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PRIMARY IMMUNE SYSTEM RESPONDERS TO *NUCLEUS PULPOSUS* CELLS: EVIDENCE FOR IMMUNE RESPONSE IN DISC HERNIATION

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Abstract

Although intervertebral disc herniation and associated sciatica is a common disease, its molecular pathogenesis is not well understood. Immune responses are thought to be involved. This study provides direct evidence that even non-degenerated *nucleus pulposus* (NP) cells elicit immune responses. An *in vitro* colony forming inhibition assay demonstrated the suppressive effects of autologous spleen cells on NP cells and an *in vitro* cytotoxicity assay showed the positive cytotoxic effects of natural killer (NK) cells and macrophages on NP cells. Non-degenerated rat NP tissues transplanted into wild type rats and immune-deficient mice demonstrated a significantly higher NP cell survival rate in immune-deficient mice. Immuno-histochemical staining showed the presence of macrophages and NK cells in the transplanted NP tissues. These results suggest that even non-degenerated autologous NP cells are recognized by macrophages and NK cells, which may have an immunological function in the early phase of disc herniation. These findings contribute to understanding resorption and the inflammatory reaction to disc herniation.

Keywords: *Nucleus pulposus*, immune response, macrophage, natural killer cell, intervertebral disc, autoimmunity.

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Introduction

Resorption of herniated *nucleus pulposus* (NP) is a clinically demonstrated phenomenon during intervertebral disc herniation. In understanding the undefined pathogenesis of intervertbral disc herniation and sciatica, clarifying the molecular events that occur in resorption of NP is important. Nachemson (1969) reported decreased pH levels within and around a herniated lumbar disc and speculated that sciatica was caused by an inflammatory reaction surrounding the nerve root. Subsequently, various inflammatory chemical factors secreted from herniated NP, including tumor necrosis factor (TNF)-α (Weiler *et al.*, 2005; Le Maitre *et al., 2007*), interleukin (IL)-1β (Le Maitre *et al., 2007*) and nitric oxide (NO) (Katsuno *et al.*, 2008), have been implicated as causes of sciatica (McCarron *et al*, 1987; Geiss *et al.*, 2007). Further, the production of matrix metalloproteinases (MMPs) has been implicated in the resorption of the herniated NP (Doita *et al.*, 2001).

Bobechko and Hirsh (1965) and Gertzbein *et al.* (1975) reported that herniated NP tissue is recognized as a foreign antigen that induces an autoimmune response producing inflammation. Later, immunohistochemical (IHC) analyses of human herniated discs revealed the presence of infiltrated T cells (Park *et al.*, 2001), macrophages (Park *et al.*, 2001; Virri *et al.*, 2001), and antigen-antibody complexes in the NP (Satoh *et al.*, 1999). An *in vitro* co-culture model of macrophages and NP cells also showed the infiltration of macrophages and a decreased wet weight of the NP (Haro *et al.*, 2000). The expression of IL-6, -8, -12, and interferon (IFN)-γ suggests Th1 lymphocyte activation (Kang *et al.*, 1996; Burke *et al.*, 2002; Park *et al.*, 2002). Geiss *et al.* placed autologous porcine NP in subcutaneous titanium chambers and observed the infiltration of activated T and B cells (Geiss *et al.*, 2007), including IL-4-producing Th2 cells and γδ T cells (Geiss *et al.*, 2008). These results indicate both innate and acquired immune responses to the NP. Other studies (Park *et al.*, 2001; Jones *et al.*, 2008), however, have reported that NP cells undergo apoptosis and are phagocytised by macrophages without an immune response. Ikeda *et al.* (1996) investigated infiltrated cells consisting of macrophages and a small number of T cells, and proposed that extruded or sequestrated disc material

has the potential to be absorbed by phagocytes. It remains unclear from these reports whether immune responses are truly involved in disc herniation, and if so, which immune cells initiate the immune response.

In order to investigate whether an immune response is involved in disc herniation, fundamental research on NP cells and the immune system is required. The purpose of this study is to clarify the immune response to autologous NP cells and to identify the specific immune cells that initiate an immune response by using *in vivo* and *in vitro* rat models to assess the survival of NP cells exposed to immune system cells.

Materials and Methods

In vitro studies

Preparation of rat-tail NP cells. Male Sprague-Dawley (SD) rats (Nihon Charles River Co., Kanagawa, Japan) aged 10-12 weeks, were used for the colony forming inhibition assay (CFI), and male Lewis rats (Nihon Charles River) aged 10-12 weeks were used for the cytotoxicity assay. Following sacrifice, NP tissues were dissected from the whole tail and digested in 0.05% trypsin-ethylene diamine tetraacetic acid (EDTA; Gibco, Grand Island, NY, USA) for 15 minutes. The digestate was washed, passed through a 100 μm mesh cell strainer, the NP cells were collected by mild centrifugation (500Gx4min). These experiments were approved by the Animal Research Committee of Tokai University (071095) and conducted according to the guidelines for animal experiments.

Preparation of spleen cells. Autologous spleen cells were used as effector cells for the CFI assay and isogenous spleen cells were used for the cytotoxicity assay. Briefly, the spleens were removed, mashed and passed through a 100 μm mesh cell strainer. Red blood cells were haemolysed using 0.8% NH_3Cl. The spleen cells were collected by mild centrifugation (500Gx4min). The spleen cells (10^7cells/ml) were then incubated in RPMI-1640 medium (Invitrogen, Grand Island, NY, USA) with 15% foetal bovine serum (FBS, Qualified FBS, Invitrogen) at 37°C for five hours with IL-2 (60 IU/ml; Imunase, Shionogi, Osaka, Japan).

Purification of T cells, natural killer (NK) cells and macrophages. For cytotoxicity assays, more than 10^8 of the spleen cells isolated from a Lewis rat were suspended in fluorescence-activated cell sorting (FACS) buffer (Facs Flow, Becton Dickinson (BD) Pharmingen, Tokyo, Japan) and incubated for 30 minutes at 4°C with saturating amounts of the following antibodies: CD3 (#550353, PE mouse anti-rat CD3, BD), CD4 (#550057 Pharmingen, APC mouse anti-rat CD4, BD), CD8 (#558824, Per CP mouse anti-rat CD8a, BD), CD161 (#550978, biotin mouse anti-rat CD161, BD). The labelled spleen cells were then separated into NK cells (CD161+), CD4+T cells (CD3+CD4+), CD8+T cells (CD3+CD8+), and macrophages (remaining CD3-) using a FACS Vantage (BD).

CFI assay. For the CFI assay, the suppressive effect of immune cells (effector cells) on colony formation by autologous NP cells (target cells) was assessed by a previously described method (Spitzer *et al.*, 1980). The NP cells isolated from SD rats (N=4) were immediately utilized for the assay procedures. NP cells ($6x10^3$) and autologous spleen cells were seeded for each E:T ratio of 0:1, 25:1, 50:1 and 100:1 in 6ml of 0.9% methylcellulose formation (MethoCult H4230 Stemcell Technologies, Vancouver, Canada) in a single tube, mixed completely, then we dispensed it by 1ml in 35mm dishes (n=4 for each E:T ratio). The dishes were incubated at 37°C in 5% CO_2 and full humidity for 14 days without medium replacement, after which the number of NP colonies was scored at least twice for each dish using a tally board on the bottom of the dishes.

Cytotoxicity assay. For the cytotoxicity assay, NP cells from Lewis rats (N=2) were monolayer cultured in RPMI-1640 medium with 15% FBS for 10 days. The cells were labeled using calcein-AM (Dojin Chemical Institute, Kumamoto, Japan) for 60 minutes at 37°C without serum, washed, and seeded into 96-well V-bottomed plates (#4914, Matrix Technologies, Hudson, NH, USA) at $1x10^4$ cells/ well. Suspensions of purified isogenous NK cells, CD4+ T cells, CD8+ T cells, or macrophage cells were then added to wells at E:T cell ratios of 0:1, 25:1, 50:1 and 100:1 in a final volume of 200 μL/well in RPMI-1640 medium without serum (n = 4 for each ratio). The plate was centrifuged, then incubated in humidified air for eight hours at 37°C. After incubation, the plates were centrifuged and 100 mL of supernatant from each well was moved to another 96 well flat-bottomed plate in the same pattern, and was measured using a fluorescent plate reader (λ_{ex}=485 nm, λ_{em}=520 nm, Beckman Coulter, Brea, CA, USA). Cytotoxic activity was determined according to a modification of the ^{3}H-uridine labelling method described by Wang *et al.* (1993). Cytotoxicity was calculated as:

$$\%\text{cytotoxicity} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{total release} - \text{spontaneous release}} \times 100 \quad (1)$$

Total release was obtained by detergent solubilisation in the presence of 1% Triton X-100 (GE Healthcare Japan, Tokyo, Japan). Spontaneous release means the fluorescence release of the pure NP cell groups. Fibroblastic cells from the *anulus fibrosus* was also analyzed as negative control.

In vivo study

For *in vivo* studies, intact rat NP tissues were transplanted with PBS into immunodeficient mice and wild type rats. The survival rate of the NP cells in the transplanted tissue was measured using the bioluminescence imaging (BLI) method described below to estimate the influence of immunity on NP cell survival. IHC staining was done on the NP tissues from the rat model to detect attracted immune cells, which would indicate the initiation of an immune response.

Transplantation of NP for the BLI study

For the BLI analysis, four male Lewis rats 10-12 weeks of

age were used as recipients of NP tissues for the Lewis to Lewis (Lew-Lew) group and four male 10-12-week-old NOD/Shi-*scid* mice (Nihon Charles River) served as recipients of NP tissues for the Lewis to NOD (Lew-NOD) group. Transgenic (Tg) male Lewis rats (8-10 weeks of age) whose tissues express luciferase produced by repeated crossing of Tg rats and confirmed in the *Organ Replacement Research Department in Jichi Medical University* were used as NP tissue donors. 100 μg of NP tissues were injected with 100 μL of PBS under the abdominal skin of recipients under general anaesthesia using 2-3% isoflurane. One donor was used for each recipient. The BLI study was conducted using a IVIS system (Xenogen Corp., Hopkinton, MA, USA) with LivingImaging acquisition and analysis software. Briefly, animals were anesthetized with isoflurane and given 125 mg/kg D-luciferin substrate (Biosynth AG, Staad, Switzerland). The animals were then placed in a light-tight chamber for imaging with a CCD camera. The photon counts from the peak luciferase activity were recorded. Luciferase activity was measured as photons emitted/second. Imaging studies were performed immediately after transplantation and at day 7, day 14 and day 21.

IHC staining

For IHC staining, male Lewis rats (n=6) were newly used as recipients of NP tissues. The transplantation procedure was the same as for the Lew-Lew group described above and one donor was used for each recipient (n=6). Two recipients were sacrificed at 5, 10, and 40 days after transplantation. In addition, two NOD mouse in BLI study was sacrificed at 26 days after transplantation. After fixation with 10% formalin for three days, a paraffin block was made though an alcohol-xylene-paraffin graded series. Five-micron thick paraffin sections were cut sagittally from the epidermis to the peritoneal membrane across the transplantation site, deparaffinized 5-μm sections first were rehydrated through xylene and graded alcohol series. For double-staining immunofluorescence, tissue slides were incubated overnight at 4°C with a primary monoclonal antibody to keratan sulphate (KS) (#270427-1, mouse anti-KS, Associates of Cape Cod, Falmouth, MA, USA), diluted 1:100 in PBS with 1% BSA, followed by incubation in darkness at room temperature for three hours with Alexa Fluor 488-conjugated anti-mouse IgG diluted 1:200. After washing with PBS, the slides were incubated overnight at 4°C in darkness with diluted (1:100) primary antibodies for rat T cells (#550353, PE mouse anti-rat CD3, BD), macrophages (#sc-9139, rabbit anti-rat CD68, Santa Cruz Biotechnology, CA, USA), or NK cells (#550978, Biotin mouse anti-rat CD161, BD). After washing with PBS, the slides with CD68 were incubated for 60 minutes in room temperature with anti-rabbit goat Alexa 594 antibody (Invitrogen); slides with CD161 staining were incubated for one hour with streptavidin-Alexa 594 (Invitrogen). All slides were then covered with Vectashield mounting medium with DAPI (H-1500, Vector Laboratories, Burlingame, CA, USA). Sample sections of day 5, day 10 and day 40 were also stained with HE and Safranin-O.

Data Analysis

All data are given as the mean ± standard deviation (SD). The statistics were processed by Excel Stat 2006 (SSRI, Tokyo, Japan). Two-factor analysis of variance (ANOVA) was employed to analyze the *in vitro and in vivo results*. The Mann-Whitney U-test was used to compare the results of the two groups in CFI assay. When significant differences were revealed by the ANOVA, *post hoc* comparisons were done. Statistical significance was defined as $p < 0.05$.

Results

***In vitro* study**

CFI assay. Spleen cells from SD rats were used as effector cells for autologous tail NP target cells. Colony formation assays showed two types of colonies that were identified as CFU-A (adherent) and CFU-NA (non-adherent) when counting colonies. Without effector cells (E:T cell ratio of 0:1), NP cells ($1x10^3$) formed CFU-NA colonies ranging in numbers from 82-118 (94.8±18.1) and CFU-A colonies ranging from 39-60 (48.3±9.6). When effector cells were added, NP cells ($1x10^3$) with an E:T cell ratio of 25:1 yielded CFU-NA colonies ranging from 26-31 (29.0±2.2) and CFU-A colonies ranging from 22-38 (28.3±6.8), an E:T cell ratio of 50:1 resulted in CFU-NA colonies ranging from 19-26 (22.8±3.0) and CFU-A colonies ranging from 19-34 (26.8±6.6) and an E:T cell ratio of 100:1 produced CFU-NA colonies ranging from 19-25 (21.0±2.8) and CFU-A colonies ranging from 18-27 (21.0±4.2). The suppressive effect of spleen cells was apparent (Fig. 1A). CFU-NA colonies were affected stronger than CFU-A colonies (Table 1). Microscopic examinations of CFU-NA

Table 1 Percentage of the number of colonies to the control (E:T cell ratio = 0:1)

E:T cell ratio	0:1	25:1	50:1	100:1
CFU-NA (%)	100	30.6±2.3	24.0±3.2	22.2±3.0
CFU-A (%)	100	58.5±14.2	55.4±13.7	43.5±8.8
p-value		p=0.021	p=0.021	p=0.019

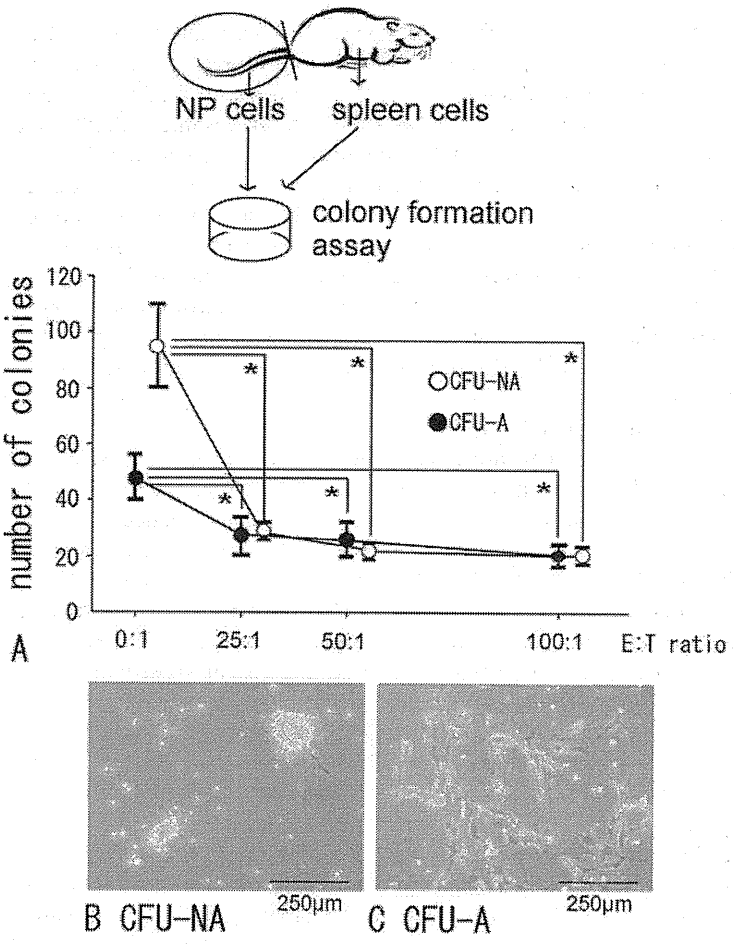

Fig. 1. (A) Results of CFI assay *in vitro*. Numbers of CFU-NA (open circle) and CFU-A (closed circle) colonies in the E: T ratio of 0:1, 25:1, 50:1 and 100:1 were counted at day 14. Colony formation of NP cells was suppressed by the addition of autologous spleen cells in both groups ($*p < 0.05$ compared with that in the E:T ratio of 0:1). (B) Attraction of spleen cells to CFU-NA. (C) Attraction of spleen cells to CFU-A. The larger number of spleen cells attracted to CFU-NA than to CFU-A supports the result in our current study that CFU-NA is more sensitive to autologous spleen cells.

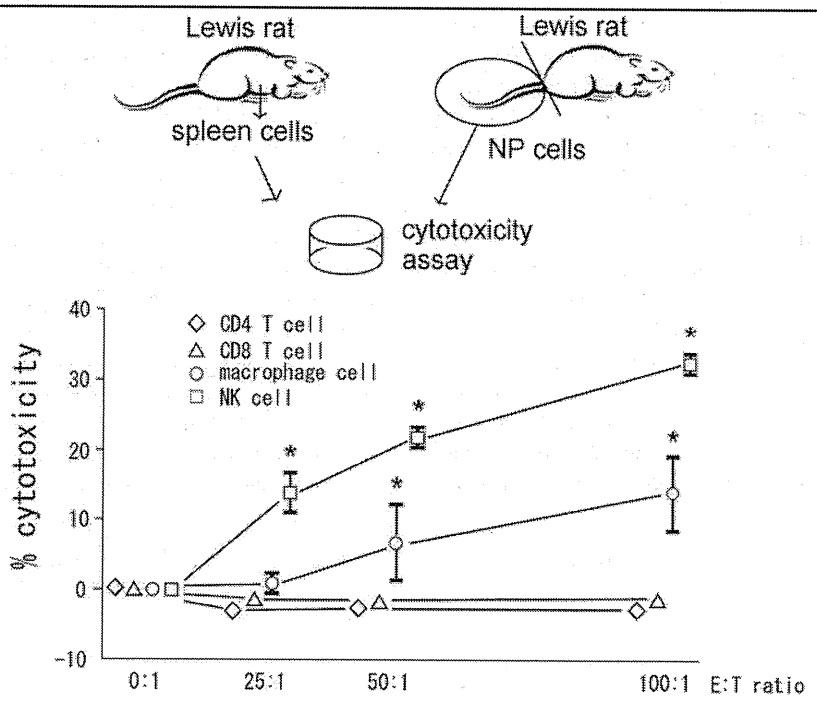

Fig. 2. Results of cytotoxicity assay *in vitro*. Cytotoxicity was calculated as follows,

$$\%\text{cytotoxicity} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{total release} - \text{spontaneous release}} \times 100$$

0%=no cytotoxicity, 100%=maximum cytotoxicity as strong as detergent agent.
Cytotoxicity caused by NK cells and macrophages was suggested as a result of 8 hrs coculture ($*p < 0.05$ compared with that in the E:T ratio of 0:1).

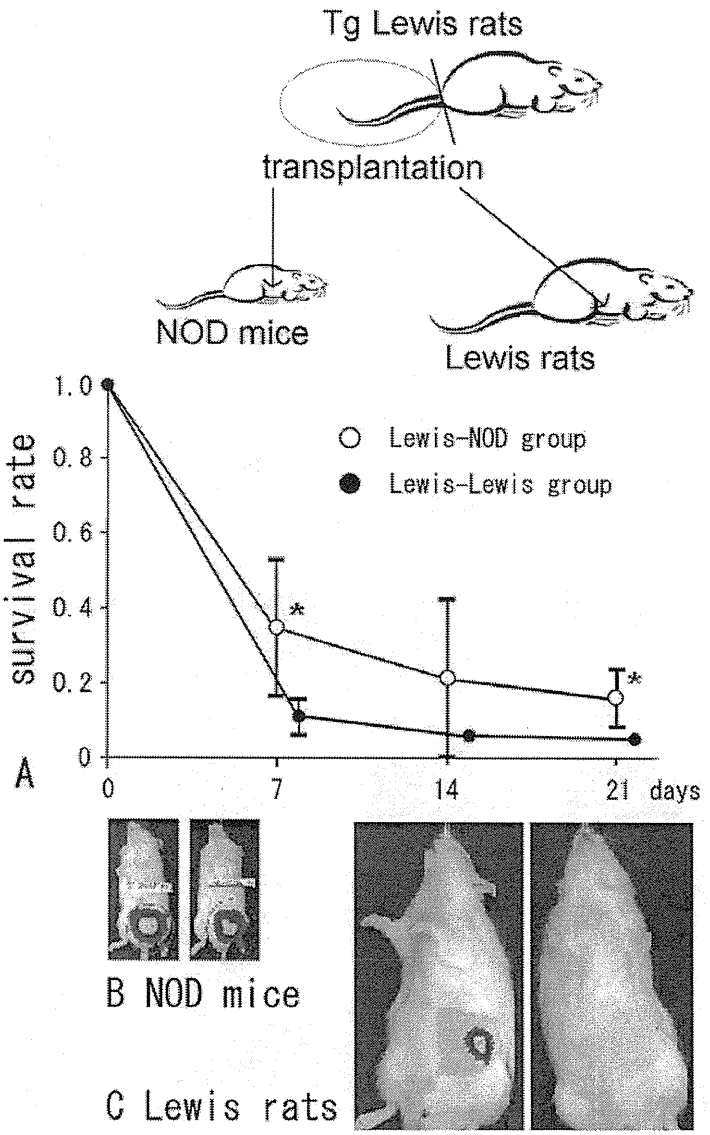

Fig. 3. (A) Survival rate of transplanted NP cells in Lewis rats and NOD mice (n=4, each). Closed circle indicates Lewis-Lewis group and open circle indicates Lewis-NOD group. Because intensity of luminescence is positively linear to the number of NP cells (data not shown), survival rate of NP cells was calculated as follows:

$$\text{Survival rate} = \frac{\text{Intensity of luminance after 7, 14 or 21 days}}{\text{Intensity of luminance just after transplantation}} \quad (2)$$

so that baseline value of survival rate (day 0) is "1". The survival rate was higher in the Lewis-NOD group than in the Lewis-Lewis group (*$p < 0.05$). (B) BLI imaging of NOD mouse at day 0 (left) and at day 90 (right). (C) BLI imaging of Lewis rat at day 0 (left) and at day 21 (right). NP cells hardly survived at day 21.

(Fig. 1B) and CFU-A (Fig. 1C) colonies revealed that larger numbers of spleen cells were attracted to CFU-NA colonies than to CFU-A colonies, further indicating that CFU-NA colony formation was more sensitive to the presence of spleen cells.

Cytotoxicity assay. From 10^8 Lewis rat spleen cells, $3.0x10^7$ CD4+T cells, $2.0x10^7$ CD8+T cells, $1.0x10^7$ macrophages and $6.0x10^6$ NK cells were sorted by FACS with data showing that more than 95% of the cells were alive.

Cytotoxicity to autologous NP cells was proportional to the E:T cell ratio in NK cells and macrophages (Fig. 2). At an E:T cell ratio of 100:1, cytotoxicity was 31-35% in NK cells and 9-20% in macrophages. Significant cytotoxicity was observed in NK cells at E:T cell ratios of 25:1 or more ($p < 0.0001$) and in macrophages at E:T cell ratios of 50:1 or more compared to the corresponding values in the absence of effector cells ($p = 0.001$ at 50:1; $p < 0.0001$ at 100:1) (Fig. 2). CD4+T cells and CD8+T cells did not have cytotoxic effects on NP cells.

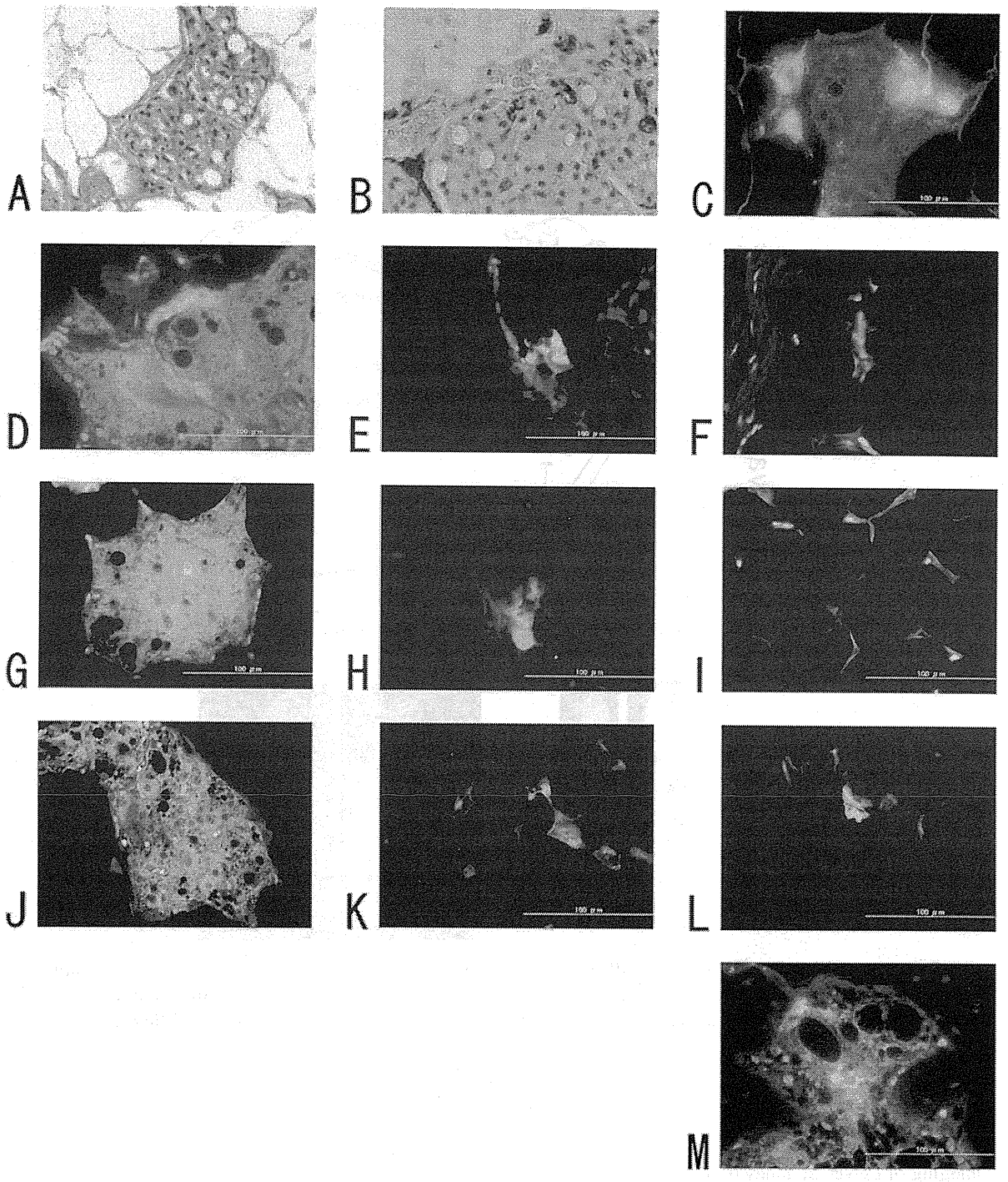

Fig. 4. Histological analysis of NPs at the transplant site of recipient rats. HE (A), safranin-O (B) and keratan sulphate IHC (C) staining indicate the presence of transplanted NPs at day 5. Safranin-O stains the proteoglycan of NPs (Red), and keratan sulphate is specific extracellular matrix of NPs (Green). Keratan sulphate was stained even 26 days after transplantation in the recipient of NOD mouse (M).

CD3 (D~F), CD68 (G~I) and CD161 (J~L) (Red) are immunohistochemically double-stained with keratin sulphate (Green). D, G, and J are the results at day 5; E, H, and K at day 10; F, I, and L at day 40. Keratan sulphate decreased dependent on time, NK cells and macrophages decreased simultaneously.

To summarize, the results of the *in vitro* CFI and cytotoxicity assays revealed the presence of a spleen cell population that had cytotoxic effects on autologous NP cells. This cytotoxic spleen cell population is composed of sub-populations of NK cells and macrophages. Furthermore, as a negative control of cytotoxicity assay, we used fibroblast-like cells of *annulus fibrosus* origin. The result was that the fibroblast-like cells was tolerant to isogeneous spleen cells, however *nucleus pulposus* cells was sensitive. This result raised our test hypothesis that NP is sensitive to specific immune cells.

***In vivo* study**

BLI study. We performed a BLI study to investigate immunological responses to transplanted NP tissues *in vivo*. The BLI evaluation showed a significantly higher survival rate for transplanted NP cells in the Lew-NOD group compared to that in the Lew-Lew group totally ($p = 0.036$), at day 7 (0.35±0.18 *vs.* 0.11±0.05; $p = 0.042$) and at day 21 (0.16±0.08 *vs.* 0.03±0.02; $p = 0.037$) (Fig. 3A). After 90 days, up to 13% of the transplanted NP cells had survived in the Lew-NOD group (Fig.3B). NP cells transplanted into Lewis rats (Lew-Lew) did not survive past 21 days, when luminescence at the NP cell transplant site had decreased to near background levels (Fig.3C).

IHC staining. Because our results showed that transplanted NP cell survival was reduced in association with an immunological reaction, we used immunological staining to identify which types of immune cells had infiltrated. Transplanted NP tissues in rats at day 5 existed mainly in the loose subcutaneous fat tissue as an agglomeration of cells with a bubble-like extracellular matrix (Fig. 4A). Safranin-O staining showed the presence of red-stained proteoglycans (Fig. 4B), and fluorescent green-stained keratan sulphate, both of which are constituents of the extracellular matrix of the NP (Fig. 4C). We observed transplanted NP tissues at day 5 (Fig. 4D, G, J), however, the amount of transplanted NP tissue was markedly decreased at day 10 in the Lewis rats, while NP tissue was obviously present in NOD mice recipients even at day 26 (Fig. 4M). From the IHC evaluation of immune cells in Lewis rats, no CD3 positive T cells attraction were observed subcutaneously from day 5 to day 40 (Fig. 4D, E, F). Attraction of NK cells and macrophages was observed at days 5 and 10 around the outgrown NP tissues (Fig. 4G, H, J, K); however, neither NP tissues nor immune cells were observed at day 40 (Fig. 4I, L). The numbers of NK cells and macrophages in microscope fields that included agglomerated NP cell clusters decreased with time (Table 2).

Discussion

The precise mechanism of immunological involvement in the pathology of disc herniation has not been defined. We performed two immunological assays, the CFI and the cytotoxicity assay, using co-cultured NP and immune cells. We also developed an *in vivo* subcutaneous transplantation model and measured the survival rate of transplanted NP cells using the BLI method. IHC at the transplant site of the recipient rats was used to identify the immune cells.

The suppression of NP cell colony formation was observed to be dependent on the effector:target (E:T) cell ratio. We found that non-adherent (CFU-NA) colonies were more strongly suppressed by immune cells than adherent (CFU-A) colonies. Because NP cells are known to be heterogeneous (Chelberg *et al.*, 1995), this difference in colony formation may reflect the different epitopes recognized by immune cells, or possible differences in the immune privilege function, like the presence or absence of Fas ligand. Based on these possibilities, about 20% of the NP cell population were alive even at E:T cell ratio of 100:1, which appears to differ immunologically from other NP cells. Of particular interest was the assessment of direct

Table 2 The number of immune cells in a microscope field (x40)

	Day 5	Day 10	Day 40
T cell	None	None	None
NK cell	2-5	1	None
Macrophage	4-7	1-3	None

This table indicates the number of representative agglomerated NP cell clusters in the tissue specimen of transplanted site in Lewis rats. NK and macrophage cells were observed in the transplanted site in the early phase, whereas T cells were not observed. In day 40, neither NP tissues nor immune cells were observed.

cytotoxic function of the immunological cell types. The results of the cytotoxicity assays of isolated T, NK, and macrophage cells demonstrated that only the NK and macrophage cells had cytotoxic activity on NP cells. The target molecules and their location on the NP cells remain undefined; further biological and immunological studies are necessary.

The results of the BLI study showed differences in the survival rate of NP cells in the transplanted NP tissues between the Lewis rat and NOD mouse recipients. NP cells are known to undergo apoptosis (Park *et al.*, 2001), and intervertebral disc cells are thought to be able to behave as competent phagocytes (Jones *et al.*, 2008). However, these results do not explain the different survival rates of NP cells in the current study. Because the survival rate of NP cells was higher in immunodeficient mice than in Lewis rats, immunological functions are implicated. NOD/shi-scid mice lack mature lymphocytes, and have macrophage dysfunction, a reduced level of NK cell activity and absence of circulating immune components compared to wild-type mice. These factors may account for the difference in NP cell survival rate between NOD mice and Lewis rats in our study.

We also detected the infiltration of specific immune cells into the NP transplant sites; these results definitively demonstrate the immunological activity of these cell types against NP tissues. Macrophages and NK cells, but not T cells, were detected, although the presence of T and B cells in isolated human herniated discs and in experimental porcine models has been previously reported (Geiss *et al.*, 2007). Our results suggest an early immunological response after normal NP tissues were exposed to the immune system. Thus, macrophages and NK cells were observed on days 5 and 10 when residual NP was present, but not on day 40 when the transplanted NP had disappeared. This finding supports the presence of an immunological response to transplanted NP tissues.

In our IHC study, CD68 positive cells did not resemble the appearance of resident chondrocytes. Although Jones *et al.* (2008) suggested that CD68 positive cells were transformed resident intervertebral disc cells, based on their morphology, the results of our study show that these macrophages are not transformed resident cells but rather are infiltrating cells.

Autologous tissues are generally not recognized as foreign by the immune system. The NP is an immune-privileged tissue isolated from the immune system (Hiyama *et al.*, 2008) and it, like similarly isolated tissues, including the eye and testis, can produce inflammatory autoimmune responses (Wildner and Diedrichs-Möhring, 2004; Schuppe and Meinhardt, 2005). Another possible trigger for autoimmunity is innate immunity, which is induced by chemical factors without specific antigen-antibody responses, leading to rapid immune responses to pathological microbe antigens. Because the NP cell produces chemical factors and the carbohydrate structure of the extracellular matrix produced by NP cells may mimic that of pathological microbe antigens, the NP may trigger an innate immunity response (Bárdos *et al.*, 2005).

In the *in vivo* transplantation model, we utilized a xenogeneic model because the mouse is too small to obtain enough donor NP cells. The use of NOD mice as recipients is well established for evaluating the effects of immunodeficiency. In addition, the xenogeneic transplantation model is commonly used for immunological evaluation (Yoshino *et al.*, 2000).

In conclusion, even non-degenerated NP cells elicit an immune response, and macrophages and NK cells in particular are shown to have an early immunological function when NP cells are exposed to the immune system. While these results may not be directly applicable to the human, this study provides important information for understanding the pathophysiological mechanism of disc herniation.

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References

Bárdos T, Szabó Z, Czipri M, Vermes C, Tunyogi-Csapó M Urban MR, Mikecz K, Glant TT (2005) A longitudinal study on an autoimmune murine model of ankylosing spondylitis. Ann Rheum Dis **64**: 981-987.

Bobechko WP, Hirsh C (1965) Auto-immune response to *nucleus pulposus* in the rabbit. J Bone Joint Surg Br **47**: 574-580.

Burke JG, Watson RWG, McCormack D, Dowling FE, Walsh MG, Fitzpatrick JM (2002) Spontaneous Production of Monocyte Chemoattractant Protein-1 and Interleukin-8 by the Human Lumbar Intervertebral Disc. Spine **27**: 1402-1407.

Chelberg MK, Banks GM, Geiger DF, Oegema TR Jr (1995) Identification of heterogeneous cell populations in normal human intervertebral disc. J Anat **186**: 43-53.

Doita M, Kanatani T, Ozaki T, Matsui N, Kurosaki M, Yoshiya S (2001) Influence of macrophage infiltration of herniated disc tissue on the production of matrix metalloproteinases leading to disc resorption. Spine **26**: 1522-1527.

Geiss A, Larsson K, Rydevik B, Takahashi I, Olmarker K (2007) Autoimmune Properties of *Nucleus Pulposus*: An Experimental Study in Pigs. Spine **32**: 168-173.

Geiss A, Larson K, Junevik K, Rydevik B, Olmarker K (2008) Autologous *nucleus pulposus* primes T cell to develop into interleukin-4-producing cells: an experimental study on the autoimmune properties of *nucleus pulposus*. J Orthop Res **27**: 97-103.

Gertzbein M, Tail M, Gross A, Falk R (1975) Autoimmunity in degenerative disc disease of the lumbar spine. Orthop Clin North Am **6**: 67-73.

Haro H, Craford HC, Fingleton B (2000) Matrix metalloproteinase-7–dependent release of tumor necrosis

factor-α in a model of herniated disc resorption. J Clin. Invest., **105**: 143-150.

Hiyama A, Mochida J, Iwashina T, Omi H, Watanabe T, Serigano K,Tamura F, Sakai D (2008) Transplantation of mesenchymal stem cells in a canine disc degeneration model. J Orthop Res **26**: 589-600.

Ikeda T, Nakamura T, Kikuchi T, Umeda S, Senda H, Takagi K (1996) Pathomechanism of spontaneous regression of the herniated lumbar disc: Histologic and immunohistochemical study. J Spinal Disord **9**: 136-140.

Jones P, Gardner L, Menaga J, Williams G, Roberts S (2008) Intervertebral disc cells as competent phagocytes *in vitro*: implications for cell death in disc degeneration. Arthritis Res Ther **10**: R86.

Kang JD, Georgescu HI, McIntyle-Larkin L, Stefanovic-Racic M, Donaldson WF 3rd, Evans CH (1996) Herniated lumbar intervertebral discs spontaneously produce matrix metalloproteinases, nitric oxide, interleukin-6, prostaglandin E2. Spine **21**: 271-277.

Katsuno R, Hasegawa T, Iwashina T, Sakai D, Mikawa Y, Mochida J (2008) Age-related effects of cocultured rat *nucleus pulposus* cells and macrophages on nitric oxide production and cytokine imbalance. Spine **33**: 845-849.

Le Maitre CL, Hoyland JA, Freemont AJ (2007) Catabolic cytokine expression in degenerate and herniated human intervertebral discs: IL-1**b** and TNF**a** expression profile. Arthritis Res Ther **9**: R77.

McCarron RF, Wimpee MW, Hudkins PG, Laros GS (1987) The inflammatory effect of *nucleus pulposus*. A possible element in the pathogenesis of low back pain. Spine **12**: 760-764.

Nachemson A (1969) Intradiscal measurements of pH in patients with lumbar rhizopathies. Acta Orthop Scand **40**: 23-42.

Park JB, Chang H, Kim KW (2001) Expression of Fas ligand and apoptosis of disc cells in herniated lumbar disc tissue. Spine **26**: 618-621.

Park JB, Chang H, Kim YS (2002) The pattern of interleukin-12 and T-helper types 1 and 2 cytokine expression in herniated lumbar disc tissue. Spine **27**: 2125-2128.

Satoh K, Konno S, Nishiyama K, Olmarker K, Kikuchi S (1999) Presence and distribution of antigen-antibody complexes in the herniated *nucleus pulposus*. Spine **24**: 1980-1984.

Schuppe HC, Meinhardt A (2005) Immune privilege and inflammation of the testis. Chem Immunol Allergy **88**: 1-14.

Spitzer G, Verma DS, Fisher R, Zander A, Vellekoop L, Litam J (1980) The myeloid progenitor cell – its value in predicting hematopoietic recovery after autologous bone marrow transplantation. Blood **55**: 317-323.

Virri J, Gronblad M, Seitsalo S, Habetemarian A, Kappa E, Karahaju E (2001) Comparison of the prevalence of inflammatory cells in subtypes of disc herniations and associations with straight leg raising. Spine **26**: 2311-2315.

Wang XM, Terasaki PI, Rankin GW Jr., Chia D, Zhong HP, Hardy S (1993) A new microcellular cytotoxicity test based on calcein AM release. Hum Immunol **37**: 264-270.

Weiler C, Nerlich BE, Boos N (2005) Expression and distribution of tumor necrosis factor alpha in human lumbar intervertebral discs: A study in surgical specimen and autopsy controls. Spine **30**: 44-53.

Wildner G, Diedrichs-Möhring M (2004) Autoimmune uveitis and antigenic mimicry of environmental antigens. Autoimmun Rev **3**: 383-387.

Yoshino H, Ueda T, Kawahata M, Kobayashi K, Ebihara Y, Manabe A, Tanaka R, Ito M, Asano S, Nakahata T, Tsuji K (2000) Natural killer cell depletion by anti-asialo GM1 antiserum treatment enhances human hematopoietic stem cell engraftment in NOD/Shi-scid mice. Bone Marrow Transplantation **26**: 1211-1216.

Human Nucleus Pulposus Cells Significantly Enhanced Biological Properties in a Coculture System with Direct Cell-to-Cell Contact with Autologous Mesenchymal Stem Cells

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ABSTRACT: Activated nucleus pulposus (NP) cells can be reinserted into the disc to inhibit intervertebral disc degeneration. Experimental studies in animals showed that using a coculture system with direct cell-to-cell contact with mesenchymal stem cells (MSCs) significantly upregulated the biological activity of NP cells. The purpose of this study is to determine whether this activation of NP cells by autologous MSCs is applicable to human cells in vitro. Human NP tissue was obtained from surgical specimens and MSCs from bone marrow of 10 subjects. Six-well culture plates and inserts were used for culture; 1.0×10^4 NP cells were seeded onto each insert and incubated alone, in standard coculture with 1.0×10^4 MSCs, or cocultured with direct cell-to-cell contact. NP cell proliferation, DNA synthesis, and proteoglycan (PG) synthesis were evaluated. Chromosome abnormalities in the activated NP cells and tumorigenesis of the cells were evaluated in an additional 10 patients by microscopic examination for segmented cells and histological assessment of activated cells transplanted into nude mice. Cell proliferation, DNA synthesis, and PG synthesis were significantly upregulated. The positive effects of the coculture system with direct cell-to-cell contact seen in animal studies were also confirmed in human cells. Chromosome abnormalities and tumorigenesis were not observed in the activated NP cells. In conclusion, a coculture system with direct cell-to-cell contact demonstrated a significant positive effect, enhancing the biological properties of human NP cells, as it did in animal models. These results should prove useful for conducting trials leading to the clinical use of activated NP cell transplantation. © 2009 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 28:623–630, 2010

Keywords: intervertebral disc; disc degeneration; nucleus pulposus; mesenchymal stem cells; coculture

The fact that working people often suffer from spinal disorders poses a severe societal problem. According to United States health care statistics, the lifetime incidence of low back pain is between 60% and 80%.[1] Lumbar disc degeneration can cause low back pain and is not clinically reversible. The mechanism that produces the pathogenesis of disc degeneration has not yet been elucidated. Various nonsurgical methods developed to treat disc degeneration include gene therapy, the injection of growth factors, tissue engineering, and cell transplantation.[2–11]

Gene therapy using adenovirus-mediated transfer has been reported in an in vivo rabbit intervertebral disc model[2] and in human intervertebral disc cells in vitro.[3] Masuda et al. have reported that recombinant human osteogenic protein-1 is effective in promoting matrix synthesis and formation by rabbit nucleus pulposus (NP) and annulus fibrosus (AF) cells.[4] To date, none of these therapies have been applied clinically due to problems with ethics, safety, and the difficulty of controlling cell proliferation.

NP tissues play a very important role in the function and morphology of the disc. NP cells are alive in an avascular space, and thus the loss of characteristics of NP seem to be a trigger which can cause disc degeneration.[12] As disc degeneration accelerates, the relative amount of water and proteoglycan (PG) in the degenerating disc is reduced and the structure of the disc gradually collapses. The mechanism of the decline of human NP cell activity in this progression has not been clearly defined. Evans noted that disc cells from patients with more degenerative discs are poorly suited for repair.[13]

Some researchers have suggested that the NP itself is a trigger for disc degeneration. Mochida et al. reported that a large amount of the central portion of the surgically treated discs showed significantly more progressive degeneration than other discs in patients 10 years after a percutaneous nucleotomy.[14] We have primarily focused our research efforts on disc regeneration, specifically on the repair of the NP using cell therapy. Animal studies have revealed that the use of autologous NP cells can maximize repair activity. Nishimura and Mochida reported that reinsertion of autologous NP tissues decelerated disc degeneration.[15] Okuma et al. found the biological viability of NP cells to be upregulated using a coculture system with AF cells.[16] In an in vivo animal model, they reported that the reinsertion of activated NP cells into degenerated discs decelerated further disc degeneration.[16] However, it is clinically difficult to quickly obtain sufficient fresh NP cells for transplantation. To overcome this problem, Yamamoto et al. cocultured NP cells with bone marrow-derived stromal cells (BMSCs) in direct cell-to-cell contact; this coculture system significantly activated the biological properties of NP cells, such as cell proliferation, DNA synthesis, and PG synthesis.[17]

Before this technique is applied clinically to autologous NP cell therapy, it is necessary to evaluate the use of the coculture system clinically. For a useful clinical application, an ex vivo expansion to appropriate cell numbers in a short-term in vitro culture system is

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required. However, human NP cells are difficult to culture and expand.[18] Therefore, we conducted a preclinical study using human surgical disc materials from 10 clinical cases with autologous MSCs. In addition, the safety of activated human NP cells was evaluated in vitro for chromosomal abnormalities and tumorigenesis in an additional 10 cases.

MATERIALS AND METHODS

Clinical Cases

After obtaining informed consent, 10 subjects undergoing surgery for a burst fracture ($n = 3$), lumbar disc herniations ($n = 6$), and spondylolysis ($n = 1$) were enrolled in this study. The age of patients ranged from 18 to 59 years (Table 1). The extent of disc degeneration in each subject was graded on magnetic resonance images (MRIs) using Pfirrmann's classification. In Pfirrmann's classification, the distinction between the NP and AF becomes less clear with increasing disc degeneration.[19]

Cell Isolation

During surgery, lumbar discs were removed, the NP and AF tissues were carefully identified, and the tissue from the NP was dissected. In three cases of burst fracture, in which the upper endplate with fractured vertebral body was injured and the bottom endplate was intact, we harvested the nucleus tissue from the disc with the intact endplate, observing that the disc was primarily intact as well. In degenerated cases, it was difficult to distinguish macroscopically between NP and AF. Therefore, we harvested the cells from the central nucleus region composed of primarily nucleus pulposus cells with possible contamination of inner AF cells. We regarded these cells as "NP cells" in this study.

The NP cells were enzymatically released from NP tissues by digestion using 0.27% pronase (Kakenseiyaku, Tokyo, Japan) for 1 h followed by 0.025% collagenase (Boehringer, Mannheim, Germany) for 2 h. The digested cells were washed twice with normal saline solution, centrifuged at 1,500 rpm for 5 min, and primarily seeded at 3,000 to 5,000 cells/cm^2. The viable adherent NP cells were first cultured in a 100 cm^2 culture flask containing DMEM/F-12 (Gibco, Grand Island, NY) with 10% autologous serum that was obtained during surgery.

Mononuclear cells were isolated from 50 ml of bone marrow blood aspirated from the vertebral body or iliac bone during surgery using 5% dextran in normal saline solution (NS), and allowed to settle for 1 h. The top of the serum was gradient-centrifuged at 3,000 rpm for 20 min and washed twice with NS at 1,500 rpm for 5 min. The middle fraction was selected and cultured in a 100 cm^2 culture flask containing the same medium. The adherent cells were considered to be mesenchymal stem cells. The expression of CD29, CD44, and CD105, and the absence of hematopoietic markers such as CD14, CD34, and CD45 were investigated by flow cytometry.

Cell Culture Method

Primary NP cells and MSCs were harvested following 4 days of monolayer culture. A six-well culture plate and inserts (Becton Dickinson, Franklin Lakes, NJ), containing a polyethylene terephthalate track-etched membrane with 0.4 μm pores at the bottom, was used for coculture. NP cells were seeded onto each culture insert at 1.0×10^4 cells and divided into three groups: Group A, monoculture of NP cells; Group B, equal numbers of NP cells and MSCs for standard coculture; and Group C, equal numbers of NP cells and MSCs cocultured with direct cell-to-cell contact. DMEM/F12 with 10% autologous serum was the culture medium (Fig. 1). Both NP cells and MSCs that were used for this study (Table 1) were cultured for one passage, and were seeded at 1.0×10^4 cells onto each insert that has 4.2 cm^2 of available culture area.

Evaluation

After 3, 5, and 7 days of culture, NP cell proliferation, DNA synthesis, and PG synthesis were evaluated for each group.

Measurement of Cell Proliferation

Cell proliferation was determined by the WST-8 assay using the Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technology, Gaithersburg, MD). Briefly, NP cells from each of the three groups were added to a 96-well plate. After adding 10 μl of the prepackaged CCK-8 solution, the plate was incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 2 h. The plate was read on a spectrophotometer at 450 nm and the cell counts were determined using a calibration curve.

Measurement of DNA Synthesis

DNA synthesis was determined by the uptake of ^{3}H-thymidine. Cultures were labeled with 74 kBq of ^{3}H-thymidine per well for 3 h. Cells were washed twice with phosphate buffered saline (PBS), and 2 ml of 10% trichloroacetic acid (TCA) was added to each well. The cultures were centrifuged (3,000 rpm for 10 min) and the supernatant was removed. This procedure was repeated five times, and the TCA-insoluble material was collected and dried with 70% ethanol, and treated overnight

Table 1. Clinical Cases

Case	Age	Sex	Disease	Operation	Disc Level	Disc-Grading
1	30	F	Burst fx	ASF	L1-L2	Grade 2
2	21	M	LDH	PN	L5-S1	Grade 3
3	34	M	LDH	PLIF	L5-S1	Grade 3
4	43	F	LDH	Herniotomy	L5-S1	Grade 4
5	18	M	LDH	Herniotomy	L3-L4	Grade 3
6	59	M	Spondylolysis	PLIF	L5-S1	Grade 4
7	32	M	Burst fx	ASF	L2-L3	Grade 2
8	46	M	Burst fx	ASF	L3-L4	Grade 2
9	55	F	LDH	Herniotomy	L4-L5	Grade 4
10	32	M	LDH	Herniotomy	L4-L5	Grade 3

LDH, lumbar disc herniation; ASF, anterior spinal fusion; PN, percutaneous nucleotomy; PLIF, posterior lumbar intervertebral body fusion.

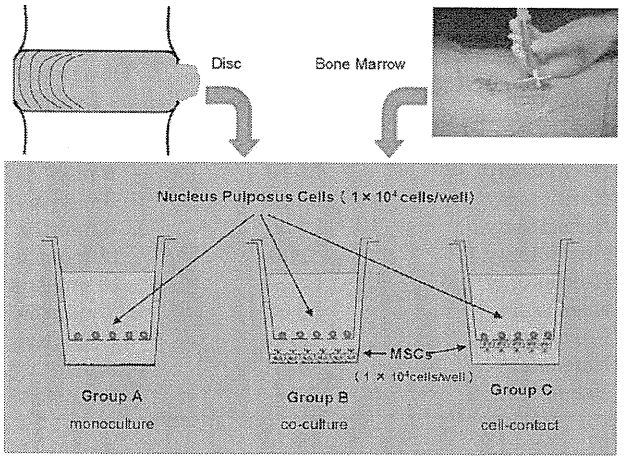

Figure 1. Study design: During surgery, the lumbar disc was removed, and 50 ml of autologous bone marrow was taken from the same patient. Nucleus pulposus (NP) cells and mesenchymal stem cells (MSCs) were isolated by enzymatic digestion and gradient centrifugation, respectively, and utilized for the coculture system. Group A, NP cells alone (in monolayer cultured on the culture insert membrane); Group B, NP cells (in monolayer on the culture insert membrane) cultured with MSCs (in monolayer on the culture plate well), a conventional coculture system; and Group C, NP cells (in monolayer on top of the culture insert membrane) cocultured with MSCs (in monolayer on the bottom of the culture insert membrane) with direct cell-to-cell contact.

with 1 ml of solvent (Solvable™; Packerd, Meriden, CT) at 45°C. Liquid scintillation cocktail (10 ml; Atomlight™; Packerd) was added to count the emissions with a scintillation counter (Beckman LS4800, Fullerton, CA). Radioactivity, expressed as disintegrations per minute (DPM), was divided by the number of cells determined by the Cell Counting Kit-8 in each culture method, as described above, at the same time. The radioactivity of each sample was expressed as DPM per cell.

Measurement of PG Synthesis

The incorporation of ^{35}S-sulfate was used to measure PG synthesis. The cultures were labeled with 370 kBq of ^{35}S-sulphate per well for 3 h. Subsequent PBS washes, TCA treatment, drying, and scintillation counting were carried out using the same procedure as that used for the ^{3}H-thymidine uptake.

Statistical Analysis

All results are presented as the mean ± SD. Statistical significance was considered to be at $p < 0.05$. The significant difference between three groups was determined using an ANOVA and Fisher's LSD post hoc test at three different time points among three groups.

Scanning Microscopic Findings of Chromosomes

In an additional 10 clinical cases, patients with lumbar disc herniation were enrolled in the study after obtaining informed consent (Table 2). Using the same methods of cell isolation from surgical specimens, primary NP cells and MSCs were obtained following monolayer culture for 4 days. Both cell types were then cocultured with direct cell-to-cell contact, as described above for Group C for 3 days. After a total of 7 days of in vitro culture, the activated NP cells were seeded into another culture flask with DMEM/F12 and 10% autologous serum.

One percent colchicine was then added to the medium to inhibit mitotic activity, and the samples were fixed in acetic acid/methanol solution. The processed cells were examined by optical and fluorescent microscopy. Ten cells that were in the mitotic stage were chosen randomly for each case for the G-banding procedure. We looked for abnormalities, such as trisomy, translocations, defects, or other chromosomal abnormalities.

Tumorigenesis of Activated NP Cells

In another 10 cases listed in Table 3, five cases of lumbar disc herniation that partially overlapped with those in Table 2 were enrolled as described above. After isolating cells from the surgical specimen, primary NP cells and MSCs were obtained and monolayer cultured for 4 days. Both cell types were then cocultured with direct cell-to-cell contact for 3 days, as described above for Group C.

A sample of 5×10^5 of these cocultured cells was harvested from each culture for evaluation of clinical safety. The cells were washed twice with NS and mixed into 0.5 ml colloidal gel containing collagen, laminin, and DMEM (Matrigel, Becton Dickinson, Franklin Lakes, NJ). Four-week-old nude mice were used as an immunodeficient model to determine any tumorigenicity or carcinogenicity of the cells. The cell-containing gel was injected subcutaneously into a nude mouse where the transplanted cells gradually synthesized matrix in the colloid gel. After more than 6–13 months after transplant, a small nodule developed on the skin of the mice. After the mice were sacrificed using inhaled isofluorine, the excised skin with the nodule was fixed in 10% formalin neutral solution for 2 days, and frozen sections were prepared. The histology of hematoxylin and eosin (H&E)- and safranin–O-stained cells and matrix was checked to determine whether the cells were pathologically tumorigenic or carcinogenic.

Table 2. Clinical Cases (Examination of Chromosomes)

Case	Age	Sex	Disease	Operation	Disc Level
1	28	M	LDH	PLIF	L4-L5
2	35	M	LDH	Herniotomy	L5-S1
3	28	M	LDH	Herniotomy	L4-L5
4	27	M	LDH	Herniotomy	L5-S1
5	38	M	LDH	Herniotomy	L4-5
6	29	M	LDH	Herniotomy	L4-5
7	27	M	LDH	Herniotomy	L4-5
8	19	M	LDH	Herniotomy	L5-S1
9	23	M	LDH	Herniotomy	L4-5
10	32	M	LDH	Herniotomy	L5-S1

Table 3. Clinical Cases (Tumorigenesis)

Case	Age	Sex	Disease	Operation	Disc Level	Observation Period
1	28	F	LDH	PLIF	L4-L5	6 months
2	27	M	LDH	Herniotomy	L5-S1	6 months
3	29	M	LDH	Herniotomy	L4-L5	8 months
4	19	M	LDH	Herniotomy	L5-S1	6 months
5	23	M	LDH	Herniotomy	L4-5	13 months
6	32	M	LDH	Herniotomy	L5-S1	10 months
7	29	F	LDH	Herniotomy	L5-S1	7 months
8	37	M	LDH	Herniotomy	L4-5	8 months
9	28	M	LDH	Herniotomy	L4-S1	6 months
10	35	N	LDH	Herniotomy	L4-5	6 months

RESULTS

Cell Proliferation

The average number of NP cells per well at 3, 5, and 7 days of monoculture (Group A) or coculture (Groups B and C) was the following: Group A, 2.24 ± 0.25 ($\times 10^4$), 3.66 ± 0.50 ($\times 10^4$), and 4.36 ± 0.62 ($\times 10^4$) cells at 3, 5, or 7 days, respectively; Group B, 3.51 ± 0.66 ($\times 10^4$), 5.42 ± 0.31 ($\times 10^4$), or 6.82 ± 0.88 ($\times 10^4$) cells at 3, 5, or 7 days, respectively; and Group C, 3.96 ± 0.68 ($\times 10^4$), 7.05 ± 1.41 ($\times 10^4$), and 9.99 ± 1.86 ($\times 10^4$) cells at 3, 5, or 7 days, respectively. From the early phases of culture, significantly more cell proliferation was seen in cocultured NP cells with direct contact with MSCs (Group C), compared to monocultured NP cells (Group A) ($p < 0.001$) and NP cells cocultured with MSCs without direct contact (Group B) ($p < 0.001$) (Fig. 2A). By day 7, cell proliferation in Group C had increased about twofold compared to Group A (Fig. 2A).

On cell proliferation in Group C, there were no apparent differences dependent on the age and disc grading on MRI among the 10 cases at 3 days of coculture. However, at 5 and 7 days of coculture, NP cells from the lower-graded discs (less degenerated discs) tended to be more proliferative (Fig. 3).

In comparison to the monolayer cultures of Group A in the 10 cases, NP cells from lower-graded discs (less degenerated discs) on MRI were not always highly proliferative. Although there was a trend of cells taken from less degenerated discs having higher proliferative ability, more cases need to be investigated to fully confirm this.

DNA Synthesis

The DNA synthesis assays showed that the average DPM of ^{3}H-thymidine per cell measured, relative to the value for Group A, was 1.54 ± 0.11, 2.56 ± 0.32, or 3.15 ± 0.92 in Group B, and 2.45 ± 0.54, 4.23 ± 0.72, or 5.22 ± 1.38 in Group C at 3, 5, and 7 days, respectively, of coculture. At all time points, a significantly greater increase of DPM of ^{3}H-thymidine per cell was seen in Group C compared to Group B ($p < 0.001$) (Fig. 2B).

PG Synthesis

For the PG synthesis assay, the average DPM per cell measured for ^{35}S-sulfate, relative to the value of Group A, was 1.78 ± 0.27, 2.86 ± 0.26, or 3.25 ± 0.65 in Group B, and 2.86 ± 0.51, 4.45 ± 0.89, or 5.68 ± 1.23 in Group C at 3, 5, or 7 days of coculture. At all time points, a significantly greater increase of DPM of ^{35}S-sulfate per cell was seen in Group C compared to Group B ($p < 0.001$) (Fig. 2C).

Microscopic Examination of Chromosomes

In 10 clinical cases, NP cells cocultured for 3 days with autologous MSCs with direct cell-to-cell contact were investigated for chromosomal abnormalities. There was no evidence of abnormalities in any culture, and no cell cycles of the activated NP cells were influenced negatively (Fig. 4).

Tumorigenesis of Activated NP Cells

NP cells activated by coculture were suspended in a colloidal gel and injected subcutaneously into nude mice. After 6–13 months, a small nodule developed on the skin of the mice. The excised small nodules were colorless, soft, and a very transparent tissue. The findings from H&E-stained frozen sections showed that cell concentrations were very low and the nodule was matrix-rich in all cases. The nuclei were regularly arranged and the size of cells was uniform. We additionally stained the section with safranin-O and checked if there was any cell producing glycosaminoglycan, which may suggest cell survival and differentiation. The result was that there was no significant evidence of cells producing glycosaminoglycan (Fig. 5).

Some activated NP cells had survived over this long term, but not many remained viable in this severely deprived nutritional environment. However, a rich interstitial matrix was maintained. These results showed no evidence of tumorigenesis of activated NP cells at 6 months.

DISCUSSION

Several researchers have reported the utilization of cell-transplantation therapy for disc regeneration. Autolo-

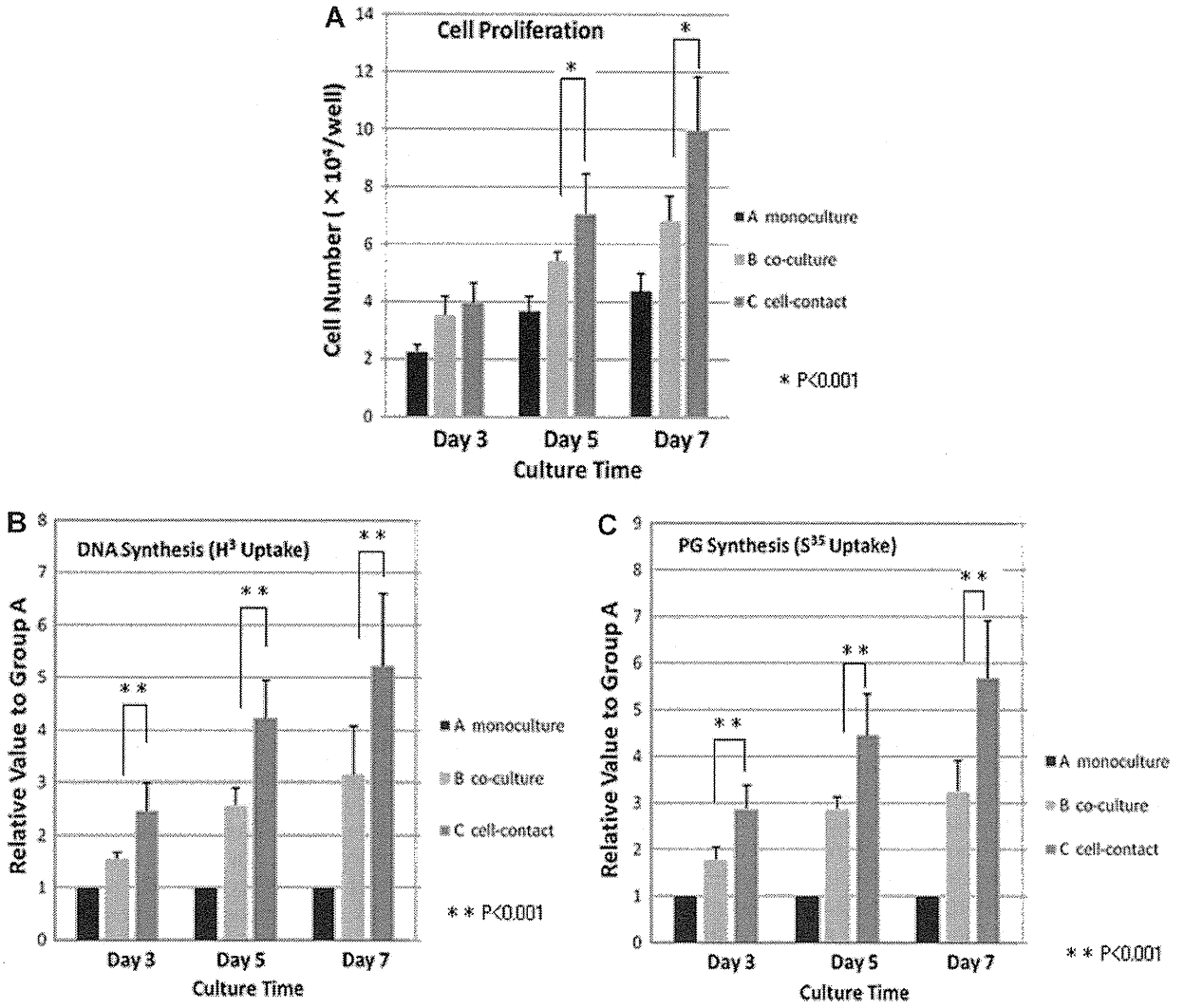

Figure 2. Nucleus pulposus (NP) cells were seeded onto culture inserts and divided into three groups: Group A, NP cell monoculture; Group B, standard coculture of NP cells and mesenchymal stem cells (MSCs); Group C, NP cells and MSCs cocultured with direct cell-to-cell contact. After 3, 5, and 7 days of culture, NP cell proliferation, DNA synthesis, and proteoglycan (PG) synthesis were evaluated for each group. (A) Results of cell proliferation of NP cells. Group C showed a significant increase in cell proliferation when compared to the other two groups at days 5 and 7. (B) Results of DNA synthesis by NP cells. Group B and Group C showed a significant increase in DNA synthesis at all time points compared to Group A. The increase in DNA synthesis by NP cells was greater in Group C than Group B. (C) Results of PG synthesis by NP cells. Group B and Group C showed a significant increase in PG synthesis at all time points when compared to Group A. The increase in PG synthesis by NP cells was greater at all time points in Group C than Group B.

gous disc cell therapies using animal models have been attempted to decelerate disc degeneration. Okuma et al. induced disc regeneration using reinsertion of activated NP cells from one disc into another disc of the same rabbit.[16] Gruber et al. harvested cells from intervertebral disc tissues of sand rats, expanded the cells in culture, and reimplanted the cells into a second disc of the same host rat.[20] Ganey et al. reported autologous disc chondrocyte transplantation in a canine model. They cultured and reimplanted chondrocytes into the same disc.[21] Significant improvement in disc height in comparison to untreated discs was observed 1 year after implantation.[21]

There are some reports of allograft cell transplantation of discs. Nomura et al. found the injection of NP tissues or cells slowed disc degeneration after 16 weeks. However, allograft treatment can induce graft versus host disease and rejection responses; therefore, these methods are unlikely to be clinically applicable.[22]

Cell transplantation using autologous disc cells as described above avoids graft–host reaction and is physiologically more natural. However, the ability of disc cells or the NP itself is not adequate to slow disc degeneration, even when the cell numbers are enhanced by monolayer culture before retransplantation. Therefore, the most serious problem for application of this method remains the difficulty of obtaining a sufficient quantity and quality of NP cells quickly in clinical situations. We believe that the priority in study design was set on a short, in vitro culture period, due to the fact

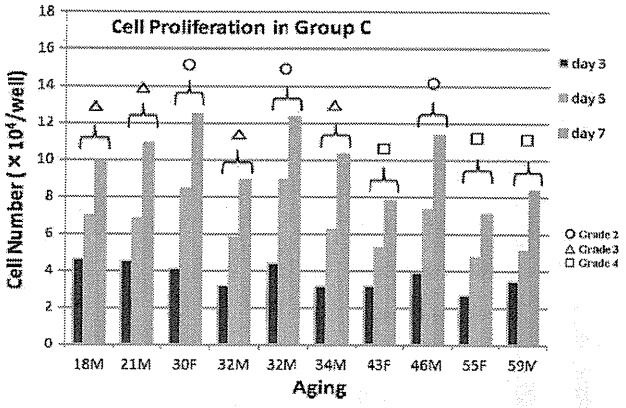

Figure 3. Cell proliferation is dependent on disc grading in Group C (Table 1). This result showed no significant differences among the 10 cases at 3 days of coculture dependent on the effects of age, the MRI disc grade. However, at 5 and 7 days of coculture, NP cells from the lower-graded discs (less-degenerated discs) tended to be more proliferative.

that prolonged culture may elicit mitotic, karyotypic changes, which may lead to tumorigenesis. It may be logical that a longer culture period maybe needed to obtain maximal effect. However, we considered that priority in thinking clinical application is, in short, an in vitro manipulation period.

Several studies have reported on the stimulatory effects of human NP cells using coculture with MSCs. Yang et al. reported that using conventional noncontact coculture, a slight significant increase was achieved for NP cell proliferation.[23] However, Le Visage et al. reported that by using a pellet coculture system with NP cells and MSCs, although there was an trend of increase in GAG production, the difference was not significant.[24] This may be a result of using progressively degenerated human NP cells, or that the pellet culture system used often for chondrocytes did not suit for NP cells and needed further investigation.

From a study done by Kawada et al., reporting that direct cellular contact enhanced cell signaling pathways for proliferation in human hematopoietic progenitor cells cocultured with feeder cells,[25] Yamamoto et al., came to

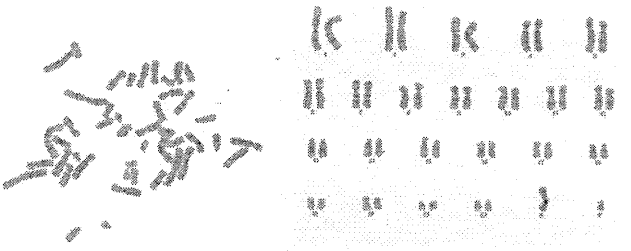

Figure 4. Microscopic examination of chromosomes (G-banding). Nucleus pulposus (NP) cells primarily cultured in monolayer for 4 days and then cocultured for 3 days with autologous mesenchymal stem cells (MSCs) with direct cell-to-cell contact were investigated for chromosomal abnormalities. Chromosomes from NP cells in a mitotic stage were observed by optical microscopy after 7 days total culture. In a randomized sample of 10 cells from each clinical case ($n = 10$), we found no abnormalities such as trisomy, translocations, defects, or other chromosomal abnormalities (Case 4, Table 2).

an idea of activating NP cells by using an coculture system having direct cell contact with autologous MSCs.[17] Coculture system having direct cell contact enhanced the viability of NP cells in vitro and decelerated disc degeneration in vivo in rabbits, in comparison to conventional noncontact coculture.[17] This technique achieved sufficient acceleration of NP cell proliferation to permit transplantation. Mochida also reported that NP cells from an adult beagle dog, activated by a coculture system with autologous MSCs with direct cell-to-cell contact, slows disc degeneration. They selected this canine model as a larger animal species with disc characteristics purported to be more like that of humans.[26]

However, there are differences between human and animal disc models, including axial loading, morphological status, and biological properties. Therefore, evidence that a coculture system between human NP cells and MSCs has an effect similar to those seen in animal models is valuable. Our study in humans demonstrates that cell proliferation was significantly enhanced, and activation of DNA synthesis and PG synthesis was achieved. The coculture system with NP cells and MSCs in direct cell-to-cell contact for only 5 days yielded an approximate fivefold activation of DNA or PG per one NP cell, compared to monolayer culture.

Another problem associated with this cell transplantation therapy concerns the safety of cells cocultured with direct cell-to-cell contact in vitro. Of course, cell treatment must be performed under strict sterile conditions. A concern has been that the sudden acceleration of the cell reproductive cycle could cause abnormalities, such as chromosomal translocations or tumorigenesis. This upregulation of viability of NP cells seemed to be related to growth factors, as reported by Yamamoto et al.[17] in the previous study. Fausto also reported that use of growth factors in tissue regeneration, likely to induce the risk for the sudden acceleration of the cell reproductive cycle, could cause some abnormalities.[27]

It was necessary to confirm that this coculture method was safe, based on analysis of chromosomes in the activated NP cells and an evaluation of any potential tumorigenesis from these cells. The results of this study confirm the safety of donor cells for transplantation at 7 days of culture (4 days-primary culture and 3 days-coculture) in vitro. This is a most important finding for promotion of clinical cell transplantation therapy in humans.

Another interest was that disc grading may possibly be related to the activity level of NP cells for this coculture system, but aging was not clearly related. It is not possible to fully state this from the small sample number. These may be areas of investigation for future studies in human samples (Fig. 3).

The results of study of these 10 clinical cases (Table 1) suggest that the activity of NP cells is affected by the intradiscal environment. For the therapeutic application of the reinsertion of activated NP cells, it therefore seems

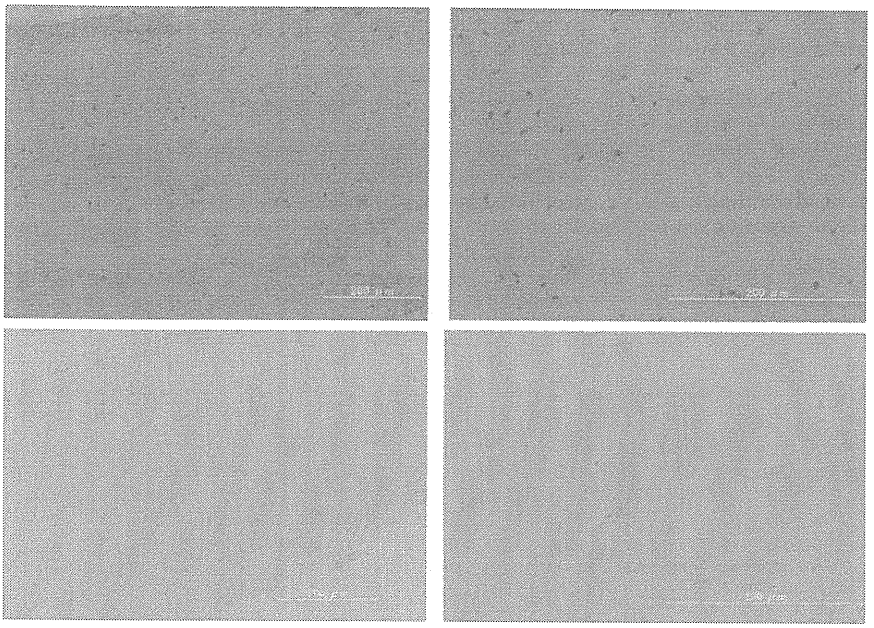

Figure 5. Histological examination of frozen sections. Nucleus pulposus (NP) cells from 10 clinical cases were primarily cultured in monolayer for 4 days and then cocultured for 3 days with autologous mesenchymal stem cells (MSCs) with direct cell-to-cell contact. Cocultured cells were suspended in a colloidal gel and injected subcutaneously into nude mice. After 6–13 months, a small nodule developed on the skin of the mice, which was excised, fixed in 10% formalin, and frozen sections were prepared. Hematoxylin and eosin (H&E)-stained cells and matrix were microscopically examined. The concentration of cells was very low in a rich matrix in all cases. The nuclei of cells were regularly arranged and not atypical. From safranin-O staining, there was no evidence of cells producing glycosaminoglycan (Case 4, Table 3).

desirable to harvest donor NP cells from a less degenerative disc.

In conclusion, a coculture system with direct cell-to-cell contact demonstrated a significantly positive effect in activating the viability of NP cells, not only in animal models, but also in humans. This finding helps to make the reinsertion of activated NP cells clinically feasible.

Furthermore, the effect of direct cell-to-cell contact with MSCs on cell proliferation was greater in NP cells from a less degenerated disc. Therefore disc grading may be one of the most important factors to make the reinsertion of activated NP cells clinically practical.

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REFERENCES

1. Waddell G., 1996. Low back pain: a twentieth century health care enigma. Spine 21:2820–2825.
2. Nishida K, Kang JD, Gilbertson LG, et al. 1999. Modulation of the biologic activity of the rabbit intervertebral disc by gene therapy. Spine 24:2419–2425.
3. Moon S-H., Gilbertson LG, Nishida K, et al. 2000. Human intervertebral disc cells are genetically modifiable by adenovirus medicated gene transfer. Spine 25:2573–2579.
4. Masuda K, Takegami K, An H, et al. 2003. Recombinant osteogenic protein-1 upregulates extracellular matrix metabolism by rabbit annulus fibrosus and nucleus pulposus cells cultured in alginate beads. J Orthop Res 21:922–930.
5. Thompson JP, Oegema TR Jr, Bradford DS., 1991. Stimulation of mature canine intervertebral disc by growth factors. Spine 16:253–260.
6. Yoon ST., 2005. Molecular therapy for the intervertebral disc. Spine J 5:280S–286S.
7. An HS, Masuda K., 2006. Relevance of in vitro and in vivo models for intervertebral disc degeneration. J Bone Joint Surg [Am] 88 (Suppl 2):88–94.
8. Anderson DG, Risbud MV, Shapiro IM, et al. 2005. Cell-based therapy for disc repair. Spine J 5:297S–303S.
9. Anderson DG, Albert TJ, Fraser JK, et al. 2005. Cellular therapy for disc degeneration. Spine 30:S14–S19.
10. Meisel HJ, Ganey T, Hutton WC, et al. 2006. Clinical experience in cell-based therapeutics: intervention and outcome. Eur Spine J 15(Suppl 3):S397–S405.
11. Alini M, Li W, Markovic P, et al. 2003. The potential and limitations of a cell-seeded collagen/hyaluronan scaffold to engineer an intervertebral disc-like matrix. Spine 28:446–454.
12. Kluba T, Niemeyer T, Gaissmaier C, et al. 2005. Human annulus fibrosus and nucleus pulposus cells of the intervertebral disc: effect of degeneration and culture system on cell phenotype. Spine 30:2743–2748.
13. Evans C., 2006. Potential biologic therapies for the intervertebral disc. J Bone Joint Surg [Am] 88 (Suppl 2):95–98.
14. Mochida J, Toh E, Nomura T, et al. 2001. The risk and benefits of percutaneous nucleotomy for lumbar disc herniation. A 10-year longitudinal study. J Bone Joint Surg 83-B:501–505.
15. Nishimura K, Mochida J., 1998. Percutaneous reinsertion of the nucleus pulposus. Spine 23:1531–1539.
16. Okuma M, Mochida J, Nishimura K, et al. 2000. Reinsertion of stimulated nucleus pulposus cells retards intervertebral disc degeneration; an in vitro and in vivo experimental study. J Orthop Res 18:988–997.
17. Yamamoto Y, Mochida J, Sakai D, et al. 2004. Upregulation of the viability of nucleus pulposus cells by bone marrow-derived stromal cells; significance of direct cell-to-cell contact in coculture system. Spine 29:1508–1514.
18. Phillips FM, An H, Kang JD, et al. 2003. Biologic treatment for intervertebral disc degeneration: summary statement. Spine 28 (15 Suppl):S99.
19. Pfirrmann CWA, Metzdorf A, Zanetti M, et al. 2001. Magnetic Resonance Classification of lumbar intervertebral disc degeneration. Spine 26:1873–1878.

20. Gruber HE, Johnson TL, Leslie K, et al. 2002. Autologous intervertebral disc cell implantation: a model using Psammomys obesus, the sand rat. Spine 27:1626–1633.
21. Ganey T, Libera J, Moos V, et al. 2003. Disc chondrocyte transplantation in a canine model: a treatment for degenerated or damaged intervertebral disc. Spine 28:2609–2620.
22. Nomura T, Mochida J, Okuma M, et al. 2001. Nucleus pulposus allograft retards intervertebral disc degeneration. Clin Orthop Relat Res 389:94–101.
23. Yang SH, Wu CC, Shih TF, et al. 2008. In vitro study on interaction between human nucleus pulposus cells and mesenchymal stem cells through paracrine stimulation. Spine 33:1951–1957.
24. Le Visage C, Kim SW, Tateno K, et al. 2006. Interaction of human mesenchymal stem cells with disc cells. Spine 31: 2036–2042.
25. Kawada H, Ando K, Tsuji T, et al. 1999. Rapid expansion of human umbilical cord hematopoietic progenitors using a novel coculture system. Exp Hematology 27:904–915.
26. Mochida J., 2005. New strategies of disc repair; novel preclinical trials. J Orthop Sci 10:112–118.
27. Fausto N., 1991. Growth factors in liver development, regeneration and carcinogenesis. Prog Growth Factor Res 3:219–234.

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Variations in gene and protein expression in human nucleus pulposus in comparison with annulus fibrosus and cartilage cells: potential associations with aging and degeneration

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Summary

Objective: Regardless of recent progress in the elucidation of intervertebral disc (IVD) degeneration, the basic molecular characteristics that define a healthy human IVD are largely unknown. Although work in different animal species revealed distinct molecules that might be used as characteristic markers for IVD or specifically nucleus pulposus (NP) cells, the validity of these markers for characterization of human IVD cells remains unknown.

Design: Eleven potential marker molecules were characterized with respect to their occurrence in human IVD cells. Gene expression levels of NP were compared with annulus fibrosus (AF) and articular cartilage (AC) cells, and potential correlations with aging were assessed.

Results: Higher mRNA levels of cytokeratin-19 (KRT19) and of neural cell adhesion molecule-1 were noted in NP compared to AF and AC cells. Compared to NP cytokeratin-18 expression was lower in AC, and alpha-2-macroglobulin and desmocollin-2 lower in AF. Cartilage oligomeric matrix protein (COMP) and glypican-3 expression was higher in AF, while COMP, matrix gla protein (MGP) and pleiotrophin expression was higher in AC cells. Furthermore, an age-related decrease in KRT19 and increase in MGP expression were observed in NP cells. The age-dependent expression pattern of KRT19 was confirmed by immunohistochemistry, showing the most prominent KRT19 immunoreaction in the notochordal-like cells in juvenile NP, whereas MGP immunoreactivity was not restricted to NP cells and was found in all age groups.

Conclusions: The gene expression of KRT19 has the potential to characterize human NP cells, whereas MGP cannot serve as a characteristic marker. KRT19 protein expression was only detected in NP cells of donors younger than 54 years.
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Key words: Intervertebral disc cells, Phenotype expression, Nucleus pulposus, Annulus fibrosus, Articular cartilage, Human, Aging.

Introduction

Degeneration of the intervertebral disc (IVD) and related spinal disorders are leading causes of morbidity, resulting in substantial pain, disability and increased health care costs[1]. The IVD comprises the highly hydrated nucleus pulposus (NP), the surrounding multilaminar annulus fibrosus (AF) and the cartilaginous endplates. Pathophysiological evidence indicates that IVD degeneration starts in the NP, where the concentration of proteoglycans and the synthesis of type II collagen decrease[2,3]. At the same time denaturation of type II collagen fibers and synthesis of type I collagen occurs[3]. As a consequence the NP loses its osmotic properties and becomes fibrotic; the disc loses its ability to transmit intervertebral forces and further degenerative processes may occur.

Regeneration of NP tissue in the early stages of degeneration may slow down or even reverse the degenerative processes and might possibly restore part of the degenerated disc. Thus, regenerative medicine and biological therapies hold great promise. In particular the therapeutic implications of stem cells have been highly anticipated by both the clinical and scientific communities[4,5]. The challenge in characterizing cellular degeneration and ultimately accomplishing cellular regeneration begins with the identification of the molecular phenotype of the cells that constitute the NP. The NP includes small cells commonly referred to as "chondrocyte-like", since they have a similar rounded morphology and synthesize similar extracellular matrix macromolecules as articular chondrocytes. Currently, no reliable markers exist to distinguish NP cells from the chondrocytes from hyaline cartilage. A cell population with the properties of articular cartilage (AC) would fail to restore the necessary function of the IVD because the requisite fluid properties unique to the IVD would not be recreated. While the ratio of proteoglycan to collagen shows a certain potential to separate disc cells, recent research has focused on the clarification of their molecular phenotype[6]. In a recent study, rat NP cells were compared with cells from the AF and AC tissues by means of large scale microarray gene expression screening. Subsequent quantitative gene expression and immunohistochemical analyses identified distinct molecules, namely glypican-3 (GPC3) and cytokeratin-19

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(KRT19), as promising candidates for NP cell characterization[7]. Similar studies in the beagle dog revealed additional potential NP marker molecules, including alpha-2-macroglobulin (A2M), cytokeratin-18 (KRT18), desmocollin-2 (DSC2), and neural cell adhesion molecule-1 (NCAM1)[8].

However, considerable developmental, anatomical, and biochemical differences among species are likely to affect the phenotypical characteristics of the disc cells[9]. In particular the presence of notochordal cells, which are regarded to be remnants of the embryonic notochord, in the NP is the cause of substantial inter-species variation. Mice, rats, rabbits, and non-chondrodystrophoid dogs retain a predominantly notochordal NP until adulthood and often throughout life, whereas bovine, ovine and chondrodystrophoid dogs closer resemble humans in that the number of notochordal cells rapidly decreases after birth[10]. Moreover, differences in tissue size, oxygen and nutrient supply, and biomechanical requirements are also likely to affect the molecular features of the cells in the disc. As a consequence, observations from animal discs will not necessarily apply to human discs. Nevertheless, animal studies are indispensable for screening purposes, since they allow investigation of normal healthy tissues with larger sample size and smaller inter-individual variability. Due to the limited availability of healthy and viable human IVD tissue, comprehensive screening is difficult in human individuals. The aim of this study therefore was to evaluate the presence and distribution of the molecules that were found to be differently expressed in disc and cartilage cell populations in various animal species in human disc cells. To account for potential variations related to aging and degeneration, cell and tissue samples from individuals of different age groups and disc degeneration grades were examined.

Materials and methods

ISOLATION OF NP, AF, AND AC CELLS

The study was approved by the medical ethical committee of the University Medical Center (UMC) Utrecht and the scientific committee from the Department of Pathology of the UMC Utrecht. Eleven patients with no known history of IVD disease were included in the study. Samples were obtained within an average of 17.5 h after death of the patient (range 6.25–23.0 h). The age of the individuals ranged from 22 to 81 years (average 46 ± 20 years; median 43 years), and the average degree of disc degeneration, assessed according to the Thompson score[11], was 2.2 ± 1.0 (median 2) (Table I). IVD tissue was harvested from segments between L1 and L5 and was separated into NP and AF tissue. To exclude any contamination by AF tissue, only the innermost part of the disc was harvested to be assigned to NP tissue, whereas the transition zone, including part of the inner AF, was entirely excluded from analysis. This is of particular importance for aged discs, where it can be difficult to clearly distinguish NP and AF tissues. AC was harvested from the patellae of the same patients. Chondrocytes were extracted from full thickness cartilage which implies mixed populations of superficial, middle and deep zone cells. Gene expression data thus represent an average cellular expression of target mRNA.

Tissue was cut into small pieces and cells were enzymatically isolated using sequential pronase (Roche) and type II collagenase (Worthington Biochemical) digestion with DNase II (Sigma) added to prevent cell clumping. AC and AF were treated with 0.2% pronase/0.004% DNase for 1 h, then with 200 U/mL collagenase/0.004% DNase over night. NP was treated with 0.2% pronase/0.004% DNase for 1 h, then with 100 U/mL collagenase/0.004% DNase for 8 h, stirring at 37°C in humidified atmosphere. After enzymatic isolation cell suspensions were filtered through a 70 μm cell strainer, washed twice with Dulbecco's Modified Eagles Medium, and lysed in TRI Reagent (Molecular Research Center, Cincinnati, OH). Samples were stored at −80°C until RNA isolation.

Table I

Patients included for gene expression analysis of NP, AF, and AC cells. IVD tissue was harvested from discs between L1 and L5 and separated in NP and AF; AC was harvested from the patella joint surface. Cartilage quality was macroscopically assessed and was without detectable changes for patients 1–9. Slight degenerative changes were detected in patient 10, and signs of osteoarthritis in patient 11

Patient number	Age	Gender	Thompson grade
1	22	M	1
2	25	M	1
3	25	M	1
4	32	M	1–2
5	40	M	2
6	43	M	2
7	46	M	2–3
8	56	M	3
9	61	F	3
10	72	M	4
11	81	F	3

RNA EXTRACTION AND REAL TIME RT-PCR

RNA was isolated using a modified TriSpin method[7,12]. Briefly, bromochloro-propane (Sigma) was added to the lysate, phases were separated, and ethanol (Merck) added to the aqueous phase. Total RNA was extracted using the SV Total RNA Isolation System (Promega), which includes an on-column DNAse digestion, and eluted in 100 μl of RNase-free water. TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA) were used for cDNA synthesis. PCR was performed with an SDS 7500 real time PCR instrument using TaqMan Gene Expression Master Mix (all from Applied Biosystems) and standard thermal conditions (10 min 95°C for polymerase activation, followed by 45 cycles of 95°C for 15 s and 60°C for 60 s). Primer-probe systems, purchased as Gene Expression Assays, were from Applied Biosystems. Genes that were previously found to be differentially expressed in NP compared to AF and/or AC cells in the rat and/or chondrodystrophoid dog were chosen for analysis (Table II)[7,8,13]. Expression of target genes was normalized to the 18S ribosomal RNA as the endogenous control. Relative mRNA levels were calculated according to the 2^(delta Ct) method and presented as log(2) transformed values[14,15].

IMMUNOHISTOCHEMISTRY

For histological analysis human IVD tissue was obtained as part of a standard postmortem procedure, in which a section of the lumbar and thoracic spine is removed for diagnostic purposes. IVD samples were stored in the UMC Utrecht Biobank of the Department of Pathology. Collection and analysis of the IVDs was approved by the medical ethical committee of the UMC Utrecht and the scientific committee of the Department of Pathology of the UMC Utrecht. Samples were obtained within a mean of 17.7 h after death of the patient, 95% within 24 h after death. Between death and tissue collection the deceased patients were kept at the mortuary at 4°C. From all patients the IVD between the fourth and fifth lumbar vertebra (spinal motion segment L4–L5), including the adjacent endplates, was obtained. The grade of degeneration was scored by three individual observers using the classification of Thompson *et al.*[11]. After individual scoring the values were averaged; outliers, i.e., differences of more than 1 Thompson grade, were re-evaluated by the three observers at a consensus meeting.

The expression and localization of KRT19 and matrix gla protein (MGP) in IVD tissue was evaluated in 41 human individuals aged between 3 and 86 years (average 47 ± 25 years; median 51 years, Table III). Sagittal slices of the motion segments were fixed in formalin, decalcified with Kristensen's solution (50% formic acid and 68 g/L sodium formiate) in a microwave at

Table II

Gene expression assays used for real time PCR (from Applied Biosystems)

Gene	Assay code
A2M	Hs_00163474_m1
CD24	Hs_00273561_s1
COMP	Hs_00164359_m1
DSC2	Hs_00245200_m1
GPC3	Hs_00170471_m1
KRT18	Hs_01920599_gH
KRT19	Hs_00761767_s1
MGP	Hs_00179899_m1
NCAM1	Hs_00169851_m1
PTN	Hs_00383235_m1
VIM	Hs_00185584_m1

Table III
Immunohistochemical results obtained from IVD sections from the UMC Utrecht Biobank of the Dept. of Pathology. Only discs between the fourth and fifth human lumbar vertebra (L4–L5) were assessed. Cells were classified according to their topographical position within the disc tissue

Patient number	Age	Gender	Thompson grade	KRT19	MGP				
				NP	NP	IAF	OAF	EP	AOA
1	3	F	1	++	+++	–	–	–	++
2	3	F	1	+++	N/A	N/A	N/A	N/A	N/A
3	6	F	1	–	+++	++	++	–	N/A
4	14	M	1	+++	+++	++	+++	–	+++
5	14	F	1	++	+++	+++	+++	–	N/A
6	14	F	1	–	–	–	++	–	N/A
7	17	M	2	+++	+++	–	–	–	+++
8	18	M	1	+	–	–	–	N/A	N/A
9	19	F	1	–	+++	++	++	–	+++
10	21	M	1	++	+++	+	++	–	+++
11	22	F	1	+	++	–	N/A	–	N/A
12	25	M	1	–	++	–	–	–	++
13	35	F	2	+++	++	+++	+++	–	+++
14	35	M	N/A	–	+++	++	++	–	+++
15	36	M	2	–	++	+	+	–	N/A
16	38	M	2	–	+	–	–	–	+
17	41	M	2	(+)	+++	+	+++	–	+++
18	44	M	2	–	–	–	–	–	–
19	47	F	3	–	++	–	–	–	–
20	51	F	2	–	(+)	–	+	–	+++
21	51	F	2	(+)	++	–	–	–	+
22	54	F	4	(+)	+++	–	+++	–	N/A
23	57	M	4	–	++	++	+++	–	N/A
24	59	M	4	–	+++	++	N/A	–	N/A
25	60	M	5	–	+++	++	++	–	+++
26	62	F	2	–	–	–	–	–	+++
27	62	M	5	–	+++	+++	+++	–	+++
28	63	M	3	–	++	+	–	–	N/A
29	67	F	5	–	+	N/A	N/A	N/A	N/A
30	68	F	5	–	N/A	N/A	N/A	+	++
31	70	M	4	–	++	–	–	–	++
32	71	M	3	–	++	–	–	N/A	NA
33	72	M	N/A	– (*)	(+)	–	–	–	NA
34	72	M	5	–	+++	–	+++	+	+++
35	73	F	3	–	+++	+++	–	–	N/A
36	74	F	3	–	++	–	–	–	+++
37	76	M	3	–	++	+++	–	–	+++
38	76	F	5	–	+	(+)	–	–	+++
39	80	F	5	– (*)	+++	++	N/A	N/A	+++
40	82	F	4	–	++	–	–	–	N/A
41	86	F	4	–	+	–	–	–	N/A

Grading scheme: (+) = 1–2 positive cells; + = 3–4 positive cells; ++ = 5–10 positive cells; +++ = >10 positive cells per field of view. For analysis a Zeiss Axioplan2 microscope equipped with a 20× objective (Neofluar) and a 10× ocular was used. IAF: Inner annulus fibrosus; OAF: Outer annulus fibrosus; EP: Cartilaginous endplate; AOA: Attachment of outer annulus fibrosus; N/A: not available. KRT19 positive cells were only detected in the NP and in 2 cases of severe degenerative disc changes in the EP (*).

150 W and 50°C for 6 h, dehydrated in graded ethanol series, and embedded in paraffin[16]. Sections were deparaffinized, treated with 3% hydrogen peroxide in methanol for 30 min and then with heated (95°C) citrate buffer (10 mM sodium citrate, 0.05% Tween20, pH 6.0) for 20 min for antigen retrieval. Then they were blocked with 5% normal horse serum for 1 h, and were incubated with mouse monoclonal anti-KRT19 antibody (clone A53-B/A2; cat. no. EXB-11-120, Exbio, Praha, CZ) or mouse monoclonal anti-MGP antibody (clone 52.1C5D; cat. no. ALX-804-512, Alexis Biochemicals, Lausen, CH) at a concentration of 5 μg/ml over night at 4°C. Negative control sections were incubated without primary antibody. Biotinylated secondary anti-mouse antibody (dilution 1:200; Vectastain ABC-kit *Elite*, cat. no. PK-6102, Vector Laboratories, Burlingame, USA) was applied, followed by ABC complex, and chromogen development using diaminobenzidine (DAB Kit, cat. no. SK-4100, Vector Laboratories, Burlingame, USA). Sections were counterstained with Mayer's haematoxylin.

STATISTICAL ANALYSIS

Differences in relative gene expression levels between paired NP and AF and paired NP and AC were assessed by the Wilcoxon Signed Ranks test. Correlations between relative gene expression and age or disc degeneration grade were determined using the Pearson correlation analysis. $P < 0.05$ was considered as significant.

Results

GENE EXPRESSION

In both the NP vs AF and the NP vs AC comparisons, pronounced gene expression differences were observed for KRT19. Levels of KRT19 mRNA were constantly higher in the NP than in both AF and AC cells, although the extent of up-regulation varied between individuals. NCAM1 expression was also increased in NP compared to AF and AC cells, while the expression of A2M and DSC2 was higher in NP than in AF cells and KRT18 expression was higher in NP than in AC cells. On the other hand, mRNA