Safranin-O staining group and the other two groups (19.3 vs 55, 43.4, p = 0.021; Table 3). However, there was no statistical correlation between the degree of Safranin-O staining and MR grading of disc degeneration (Chisquare test, p = 0.417; Table 4). There was no statistically significant difference in the category of age and the distribution of MR grade between the cell cloning positive and negative group (Table 5).

Only six of eighteen surgical specimens and none in autopsied cases showed positive apoptotic cells in TUNEL staining. Therefore, a statistical analysis for the correlation between TUNEL staining and disc degeneration was not performed.

Discussion

The degree of degeneration has widely been documented by the grading systems based on its morphologic change in the evaluation of intervertebral disc degeneration[7,19,20,24]. A comprehensive knowledge of the changes in the biologic behavior of the cells and in the matrix component of the disc is a new challenging area in the research of pathophysiologic mechanism of the disc degeneration. Therefore, a histochemical grading system of disc degeneration that could explain and pair with the degree of changes in the disc morphology, whether by its gross look or its appearance in the magnetic resonance imaging(MRI), is required. However, a microscopic or histochemical grading system of disc degeneration that is suitable to match these morphologic changes has not been established yet. As an alternative, the degree of apoptosis has previously been proposed as a scale of disc degeneration by many investigators. In the sequence of these proposals, some authors have reported correlation between the degree of apoptosis and the age. However, they found no direct correlation between the degree of apoptosis and the degree of disc degeneration[3,9,17,18].

Collagens and proteoglycans are the primary structural components of the intervertebral disc macromolecular framework. The matrices of disc components differ significantly in their relative amounts of these two structural macromolecules[5,22,23]. Proteoglycan and water concentration decrease with aging. Safranin-O is a cationic dye that binds specifically to polyanions, one dye molecule to each negatively charged group of either chondroitin 6-sulphate or keratan sulphate. Due to these properties, the intensity of Safranin-O staining correlates positively with the fixed charge density in the cartilage matrix

Table 4. Relation between Safranin-O staining and MR grading

		MR grading			Total
		III	IV	V	
Safranin – O staining	Normal	1	–	–	1
	Moderate reduction	1	5	1	7
	Faint	3	4	3	10
Total		5	9	4	18

There was no statistical significance between the degree of Safranin-O staining and MR grading of disc degeneration (Chi-square test, P>0.05)

Table 5. The degree of correlation between age, MR grading, the degree of staining by Safranin-O, and cell cloning determined by p-value

	Age	MR grade	Safranin-O	Cloning
Age		0.164	0.021	0.141
MR grade	0.164	–	0.417	0.892
Safranin-O	0.021	0.417	–	0.531
Cloning	0.141	0.892	0.531	–

and has been used for semiquantitive estimation of proteoglycans in cartilage tissue. The Safranin-O staining was included in histological-histochemical grading system of articular cartilage by Mankin et al[16].

Since Kerr et al[12] first reported in 1972 that the apoptosis is a form of cell death that is distinct from necrosis, it has been considered as a final common pathway of cell death in various conditions such as cancer, ischemia, and spinal cord injury. Gruber et al[9] pioneered the study of apoptosis in disc degeneration in 1998. They quantified the incidence of apoptotic cell death in the annulus and compared its quantity between the tissues from the diseased subjects and normal control. Their findings revealed that there is a high incidence of apoptosis in the intervertebral disc. In their another study [10], they even reported that insulin-like growth factor-1 (IGF- 1) and platelet-derived growth factor (PDGF) had anti-apoptotic effects on human intervertebral disc cells *in vitro*.

Since Gruber's report, the estimated degree of apoptosis has been considered and reflected as a scale of disc degeneration by several authors. Lotz et al[15,26] loaded mouse-tail discs *in vivo* with an external compression device and demonstrated that apoptotic cell death was proportional to the compressive stress and the time of loading. Although mechanical stress has been regarded as an important modulator of the degeneration, the underlying molecular mechanism remains unclear. Some pathologic experiment reported the induction of apoptosis by increased caspase-9 activity and decreased mitochondrial membrane potential after mechanical overload[21] while others reported the mere increase of the number of apoptotic cells depending on the weight of the load[4]. Other report demonstrated that apoptosis in the cartilaginous endplate of mouse spondylosis model increased with age and resulted in a marked decrease in cell density[3]. However, to the authors' knowledge, an apoptosis as a natural phenomenon, its incidence in the normal discs, or its degree in the various stages of disc degeneration has not been adequately clarified[11,17,18].

In the present study, apoptotic cells were detected in only six of eighteen (33%) surgical specimens and none in autopsied cases. This finding may suggest that apoptotic cell death is not a wide spread phenomenon in normal or even in the degenerated disc tissue. Although the TUNEL detection method preferentially identifies apoptotic cells, it can also label necrotic cells during the late period as well as viable cells with a high trasnscriptional activity[8,14,25]. Therefore, the TUNEL method is vulnerable to a high probability of false-positive results. The demonstration of the typical apoptotic morphology of fragmented, shrunk nuclei with condensed chromatin is required, in addition to TUNEL, to identify apoptosis[2,14].

Whether the apoptosis can be used as an index of disc degeneration, especially during the early stage of degeneration when its morphologic change is not evident, is still on a debate. Aigner T et al[1] previously had reported negatively to this suggestion in their study with human articular knee cartilage. On the contrary, there are some in vivo animal experiments that indicate the autocrine or paracrine fashion of disc tissue's response to apoptosis-triggering substances (i.e. Fas/ Fas-L, bcl2, P53), suggesting a potential mechanism for apoptosis of notochordal cells in normal animal nucleus pulposus[6,13]. In the authors' opinion, TUNEL method combined with electron microscopic study, other immunohistochemical assay (i.e. poly (ADP-ribose) polymerase (PARP) p85 immunohistochemistry) of apoptosis-triggering substances[6], or assay of apoptosis-inhibiting substances (i.e. Two mitogen-activated protein kinase (MAPK))[4] may contribute to solve that question.

The present study demonstrated significant differences only in the category of the mean age between "normal" Safranin-O staining group and the other two groups. This could be interpreted as a by-product from several pitfalls in the present study. Most of the patients in the current study had advanced degree of disc degeneration on the magnetic resonance images and therefore the range of disc degeneration included in the statistical analysis consists of only three grades, from Grade 3 to 5. Consequently, this biased distribution of the subjects may have affected the statistical results. Moreover, the degree of Safranin-O staining was categorized by an arbitrary classification scheme.

As previously noted, we could not draw any conclusion about the possible parity between the MR imaging and histochemical grading in human intervertebral disc degeneration Further studies that is based on the standardized quantitative Safranin-O or TUNEL staining estimation method and that

include earlier stages of disc degeneration are mandatory to draw more reliable conclusions regarding the effect of age and disc degeneration on the Safranin-O or TUNEL staining of disc tissue.

Conclusion

The present study suggests that the determination of the degree of degeneration in surgically obtained disc tissue per se by histochemical staining or by the degree of apoptosis was not feasible. Further studies that is based on the standardized quantitative histochemical staining estimation method and that include more diverse stages of disc degeneration are mandatory to draw more reliable conclusions regarding the effect of age and disc degeneration on the histologic staining pattern of disc tissue.

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Commentary

The biologic basis for healthy disc tissue is appropriate disc cell function but the cell biology and the cell pathology of the disc are as yet poorly understood. Various radiologic, biochemical, and structural alteration take place during the process of aging and degeneration of the intervertebral disc and these changes have been used as a scale for the grading system of disc degeneration.

The role of apoptosis in the lumbar disc degeneration has been reported by investigators but it is still controversial because apoptosis is observed in the tissue not only of normal healthy discs but also in various stages of disc degeneration.

The authors stained the disc samples with Safranin O and used TUNEL staining to detect the fragmented DNA which is known to be associated with apoptotic cell death for the investigation of apoptosis to establish the index of degeneration in the human intervertebral disc that would match the morphologic changes of the degenerated lumbar disc on MR imaging, and they suggested that there is no significant corr-

elation between the extent of apoptosis and the various gradings of MR imaging of the degenerated lumbar discs.

In this study, the numbers of disc samples were too small to draw a documented result from. As the authors pointed out, more specific staining techniques have to be developed to demonstrate the apoptotic cells in the degenerated disc tissue as there is a comprehensive understanding about the role of apoptotic cell death in disc degeneration, a new challanging area for many investigators.

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