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CD24 is expressed specifically in the nucleus pulposus of intervertebral discs

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Abstract

Intervertebral disc (IVD) consists of a soft gelatinous material in its center, the nucleus pulposus (NP), bounded peripherally by fibrocartilage, annulus fibrosus (AF). Despite the number of patients with IVD degeneration, gene expression analysis has not been undertaken in NP and therefore little is known about the molecular markers expressed in NP. Here, we undertook a microarray screen in NP with the other nine tissues to identify the specific cell surface markers for NP. Five membrane associating molecules out of 10,490 genes were identified as highly expressing genes in NP compared with the other tissues. Among them, we identified CD24, a glycosylphosphatidylinositol (GPI) anchor protein as a cell surface marker for NP. CD24 expression was also detected in the herniated NP and chordoma, a malignant primary tumor derived from notochordal cells, while it was absent in chondrosarcoma. Therefore, CD24 is a molecular marker for NP as well as the diseases of IVD.

Keywords: Intervertebral disc; Nucleus pulposus; CD24

Intervertebral disc (IVD) degeneration and concomitant herniation are the distinguishing anatomical features associated with lumbar disc disease (LDD). The molecular mechanisms leading to the onset of IVD degeneration are not well understood. Recent genetic studies in humans and mice indicate that genetic factors play an important role in the etiology and pathogenesis associated with LDD [1–3].

The IVD is composed of two discrete components termed the nucleus pulposus (NP) and the annulus fibrosus (AF). The interior structure, NP, is a soft gelatinous avascular cartilage-like tissue, derived from notochord, containing extracellular matrix proteins (ECM) rich in large proteoglycans such as aggrecan and collagens. ECM proteins play a central role in chondrocyte metabolism through regulation of growth factors and appear to be crucial for maintaining IVD homeostasis and integrity [4,5]. A recent genetic study demonstrated the role of cartilage intermediate layer protein (CILP), an ECM that acts as a modulator of LDD susceptibility through deregulation of TGFβ signaling [3].

The AF, a fibrous cartilage composed of an inner and outer coaxial lamella, is bound to the periphery of the NP. The onset of degenerative disc disease is marked by mechanical stress-induced apoptosis in the AF resulting

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in degeneration and disruption of the outer disc tissue leading to herniation of the NP [6]. This sequelae of events is consistent with the susceptibility to LDD associated with aggrecan and collagen mutations that compromise the response of IVD cells to injury [1,2].

To further delineate the mechanism of NP function in IVD degeneration, we performed a microarray screening and cluster analysis to identify cell factors that were expressed specifically in NP tissue. Based on previous findings about the properties and function of NP cells, we screened several different tissues for comparison in our microarray analysis. These tissues include: avascular tissues such as AF and tendon, mesenchymal tissues such as skeletal muscle, skin, bone, AF, and tendon; and neurogenic tissues including spinal cord and brain. We found that expression of a heat-stable antigen termed CD24, a glycosylphosphatidylinositol-anchored cell surface protein, was upregulated in NP cells in a tissue specific manner.

CD24 is expressed in neurons, preB cells, T cells, and several cancer cells [7–10]. It also functions in differentiation and activation of granulocytes and B lymphocytes [11]. CD24 deficient mice show no gross physical or behavioral abnormalities, however the homeostatic proliferation of CD24 deficient T cells is reduced indicating that CD24 may be involved in cell proliferation [12].

In this study, we found that CD24 was high in NP and was detected in herniated NP tissue. We also determined CD24 expression was upregulated in chordoma, a malignant primary tumor derived from notochord cells. In contrast, we were unable to detect CD24 expression in chondrosarcoma, malignant tumors derived from mesenchymal cells.

Materials and methods

Microarray analysis. Total RNA was extracted and pooled from 10 different tissues including NP, AF, tendon, skeletal muscle, spinal cord, brain, skin, bone, bone marrow, and peripheral blood of 8-week-old male Wistar rats using TRIzol Reagent (Qiagen GmbH, Hilden, Germany). To ensure a sufficient amount of Poly(A)$^+$ RNA for screening, we pooled NP, AF, and tendon tissue from 50 rats. For the remaining tissues, we pooled tissue from 10 rats. Synthetic polynucleotides (80-mers) representing 11,464 rat transcripts derived from 10,490 independent genes (MicroDiagnostic, Tokyo, Japan) were arrayed with a custom-made arrayer. Two micrograms of poly(A)$^+$ RNA was labeled with cyanine 5-dUTP or cyanine 3-dUTP. Hybridization and subsequent washes of arrays were performed with a Labeling & Hybridization Kit (MicroDiagnostic). Hybridization signals were measured with a GenePix 4000A scanner (Axon Instruments, Union City, CA) and then processed into primary expression ratios (ratios of cyanine 5-intensity obtained from each sample to cyanine 3-intensity obtained from the rat common reference RNA), which are indicated as 'median of ratios' by the GenePix Pro 3.0 software (Axon Instruments). Normalization was performed for the median of ratios by multiplying normalization factors calculated for each feature on a microarray by the GenePix Pro 3.0 software. The expression ratios were converted into log2 values as final expression ratios.

Animals. All animals were purchased from Japan Crea (Tokyo, Japan) or born and kept under pathogen-free conditions, and cared for in accordance with the guidelines of Keio University School of Medicine.

Rat hernia model. Posterior herniations were created between the 5th and 10th tail IVD of 8-week-old male Wistar rats. After a posterior incision above the IVD was made, the soft tissues such as posterior tendons and ligaments were separated. Then, a small incision was made in AF with subsequent compression between an upper and lower vertebral body to prepare a posterior herniation. Seven days after surgery, rats were sacrificed and the herniated discs with vertebral bodies were removed. For preparation of an IVD section, freshly isolated IVDs were embedded in rat minced liver and frozen using 2-methylbutane (Wako, Osaka, Japan) and liquid nitrogen. Frozen sections of IVD, that had not been decalcified, were obtained using a cryostat (MICROME, model HM505) equipped with a tungsten carbide knife.

Human samples. For the experimental use of the surgical samples, informed consents were obtained from the patients according to the Hospital Ethical Guideline (Keio Hospital #15-52). Specimens of seven chordomas and seven chondrosarcomas diagnosed according to conventional criteria, and three IVD tissues dissected from scoliosis patients as normal IVD were fixed in 4% paraformaldehyde/PBS, embedded in paraffin, and 4 μm sections were cut.

Cell harvest and flow cytometric analysis. NP and AF cells were macroscopically dissected from the cervical, thoracic, and lumber IVD of 8-week-old male Wistar rats. NP tissue was digested with pronase E (0.04%) (SERVA, Heidelberg, Germany) for 1 h at 37 °C and then collagenase P (0.025%) (Roche Diagnosis), for 1 h at 37 °C. To isolate AF cells, AF tissue was treated with pronase E (0.4%) for 1 h at 37 °C, followed by collagenase P (0.025%) for 4 h at 37 °C. Cells were then washed with DMEM/F12 medium (Invitrogen, Carlsbad, CA) containing fetal bovine serum (5%) (Equitech-Bio, Kerrville, TX).

Cells were stained with anti-rat CD24 (clone HIS50, BD PharMingen, San Diego, CA) followed by FITC-conjugated anti-mouse IgG (Biosource, Camarillo, CA). Flow cytometry and cell sorting was performed using FACS Vantage or FACS Calibur (Becton–Dickinson Immunocytometry Systems, San Jose, CA). NP and AF cells were also stained with May-Gruenwald-Giemsa.

Immunohistochemical analysis. Rat IVD were dissected from 8-week-old Wistar rats, fixed in formalin (10%), embedded in paraffin, and cut into 4 μm sections. Deparaffinized sections of paraffin embedded samples or cryosections of rat herniated IVD were stained with anti-rat CD24 (HIS50, BD PharMingen, San Diego, CA, diluted 100-fold) followed by FITC-conjugated anti-mouse IgG (Biosource, Camarillo, CA, diluted 200-fold) and TOTO3 (Invitrogen, diluted 750-fold) for nuclear staining. Antigen retrieval was achieved on chordomas and chondrosarcoma sections by pressure-cooking in citrate buffer (pH 6.0) for 20 min. Samples were then stained with anti-human CD24 (Ab-2, clone 24C02, Neomarkers, Fremont, CA diluted 100-fold) followed by Alexa Fluor488-conjugated anti-mouse IgG (Molecular Probes, Oregon, USA, diluted 100-fold). Immunoreactivity was detected by fluorescence microscopy (Olympus, Tokyo, Japan).

Real-time RT-PCR assay. Total RNA was extracted from NP, AF, pattela tendon, peripheral blood, tibia bone, bone marrow, brain, lens, musculus quadriceps femoris, back skins, spinal cord, white adipose tissue, articular cartilage of femur head, and medial collateral ligaments from the knees of 8-week-old male rats (RNeasy mini kit (Qiagen GmbH, Hilden, Germany) or Trizol (Invitrogen)). First strand cDNA was prepared using the first strand synthesis kit (Invitrogen) according to the manufacturer's instruction, and cDNAs were amplified using a Light Cycler FastStart DNA Master SYBR Green I (Roche diagnostics) in a Light Cycler Quick System (Roche Diagnostics, Mannheim, Germany). Relative mRNA expression levels are shown by comparison with β-actin mRNA expression. The primers used are shown below:

5′-rat β-actin 5′-TCCTAGCACCATGAAGATC-3′,
3′-rat β-actin 5′-AAACGCAGCTCAGTAACAG-3′
5′-rat CD24 5′-TGCTTCTGGCACTGCTCCTAC-3′
3′-rat CD24 5′-GGTGGTAGCATTAGTTGGATTTGG-3′
5′-human β-actin 5′-CGTGACATTAAGGAGAAGC-3′
3′-human β-actin 5′-GGAGTTGAAGGTAGTTTCG-3′
5′-human CD24 5′-GCACTGCTCCTACCCACGCAGATTT-3′
3′-human CD24 5′-GCCTTGGTGGTGGCATTAGTTGGAT-3′

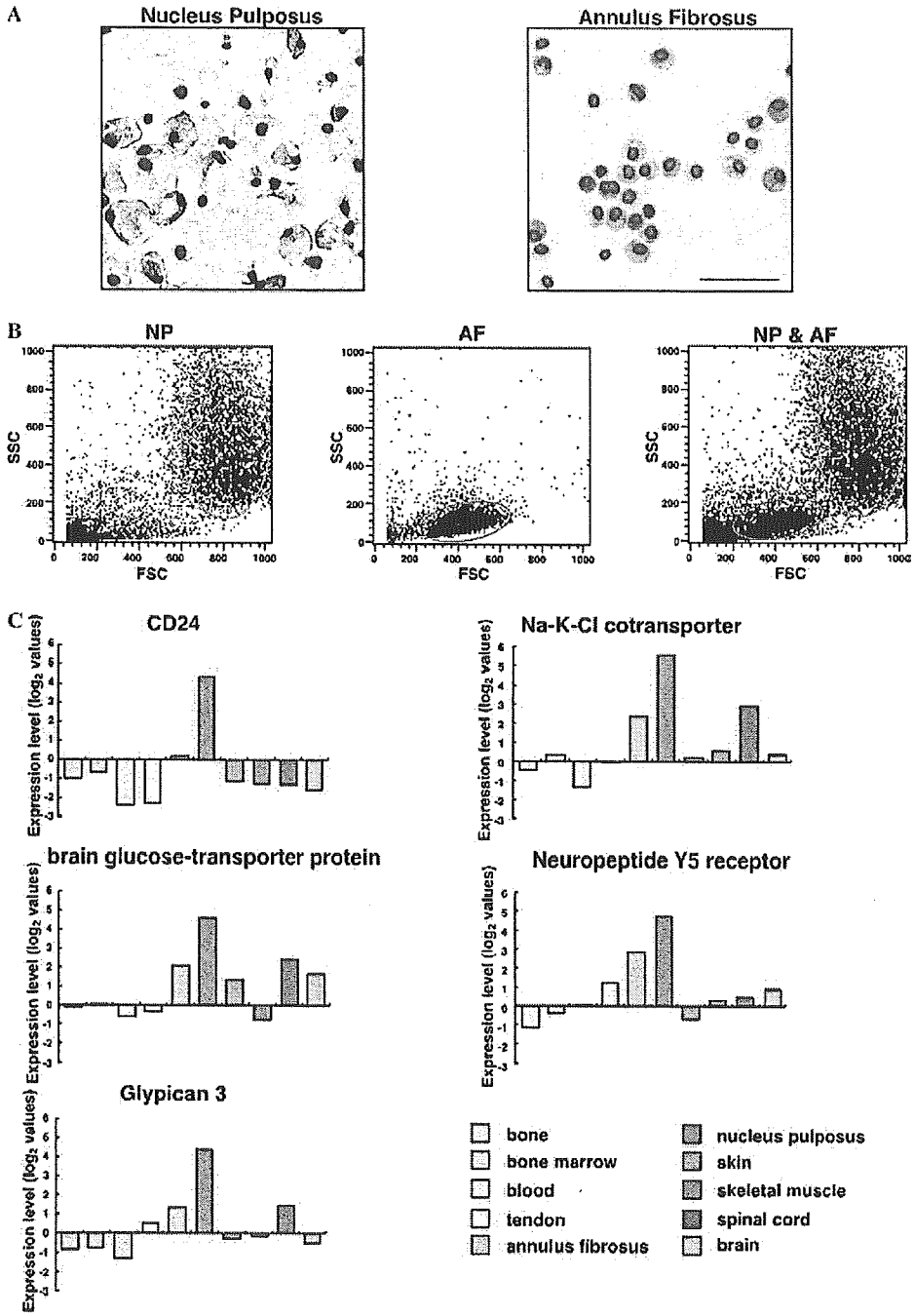

Fig. 1. Identification of NP specific cell surface molecules. Nucleus pulposus (NP) and annulus fibrosus (AF) were isolated from 8-week-old male rat IVD, and their morphology was examined by May-Grunwald-Giemsa staining (A) and flow cytometric analysis (B). Bar = 100 μm. Red and blue circles represent the NP and AF population, respectively. (C) Ten different tissues, including NP and AF, were dissected from 8-week-old male rats, and DNA array hybridization and cluster analysis were performed. Five genes were identified that were expressed highly in NP cells when compared with the other nine tissues.

Results

Identification of NP specific cell factors

To identify cell factors expressed specifically in NP cells, we performed a comparative microarray analysis of 10,490 genes in NP and AF cells, and in cells isolated from tendon, skeletal muscle, spinal cord, brain, skin, bone, bone marrow, and peripheral blood of 8-week-old male Wistar rats. We isolated NP and AF tissues from lumbar, thoracic, and cervical IVD from more than 50 rats, and pooled tissue samples prior to isolation of poly(A)$^+$ RNA. Fig. 1 shows May-Gruenwald-Giemsa staining (Fig. 1A), and flow cyto-

metric analysis (Fig. 1B) of cells isolated from NP and AF tissues. As expected, NP cells had a larger cytoplasm (FSC) and a more complex structure (SSC) when compared to AF cells. We isolated the eight other tissues including tendon, skeletal muscle, spinal cord, brain, skin, bone, bone marrow and peripheral blood from 10 rats, and pooled samples from each tissue. Poly(A)$^+$ RNA was isolated under protease-free conditions to prevent degradation and ensure that we screened a representative pool of poly(A)$^+$ RNA that accurately reflected the levels of expression in cells.

In this report, we focused our efforts on identifying an NP specific cell surface marker that could be utilized to further characterize NP cells. To that end, we chose five cell surface proteins that were specifically expressed at a high level in NP but not in AF cells, or the eight cell types we analyzed (Table 1 and Fig. 1C). These cell factors included: CD24 antigen (NM_012752), Na–K–Cl co-transporter (AF051561), brain glucose-transporter protein (M13979), neuropeptide Y5 receptor (NM_012869), and Glypican 3 (NM_012774). We selected one of the five candidate genes, CD24, for further study since, of the five genes identified, it had the highest level of specific expression in NP cells.

CD24 expression is elevated in NP cells in a tissue specific manner

To determine the relative level of expression of these genes in NP tissue, we used a semi-quantitative real-time PCR assay to determine mRNA expression in NP and AF cells, and the various cell types used in our screen (Fig. 2A). In this experiment we also examined the expression level of CD24 in three additional mesenchymal tissues: white adipose tissue (WAT), articular cartilage, and ligament cells. Our results confirmed that CD24 is specifically expressed in NP cells and that the level of expression was elevated by approximately 4-fold when compared to AF cells. We detected a very low level of CD24 expression in skin and tendon cells, and virtually no expression in the rest of the tissues we examined. We also used a commercially available CD24 antibody to confirm tissue specific expression of CD24 in NP cells using flow cytometry (Fig. 2B), and immunohistochemistry (Fig. 2C). Our results demonstrate that, based on the tissues we examined, CD24 is expressed specifically in NP cells. In addition, CD24 is a useful cell surface marker for identifying NP cells. We have also detected CD24 expression in human NP cells by RT-PCR and immunohistochemical analysis (data not shown).

CD24 is expressed in herniated NP tissue

To further investigate the role of CD24 in IVD disease, we analyzed CD24 expression in a rat model that recapitulates the pathological conditions associated with IVD disease in vivo. Posterior lumbar herniations were created between the 5th and 10th tail IVD. We were able to distinguish herniated protrusions (H) and intact NP (N) (see upper panels Fig. 3). CD24 expression was detected in the herniated protrusion as well as in the remaining NP tissue (lower panels Fig. 3). This result suggests that CD24 may also function in herniated NP tissue.

CD24 is expressed in chordoma cells

Chordoma, a primary malignant tumor of the skeleton, is considered to develop from a remnant of notochordal cells. Chordoma develops mostly in the sacrum region in

Table 1
Microarray identification of highly expressed genes in NP

Accession No.	Tissue									
	Bone	Bone marrow	Blood	Tendon	AF	NP	Skin	Muscle	Spinal cord	Tendon
NM_053518	0.2714	−0.1943	1.4249	0.5499	2.6595	6.3545	−0.2362	−0.6552	0.5039	0.9848
D45920	2.0607	4.421	0	1.6991	1.4552	6.1639	0	0.2857	4.0969	3.1089
AB020019	0.3696	0.2314	1.4082	0.5281	1.8984	5.8966	−0.4461	−0.1219	0.2485	0.585
M13518	0.5917	−0.3147	0.1648	1.542	5.5938	5.8456	−0.1047	−0.484	−0.394	−0.5564
AF051561	−0.454	0.308	−1.3364	−0.0484	2.3934	5.5614	0.2029	0.5763	2.9445	0.3896
NM_031140	1.3829	−0.456	−0.3808	2.6318	1.9309	5.3232	1.0545	−0.255	1.4942	−1.462
XM_217890	0.546	0.9298	0.514	−0.1345	1.301	5.1885	0.5772	0.519	0.4823	0.6012
X62952	1.107	0.5685	−0.8651	2.0374	1.6327	5.0932	0.8891	−0.7298	1.111	−2.0233
AA684960	−0.5951	−0.8135	−0.1959	4.0923	3.3176	4.8973	1.9355	−0.0816	−0.7346	−0.9214
AA686870	−1.9546	−3.2934	−2.7959	4.0689	3.2575	4.886	1.782	−1.2447	−1.7859	−2.0116
AA685376	−0.9828	−1.3808	−0.873	4.1059	2.9635	4.8324	1.9419	−0.0425	−1.152	−1.0262
NM_012869	−1.1392	−0.3696	0.037	1.2284	2.8584	4.7459	−0.6873	0.3208	0.4772	0.9161
AA684929	−0.8034	−0.8288	−0.0499	4.1048	3.2363	4.6141	1.9942	0.0468	−0.9828	−1.0954
M13979	−0.1187	0.0676	−0.606	−0.3364	2.0895	4.5972	1.3437	−0.7984	2.4354	1.6713
NM_012880	2.3417	−4.2379	−4.6439	0.1519	3.4848	4.4875	−3.8783	−4.7959	1.9452	−3.5395
NM_012774	−0.8494	−0.7735	−1.3004	0.514	1.3374	4.4059	−0.2934	−0.1584	1.4626	−0.482
NM_054008	1.9249	0.8237	−0.9296	1.058	0.4823	4.3906	−1.114	−0.7202	0.3161	0.1878
NM_080698	−2.4344	−2.699	−1.1779	4.5453	4.1006	4.3845	−2.9885	0.0014	−2.2042	−1.6943
NM_012752	−0.9799	−0.6804	−2.3884	−2.3004	0.1725	4.3827	−1.1297	−1.2758	−1.3147	−1.5606
AI007530	−0.035	−0.2176	0.704	3.1437	2.5969	4.3677	1.595	−0.2092	−0.3997	−0.3292

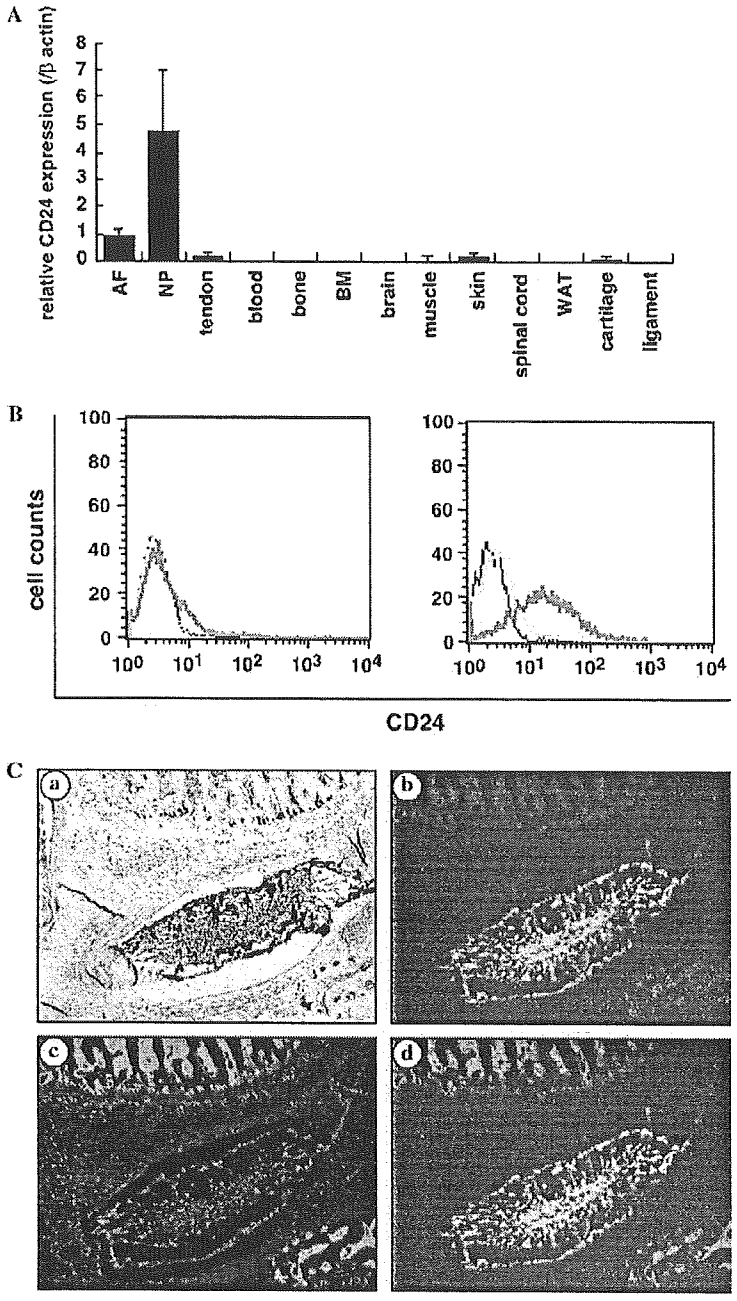

Fig. 2. Confirmation of CD24 expression in NP. (A) CD24 expression was analyzed in 13 different tissues, 10 of which were analyzed in microarray assay, by semi-quantitative real-time PCR. CD24 expression was elevated specifically in NP cells. (B) CD24 expression was analyzed in NP and AF cells using flow cytometry. CD24 expression was detected in NP but not in AF. (C) IVDs were dissected from 8-week-old male rats and stained by rabbit anti-rat CD24 antibody followed by FITC-conjugated anti-rabbit IgG. TOTO3 was used as a counter stain for nuclei. Sections were then stained and examined using phase contrast microscopy (a) and fluorescence microscopy (b–d). (b) CD24, (c) TOTO3, and (d) merged image of CD24 and TOTO3. AF, annulus fibrosis; NP, nucleus pulposus; BM, bone marrow; WAT, white adipose tissue.

elderly patients and local recurrences are frequently observed. Although chordoma cells are considered to derive from the notochord, a comparative evaluation of a tissue specific marker has not been possible. Our identification of CD24 as an NP specific cell marker allowed us to evaluate and compare CD24 expression in notochord, chordoma, and chondrosarcoma, malignant tumors that are derived from mesenchymal cells. Specimens of chordoma and chondrosarcoma were analyzed for CD24 expression (Fig. 4). CD24 expression was detected in six out of the seven chordomas, but not in the seven different chondrosarcomas we analyzed. This result suggests that chordo-

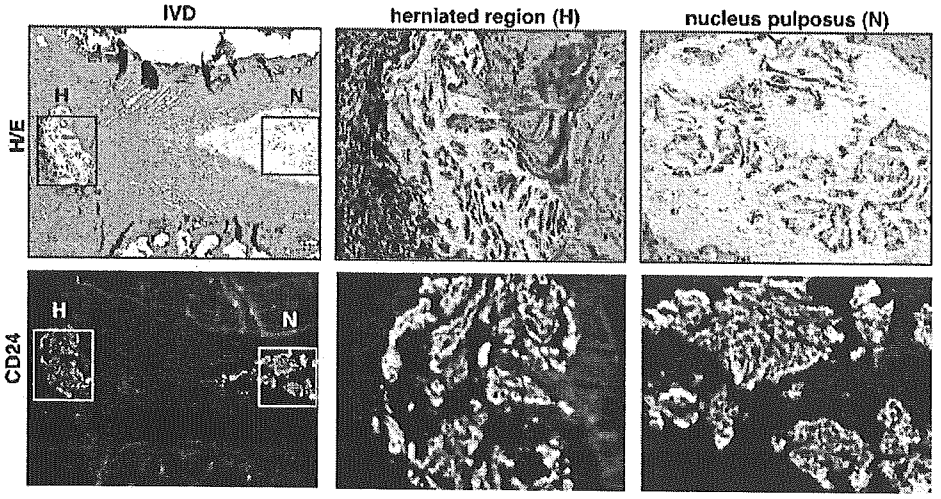

Fig. 3. CD24 expression is detected in herniated protrusion. IVD hernia model was created in 8-week-old rat tail IVDs. Seven days later, IVDs were dissected and stained by H/E (upper panel) or anti-CD24 antibody followed by FITC-conjugated anti-mouse IgG (lower panel), and observed under a fluorescence microscope.

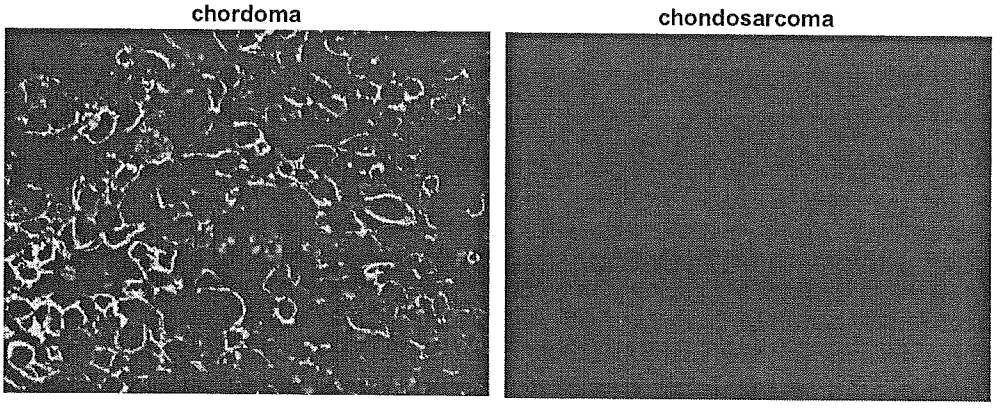

Fig. 4. CD24 expression is detected in chordoma but not in chondrosarcoma. Specimens of chordomas (left panel) and chondrosarcomas (right panel) were stained by anti-human CD24 followed by Alexa Fluor488-conjugated anti-mouse IgG. Immunoreactivity was detected by fluorescence microscopy.

mas are derived from notochordal cells in NP tissue, and that chordoma cells can be distinguished from chondrosarcoma cells by analysis of CD24 expression.

Discussion

We found that expression of CD24 is high in NP cells in a tissue specific manner. To further characterize the function of CD24 in NP tissue, we determined that CD24 is expressed in herniated NP tissue. We also determined that CD24 expression is elevated in chordoma, one of the most common malignant primary neoplasms of the skeleton, which are thought to be a remnant of notochordal cells. The similar pattern of gene expression observed between chordoma and NP supports the hypothesis that chordoma originates from notochordal cells. Interestingly, three out of the five membrane associated factors we identified in this study including: CD24, brain glucose-transporter protein, and the Na–K–Cl co-transporter are also expressed in chordoma (data not shown). Since both NP and chordoma tissues express chondrogenic extracellular matrix protein and aggrecan at high levels, they cannot be used to distinguish chordoma from other chondrogenic malignant tumors [13]. We did not detect CD24 expression in chondrosarcomas, which are malignant tumors derived from mesenchymal cells.

IVD, especially NP is rich in large chondroitin sulfate proteoglycans, which have the ability to retain water in tissues. A large proteoglycan, aggrecan, forms huge aggregates by binding to hyaluronate and links proteins to maintain tissue homeostasis. In contrast, collagens, such as collagen type 2 form a fibrous structure in cartilage. Previous reports indicate that aggrecan and collagen type 9 are involved in IVD maintenance, and more recently, signaling through TGFβ-1 was shown to be important for the expression of both collagen type 2 and aggrecan [1–3]. It

appears that loss of TGFβ-1 signaling in asporin or cartilage intermediate layer protein mutants reduces collagen type 2 and aggrecan expression, and leads to development of osteoarthritis and LDD, respectively [14].

The IVD is an avascular tissue like articular cartilage, tendon, and lens. It remains to be clarified how these avascular tissues are maintained in the absence of oxygenation. One potential explanation is that the fluid surrounding functions in maintaining these tissues under hypoxic conditions. Like NP tissue, the lens is an avascular tissue, and as seen with IVD, degeneration of lens tissue correlates with age. Interestingly, CD24 is also highly expressed in lens (data not shown), indicating CD24 may function to maintain such avascular tissues.

Identification of CD24 as an NP specific marker provides a valuable tool for future studies to ascertain the function of CD24 in the maintenance of NP homeostasis and development of chordomas.

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分担研究報告書

高齢者の腰痛症に係るより効果的かつ効率的な診断、治療、介護

及びリハビリテーション等の確立に関する研究

研究課題名：再生医療を用いた高齢者腰痛症に対する新たな治療法の開発

分担研究者：持田譲治　東海大学医学部外科学系整形外科学　教授

研究要旨：自己間葉系幹細胞(MSC)と細胞間接着を有する共培養法と自家血清を用いることで短期間に椎間板細胞を体外増幅できることが明らかになった。大動物を用いた in vivo 実験において、髄核細胞移植は椎間板変性の進行を遅延させることが可能であった。

A. 研究目的

高齢者腰痛の主たる原因となり得る椎間板変性には現在有効な治療法は存在しない。椎間板変性は多因子依存性でその成因は画一的でないが、組織学的に主因は髄核の細胞環境変化にある。すなわち髄核内の細胞代謝不均衡、栄養障害、軟骨細胞様髄核細胞を支持する脊索由来細胞の減少などにより髄核内の細胞活性が低下、細胞数が減少し相対的に合成マトリックス量が減少することで線維輪内層における形態維持が保持できなくなり始まる。我々は共培養技術を用いて髄核細胞活性を高め、椎間板へ移植することにより、変性過程の線維輪内層細胞を活性化、椎間板変性を時間的に抑制しうることを小動物にて証明した。本研究では実際の患者検体を用い髄核細胞活性化の検討、技術的に臨床応用化可能か否かを検証、さらに in vivo 実験の臨床応用前実験として大動物椎間板変性モデルを用いて細胞移植療法の効果を検討した。

B. 研究方法

自己間葉系幹細胞(MSC)と細胞間接着を有する共培養法と自家血清を用いることで短期間に椎間板細胞を体外増幅出来る為、手術検体を用いて検証した。症例数は 13 例、疾患は腰部椎間板ヘルニアが 9 例、腰椎破裂骨折が 3 例、腰椎分離すべり症が 1 例であった。患者同意の下で術中に検体から髄核細胞と MSC を自家血清添加培地下に細胞間接着を伴う共培養を行ない細胞増殖能、プロテオグリカン(PG)合成能を評価した。In vivo では Beagle 犬, 11 頭を 3 群（NC 群 2 頭：無処置 control、D 群 2 頭： 変性モデル、Tx 群 7 頭:細胞移植群）にわけ, nucleotomy を全身麻酔下に行った。髄核細胞を分離培養し、4 週後に Tx 群に透視下で経皮的に細胞移植した。評価は X 線、MRI、肉眼的、組織学的に行なった。

C. 研究結果

獲得細胞数、DNA 合成能、PG 合成能で約 5 倍と全ての評価で共培養群が優れており動物実験に準じた結果となった。大動物を用いた in vivo 実験においては実際の移植術に則して透視下で経皮的に細胞移植したところ、椎間板高と MRI での所見が変性モデルに比べ有意に保持されており、組織学的評価で線維輪内層構造がより保持されていることが確認され、術後変性の進行を遅延させることが技術的に可能であった。

D. 考察

動物実験の結果から開発した共培養法により短期間で細胞を体外培養で増幅させうることが証明された。尚、本業務は現在 Cell Processing Center での作業へと移行し、臨床応用化に向け症例数を重ねている。また移植手技は透視下で経皮的に椎

間板内へ細胞を移植することが可能であること、また術後の椎間板変性進行を時間的に抑制しうる手技であることが確認された。現在その適応症例、業務フローの検討が進められている。

E. 結論

自己間葉系幹細胞(MSC)と細胞間接着を有する共培養法と自家血清を用いることで短期間に椎間板細胞を体外増幅できることが明らかになった。大動物を用いた in vivo 実験において、髄核細胞移植は椎間板変性の進行を遅延させることが可能であった。

F. 健康危険情報

問題なし。

G. 研究発表

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3) Sakai D, Mochida J, Iwashina T, Watanabe T, Nakai T, Ando K, Hotta T. Differentiation of Mesenchymal Stem Cells Transplanted to a Rabbit Degenerative Disc Model. Spine 2005 Nov 1;30(21):2379-2387.

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7) Sakai D, Mochida J, Iwashina T, Hiyama A, Omi H, Imai M, Nakai T, Ando K, Hotta T. Regenerative effects of transplanting mesenchymal stem cells embedded in atelocollagen to the degenerated intervertebral disc. Biomaterials. 2006 Jan;27(3):335-45.

2. 学会発表：なし

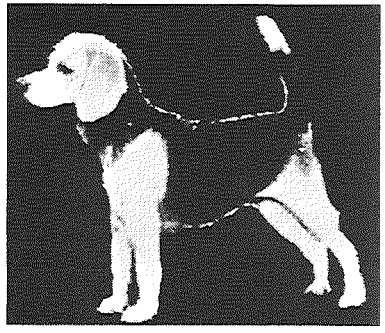
H. 知的財産権の出願・登録状況

予定していない。

Materials and method

Beagle(10~12 months, ♀)

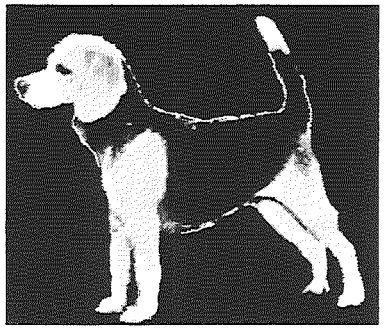
n=2



control

NC group

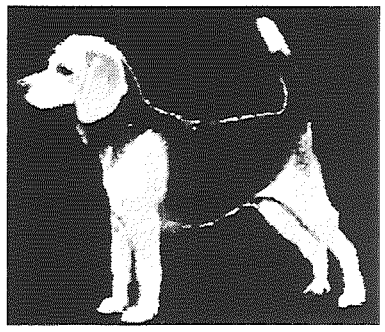
n=2



NP aspiration
(operation 1)

D group

n=6+1



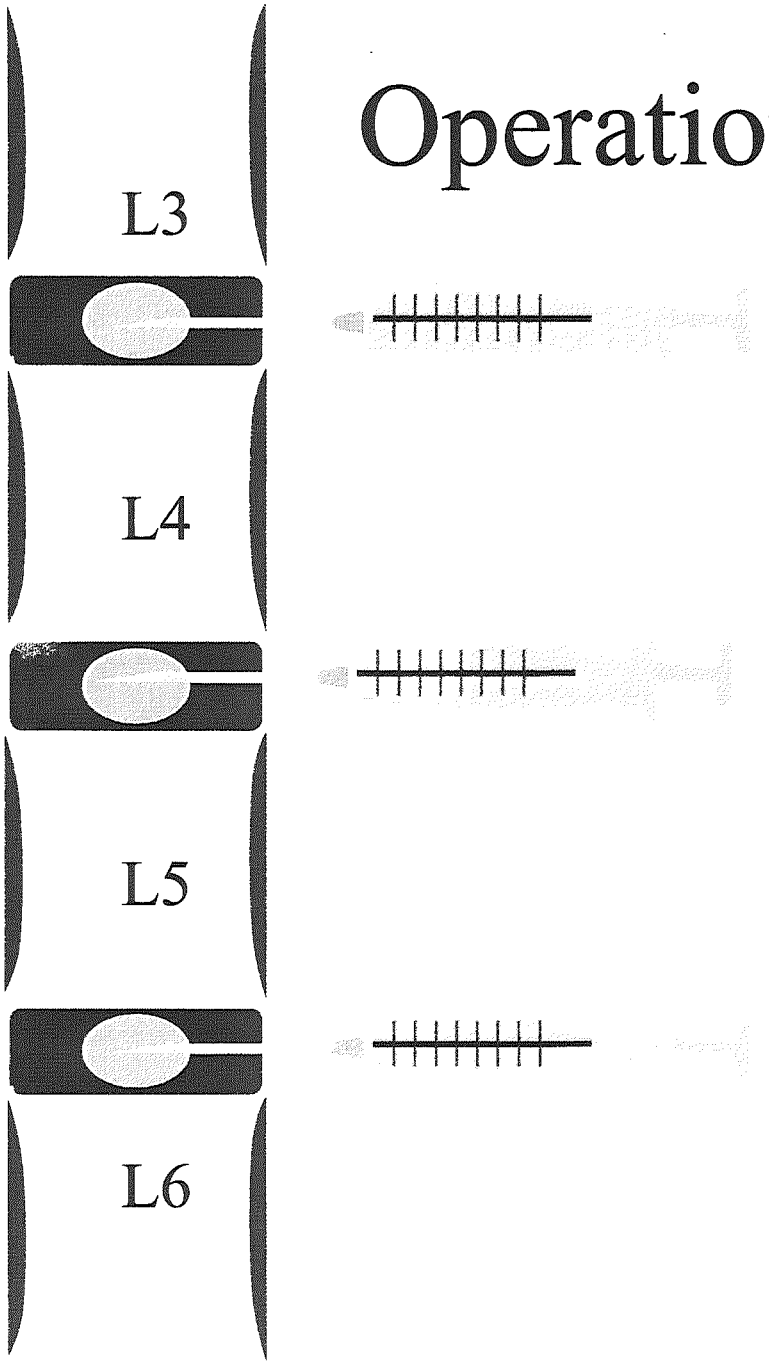
NP aspiration
(operation 1)

Cell transplantation therapy

(at 2 weeks after operation)

Tx group

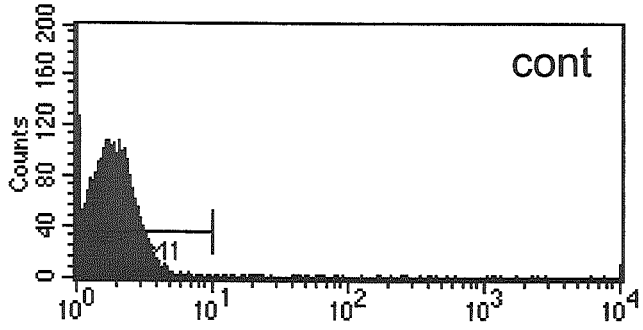
Operation 1:Disc degeneration induction

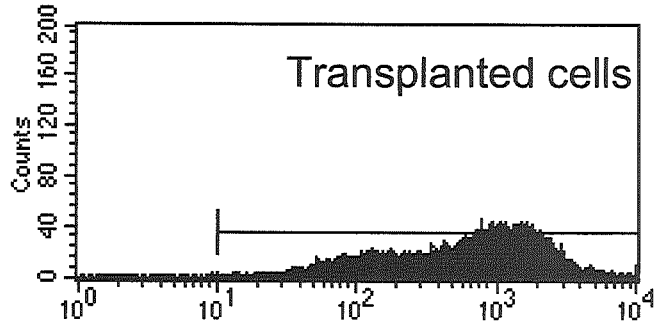


Techniques of aspiration

- antero-lateral approach
- 10 ml syringe
- 18 G needle
- only one time
- aspirated NP weight: 22.31mg (average)

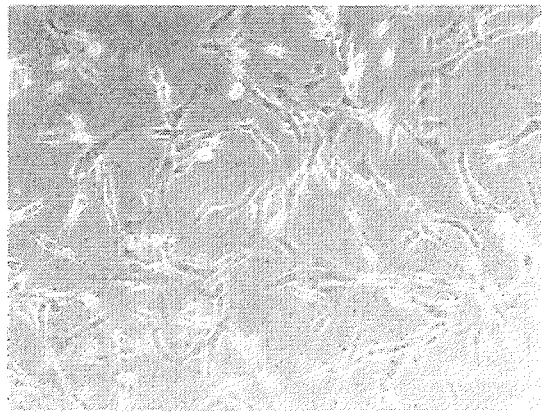
Labeling of transplanted cells with retrovirus vector expressing GFP

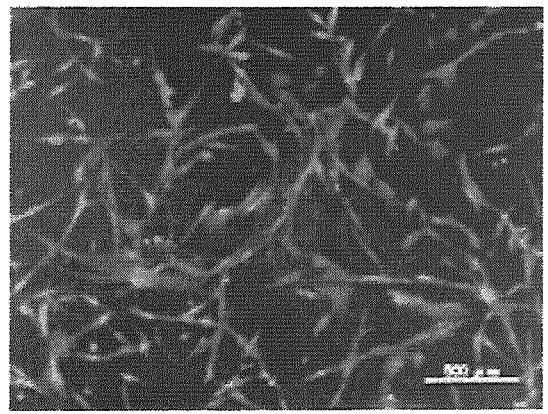




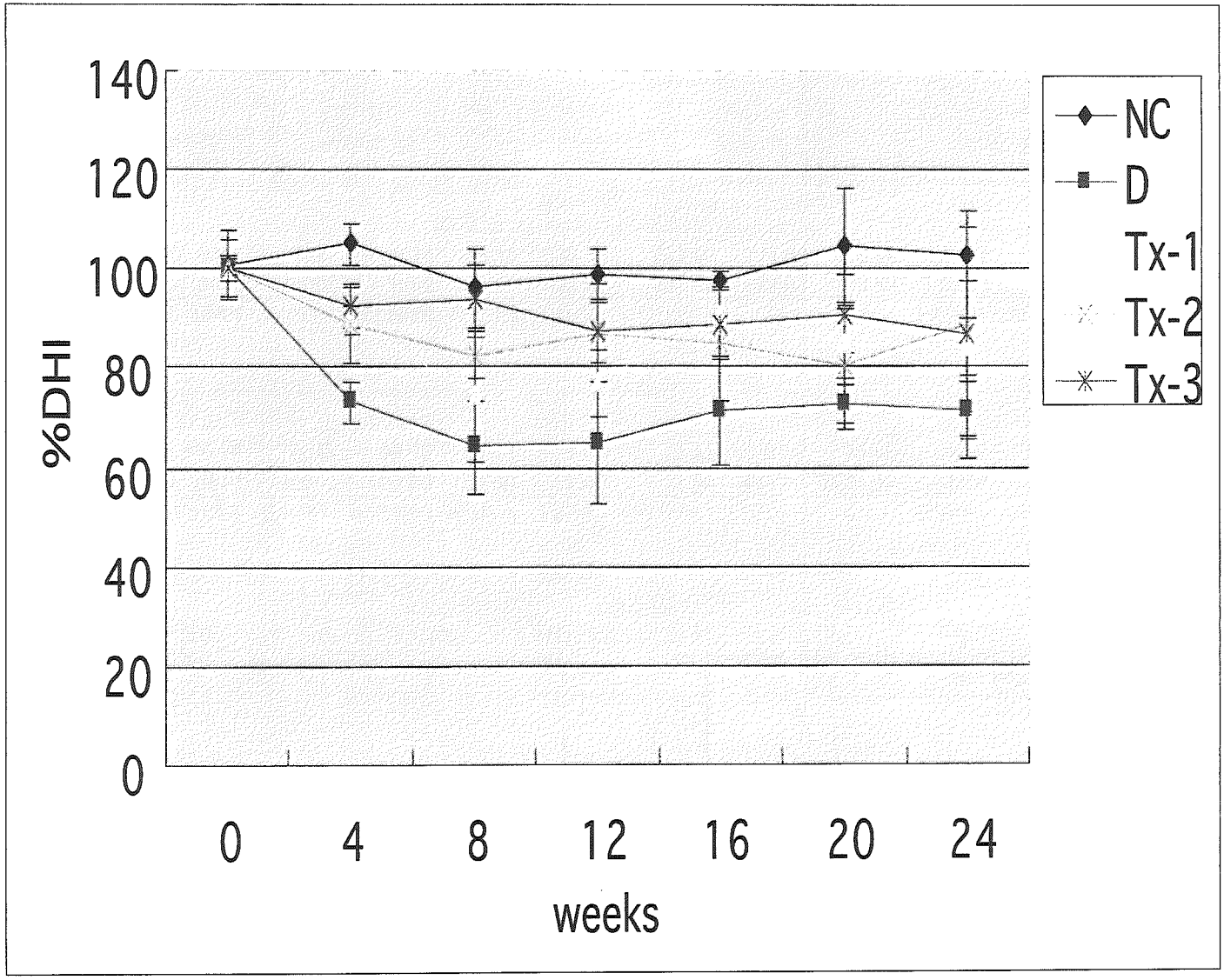
Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	SD	CV	Median	Peak	Peak Ch
All	1, 9910	10000	100.00	100.00	23.96	1.85	443.69	1851.96	1.76	780	1
M1	1, 10	9912	99.12	99.12	1.89	1.77	0.75	39.86	1.76	780	1

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	SD	CV	Median	Peak	Peak Ch
All	1, 9910	10000	100.00	100.00	1024.91	535.55	1163.03	113.48	716.92	45	729
M1	10, 9910	9919	99.19	99.19	1033.24	557.63	1164.09	112.66	723.39	45	729



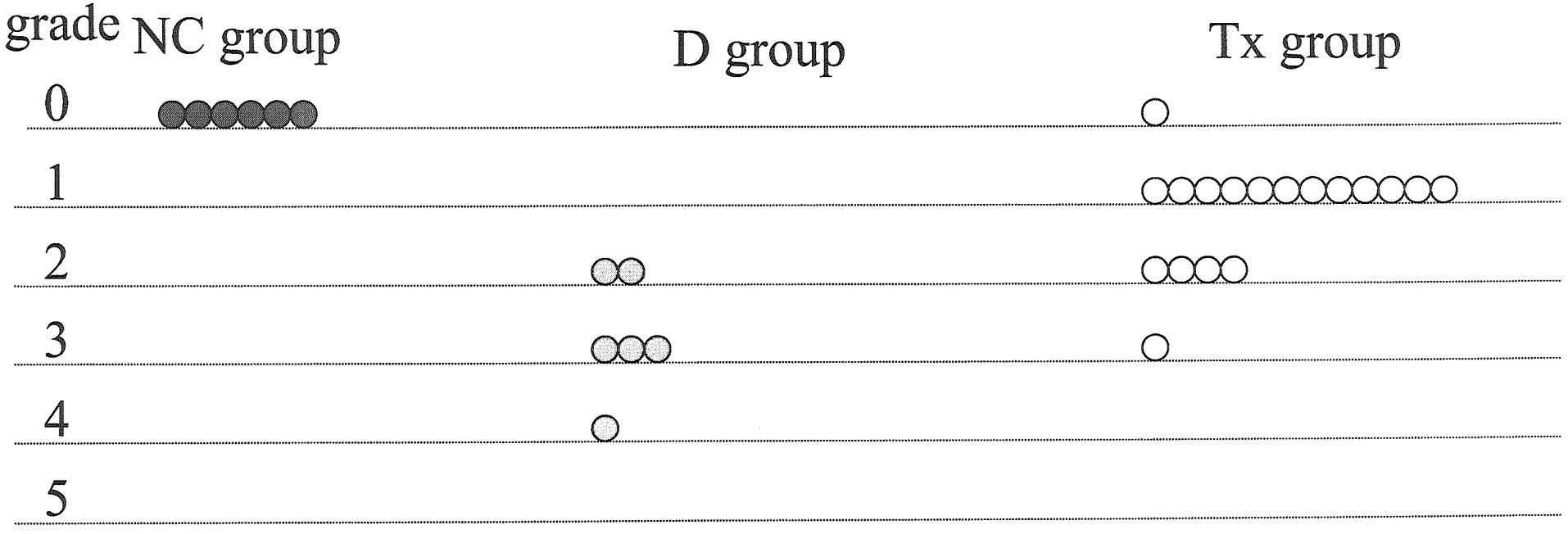


Radiographic assessment : %DHI



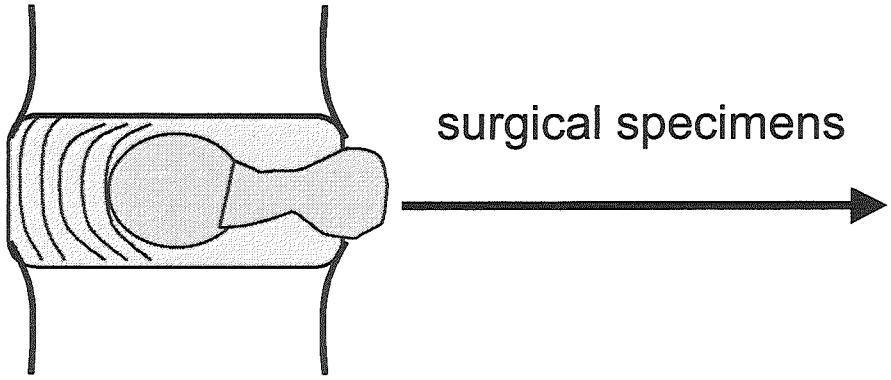
Degenerative changes in the anulus fibrosus

Grade 0: normal structure
Grade 1: mildly serpentine with rupture
Grade 2: moderately serpentine with rupture
Grade 3: severely serpentine with mildly reversed contour
Grade 4: severely reversed contour
Grade 5: indistinct

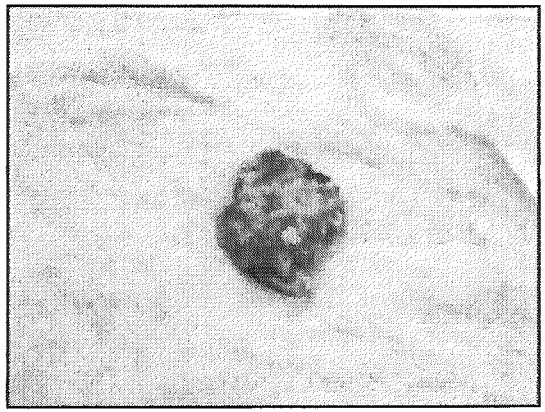


at 24weeks (22weeks after cell transplantation)

Materials and methods

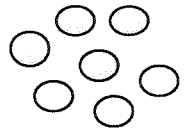


surgical specimens

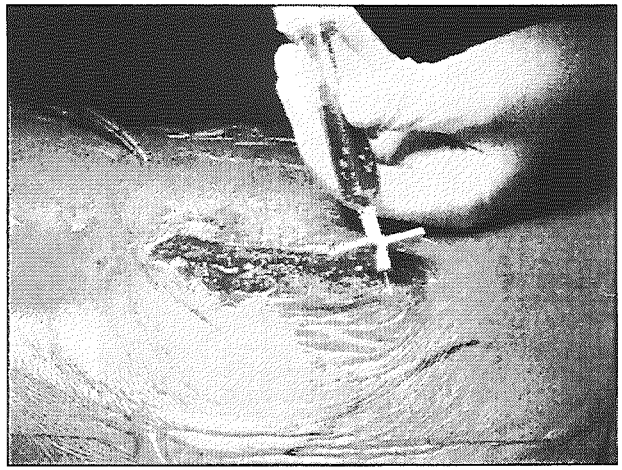


intact NP separation

digested by 0.27% pronase (1hr)
0.025% collagenase (2hrs)



NP cells



BM + 5% dextran/normal saline solution(2hrs)

↓

gradient centrifuge (450g 30min)

↓

the middle fraction were cultured

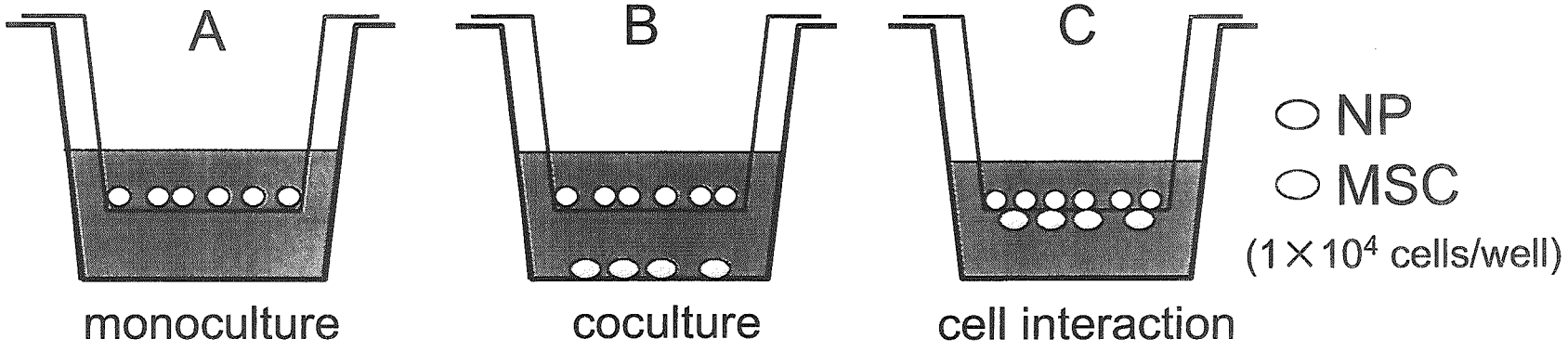
↓

CD45 negative plated cells were regarded as MSCs

↓

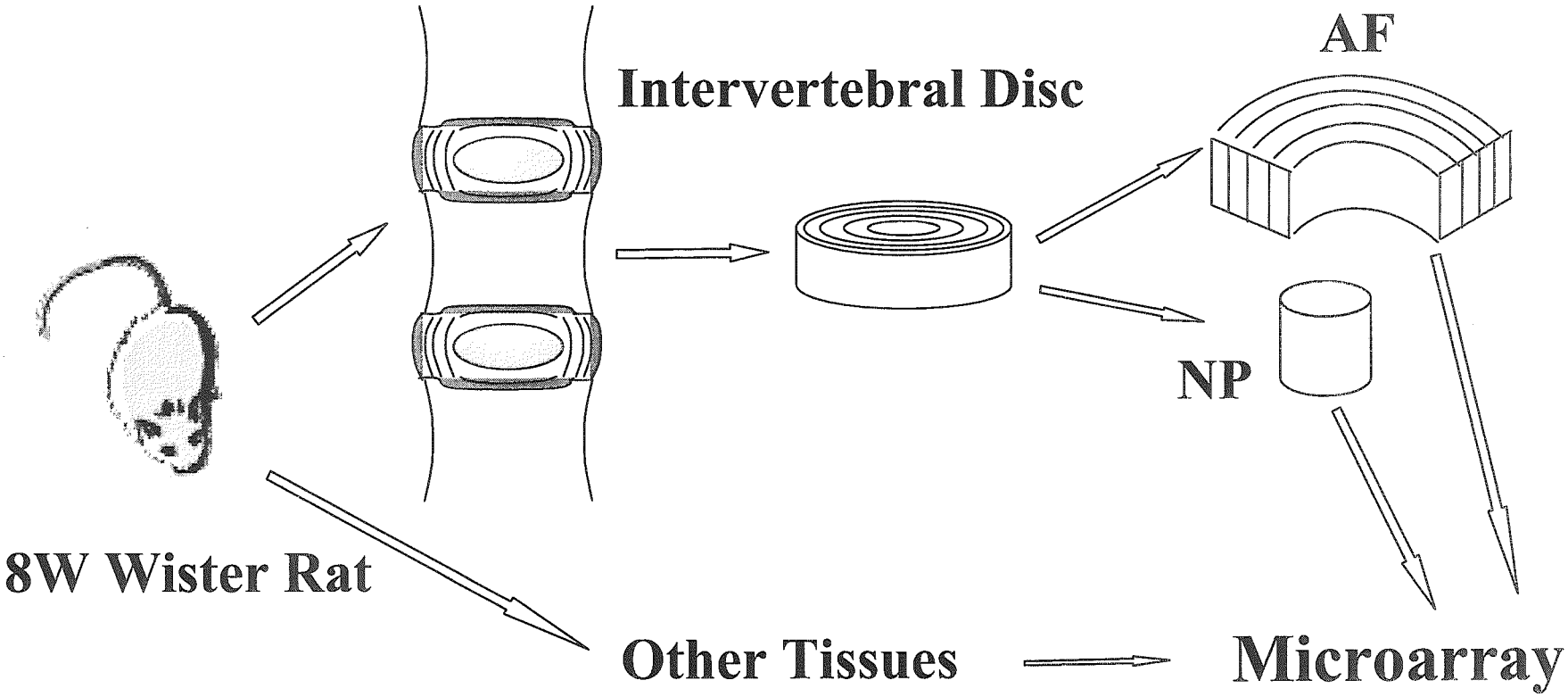
MSCs

Study design



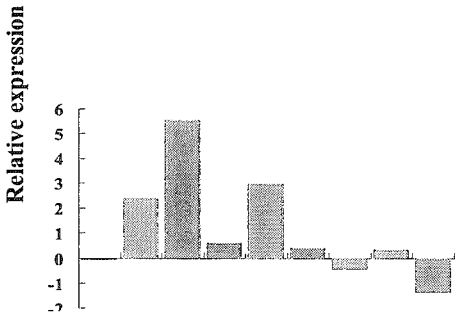
Medium : DMEM/F12　+10% autologous serum

Microarray Analysis

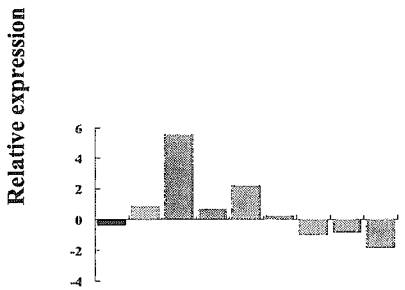


Identification of NP Specific Cell Surface Molecules

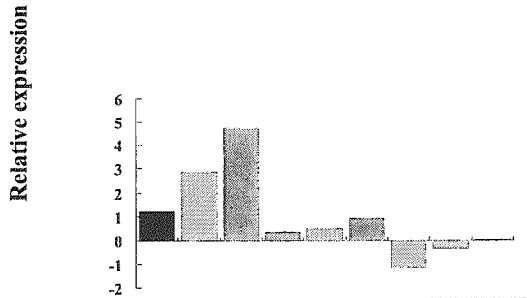
Na-K-Cl cotransporter (Nkcc1)



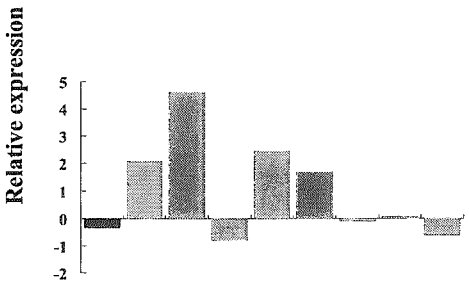
solute carrier family 12, member 2



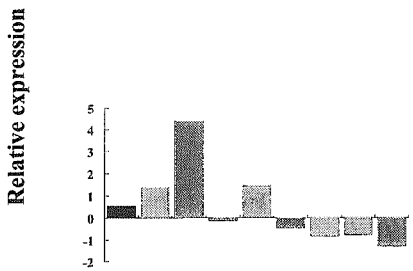
Neuropeptide Y5 receptor (Npy5r)



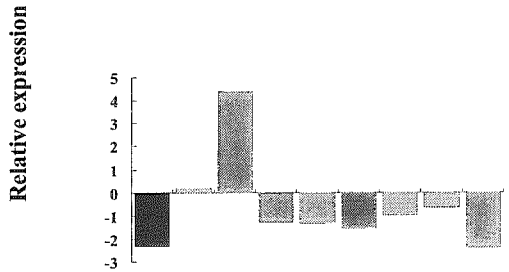
brain glucose-transporter protein

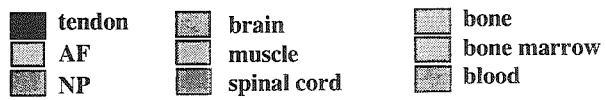


Glypican 3 (Gpc3)



CD24 antigen (CD24)





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Upregulation of the Viability of Nucleus Pulposus Cells by Bone Marrow-Derived Stromal Cells

Significance of Direct Cell-to-Cell Contact in Coculture System

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Study Design. Upregulation of the viability of nucleus pulposus cells by coculture with bone marrow-derived stromal cells using a novel culture system.

Objectives. The objective was to apply a novel coculture system having direct cell-to-cell contact between nucleus pulposus cells and bone marrow-derived stromal cells for stimulation of nucleus pulposus cells.

Summary of Background Data. Reinsertion of nucleus pulposus cells was effective for treatment of intervertebral disc degeneration. However, obtaining highly viable nucleus pulposus cells was necessary to achieve successful results. Thus, an alternative method to upregulate the biologic and metabolic viabilities of nucleus pulposus cells was desired.

Methods. Nucleus pulposus cells and bone marrow-derived stromal cells were isolated from New Zealand white rabbits. A 6-well culture plate and insert with track-etched membrane having 0.4 μm pores at the bottom were used for coculture. Nucleus pulposus cells were monocultured, cocultured conventionally (having no direct cell-to-cell contact) with bone marrow-derived stromal cells, or cocultured having direct cell-to-cell contact with bone marrow-derived stromal cells. On day 4 of coculture, nucleus pulposus cells were evaluated for proliferation using WST-8 assay, deoxyribonucleic acid synthesis by measuring [^{3}H]-thymidine uptake, and proteoglycan synthesis by measuring [^{35}S]-sulfate uptake. We also quantified cytokines in supernatants from the culture system.

Results. Cell proliferation, deoxyribonucleic acid synthesis, and proteoglycan synthesis of nucleus pulposus cells were significantly upregulated in samples cocultured having direct cell-to-cell contact. Moreover, evaluations of supernatants revealed that growth factors associated with proliferation and cellular metabolism of nucleus pulposus cells were increased.

Conclusions. Direct cell-to-cell contact in coculture system between nucleus pulposus cells and bone marrow-derived stromal cells accomplished significant upregulation in viability of nucleus pulposus cells.

Key words: intervertebral disc degeneration, nucleus pulposus cells, bone marrow-derived stromal cells, coculture. **Spine 2004;29:1508–1514**

In many cases, spinal disorders involve degeneration of intervertebral discs, often relatively early in life. The result can be back pain, sciatica, and other distressing and disabling spinal symptoms.[1] Further, disc degeneration often accelerates following lumbar surgery, exacerbating symptoms. Recently, experimental studies aiming to improve treatment of disc degeneration have captured great interest.[2–4]

One current major approach in treatment of degenerated disc is disc cell implantation. Recent study by Gruber *et al* focused on effectiveness of autologous disc cell implantation as treatment for disc degeneration in a sand rat model.[5] In our previous experimental studies, Nishimura and Mochida reported that reinsertion of autologous nucleus pulposus (NP) decelerated disc degeneration.[6] However, preparation of NP cells for reinsertion has been less than satisfactory because transplantation requires more cells than can be harvested from a single intervertebral disc. One way to obtain more NP cells was achieved by the use of coculture system. Okuma *et al* found that biologic viability of NP cells was upregulated by using coculture system with anulus fibrosus (AF) cells.[7]

From the results above, clinical trials of autologous NP cell reinsertion had been started. However, in order to achieve effective results, further upregulation of biologic and metabolic viabilities of NP cells were desired. Primary reason for necessity of further upregulation in viability of NP cells was the low cellular yields and low proliferative activity of NP cells in earlier phases of primary culture.

As methods for stimulating NP cells, there have been other effective methods represented by gene transfer or growth factor inductions.[2,8–10] Despite their effectiveness, there still is a barrier to overcome in safety and ethical issues to apply them in clinical basis. Thus, development of a technique using autologous materials in achieving highly viable NP cells is of great significance. Here, we demonstrate whether biologic and metabolic properties of NP cells are significantly upregulated by

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