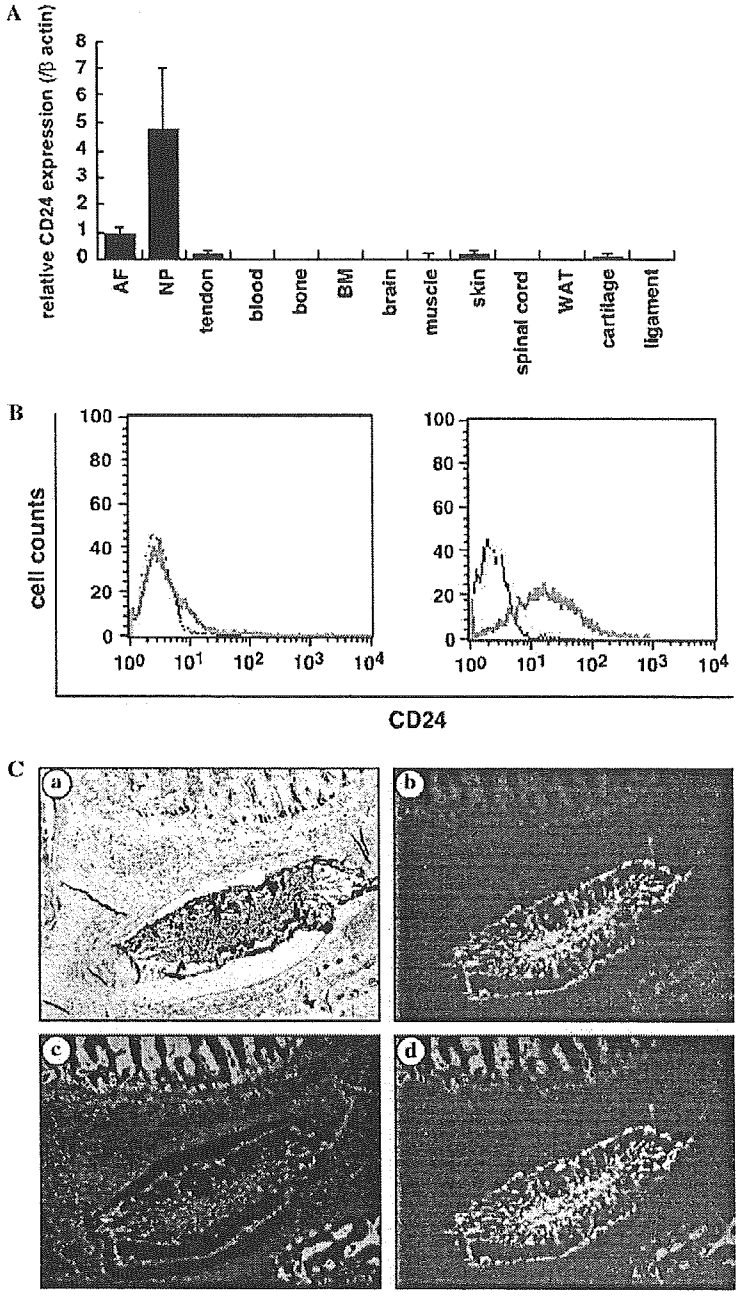

Fig. 2. Confirmation of CD24 expression in NP. (A) CD24 expression was analyzed in 13 different tissues, 10 of which were analyzed in microarray assay, by semi-quantitative real-time PCR. CD24 expression was elevated specifically in NP cells. (B) CD24 expression was analyzed in NP and AF cells using 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-

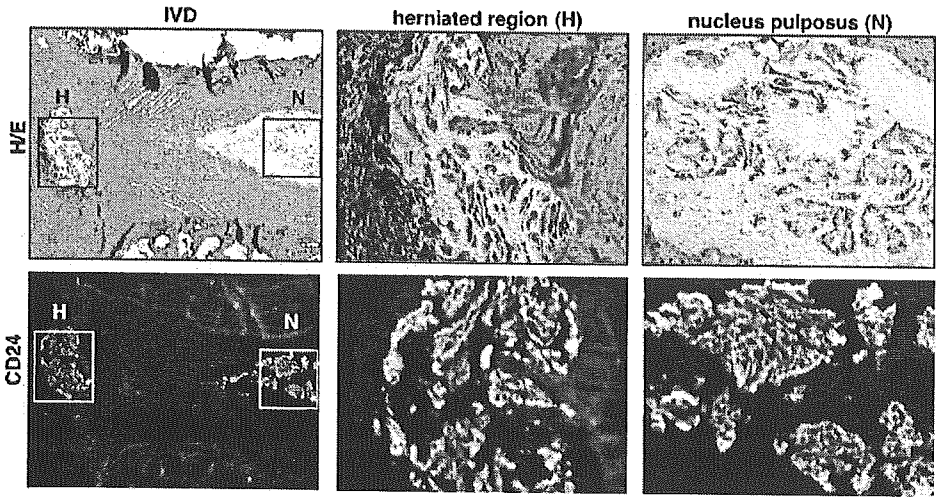

Fig. 3. CD24 expression is detected in herniated protrusion. IVD hernia model was created in 8-week-old rat tail IVDs. Seven days later, IVDs were dissected and stained by H/E (upper panel) or anti-CD24 antibody followed by FITC-conjugated anti-mouse IgG (lower panel), and observed under a fluorescence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

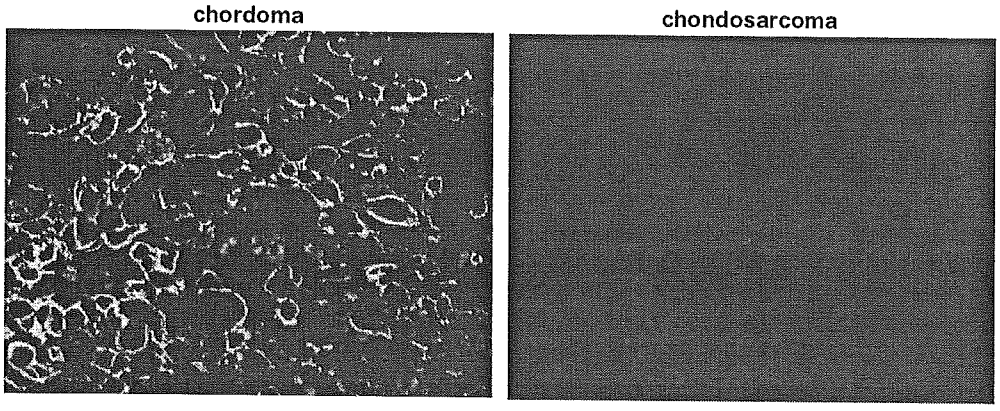

Fig. 4. CD24 expression is detected in chordoma but not in chondrosarcoma. Specimens of chordomas (left panel) and chondrosarcomas (right panel) were stained by anti-human CD24 followed by Alexa Fluor488-conjugated anti-mouse IgG. Immunoreactivity was detected by fluorescence microscopy.

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.

Acknowledgments

We thank the affected individuals for participating in this study: M. Mukai for preparation of specimens of chordoma and chondrosarcoma; Y. Sato and A. Kumakubo for technical support; E.S. Withers-Ward for manuscript preparation.

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厚生労働科学研究費補助金（健康科学総合研究事業）

分担研究報告書

再生医療を用いた高齢者腰痛症に対する新しい治療法の開発

分担研究者：持田　譲治　東海大学医学部整形外科 教授

A. 研究目的

高齢者の腰痛の原因は多彩であるが、その半数以上が椎間板変性の進行によって引き起こされる。通常の加齢変化の速度以上にその変性が進行した場合には腰痛、下肢痛などの愁訴をともなう疾患となる可能性が高い。椎間板変性は非可逆的な過程とされるが、その変性過程を時間的に遅延させる、あるいは再生方向へシフトさせる可能性について検討することは重要である。

各種椎間板変性疾患の手術時における椎間板母髄核部温存型の手技より、画像上、臨床上に良好な結果が得られてきている。この事実を基礎動物実験に応用した結果、髄核細胞が線維輪細胞を活性化させること、また活性化させた髄核細胞の変性椎間板内への再挿入によって、椎間板変性が明らかに遅延することが判明した。しかし今後の臨床応用を考える際には再挿入する髄核細胞を質、量ともに高める必要がある。本研究では骨髄間葉系幹細胞に注目し、髄核細胞の活性化への影響、さらに髄核組織そのものの導出の可能性を検討する。

B. 研究方法

実験１　骨髄間葉系幹細胞による髄核細胞の活性化ならびに椎間板変性抑制について：日本白色家兎を用いて骨髄間葉系幹細胞による髄核細胞の活性化を 3 つの培養系で比較検討した。髄核細胞($1x10^4$個)の monoculture（N 群）、同数の骨髄間葉系幹細胞との通常の coculture(M 群),細胞間接着を伴う両細胞の coculture（CI 群）の 3 群にわけ培養後の状態を検討する。M 群、CI 群で得られた活性化髄核細胞を同一固体に作成した変性椎間板内に再挿入し、その後の変性過程の変化を観察する。

実験２　骨髄間葉系幹細胞からの椎間板組織の導出について：日本白色家兎の骨髄間葉系幹細胞と髄核細胞（共に $1x10^4$ 個）を monoculture し、髄核細胞と細胞間接着を伴う coculture した骨髄間葉系幹細胞の細胞特性を各種染色性で比較検討する。また骨髄間葉系幹細胞を変性椎間板内に再挿入した際の細胞の様態を検討する。

C. 研究結果

結果１：in vitro の培養の結果 CI 群では N 群に比べ細胞数は約 10 倍に、単位細胞あたりの DNA 活性は約 20 倍に、プロテオグリカンの活性は約 15 倍に増大した。変性椎間板への活性化髄核の再挿入では CI 群、M 群の順に椎間板変性過程の抑制がみられ、細胞間接着を用いた coculture によって活性化された髄核細胞が変性椎間板のその後の変性進行を効果的に抑制することが明らかとなった。

結果２：髄核細胞と細胞間接着を伴う coculture を行った骨髄間葉系幹細胞の染色性は I 型 collagen に＋－、II 型 collagen に２＋，ケラタン硫酸プロテオグリカンに２＋、アグレカンに＋となり、骨髄間葉系幹細胞が元々有する染色性上の特性から髄核細胞の特性に明らかに変化することが示された。また、変性椎間板内に挿入された骨髄間葉系幹細胞は 8 週時には髄核腔中央部に存在し、48 週では線維輪内層に向け明らかに増殖していた。さらにその細胞染色性も in vitro で示したと同様に髄核細胞様の変化を示していた。

D. 考察

骨髄間葉系幹細胞との細胞間接着を伴う coculture で活性化された髄核細胞は、再挿入された変性椎間板内でその後の椎間板変性進行を明らかに抑制していた。自らの髄核細胞を体外で活性化して変性椎間板内に戻すこの手法は、人への

臨床応用の可能性が極めて高い方法と考えられ、現在その実施に向けてのプロジェクトが、本学倫理委員会の承認を得て進行中である。

また、骨髄間葉系幹細胞の変性椎間板内での椎間板様組織への可塑化の事実は、活性化髄核の再挿入法と同様に人椎間板変性抑制の一法として応用される可能性があると考えられた。

E. 結論

椎間板変性進行は高齢者の腰痛の原因のひとつであり、かつ大きな割合を占める。この変性抑制のために椎間板細胞再生的なアプローチが極めて有効であると考えられる。

健康危険情報

問題なし。

F. 研究発表

1. Yamamoto Y, Mochida J, Sakai D, et al. 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 2004; 14、1508-1514

2. Sakai D, Mochida J, Yamamoto Y, et al. Transplantation of mesenchymal stem cells embedded in Atelocollagen gel to the intervertebral disc: a potential therapeutic model for disc degeneration. Biomaterials 2003; 24: 3531-41

G. 知的財産権の出願・登録状況

Patent Cooperation Treaty(米国、カナダ、日本)出願中 (2003年2月25日出願)

平成17年度 厚生労働科学研究費補助金（痴呆・骨折臨床研究事業）

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

1. 論文発表：

1) Sakai D, Mochida J, Yamamoto Y, Toh E, Iwashina T, Miyazaki T, Inokuchi S, Ando K, Hotta T. Immortalization of Human Nucleus Pulposus Cells by a Recombinant SV40 Adenovirus Vector: Establishment of a Novel Cell Line for the Study of Human Nucleus Pulposus Cells. Spine. 2004 Jul 15;29(14):1515-1523.

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6) Sakai D, Mochida J, Iwashina T, Watanabe T, Suyama K, Ando K, Hotta T. Atelocollagen for culture of human nucleus pulposus cells forming nucleus pulposus-like tissue in vitro: influence on the proliferation and proteoglycan production of HNPSV-1 cells. Biomaterials. 2006 Jan;27(3):346-53.

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

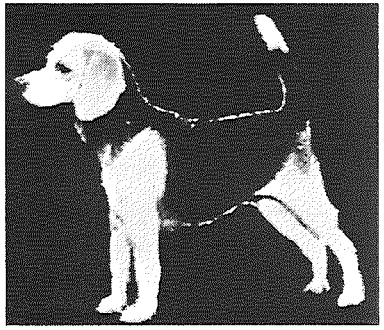
H. 知的財産権の出願・登録状況

予定していない。

Materials and method

Beagle(10~12 months, ♀)

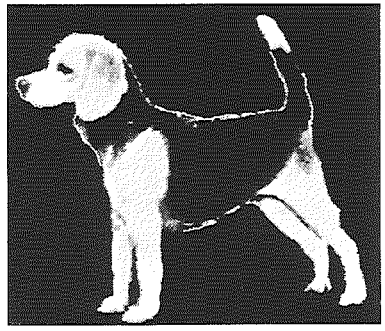
n=2



control

NC group

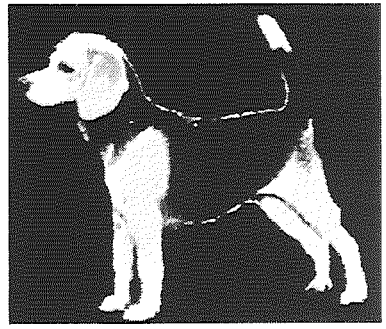
n=2



NP aspiration
(operation 1)

D group

n=6+1



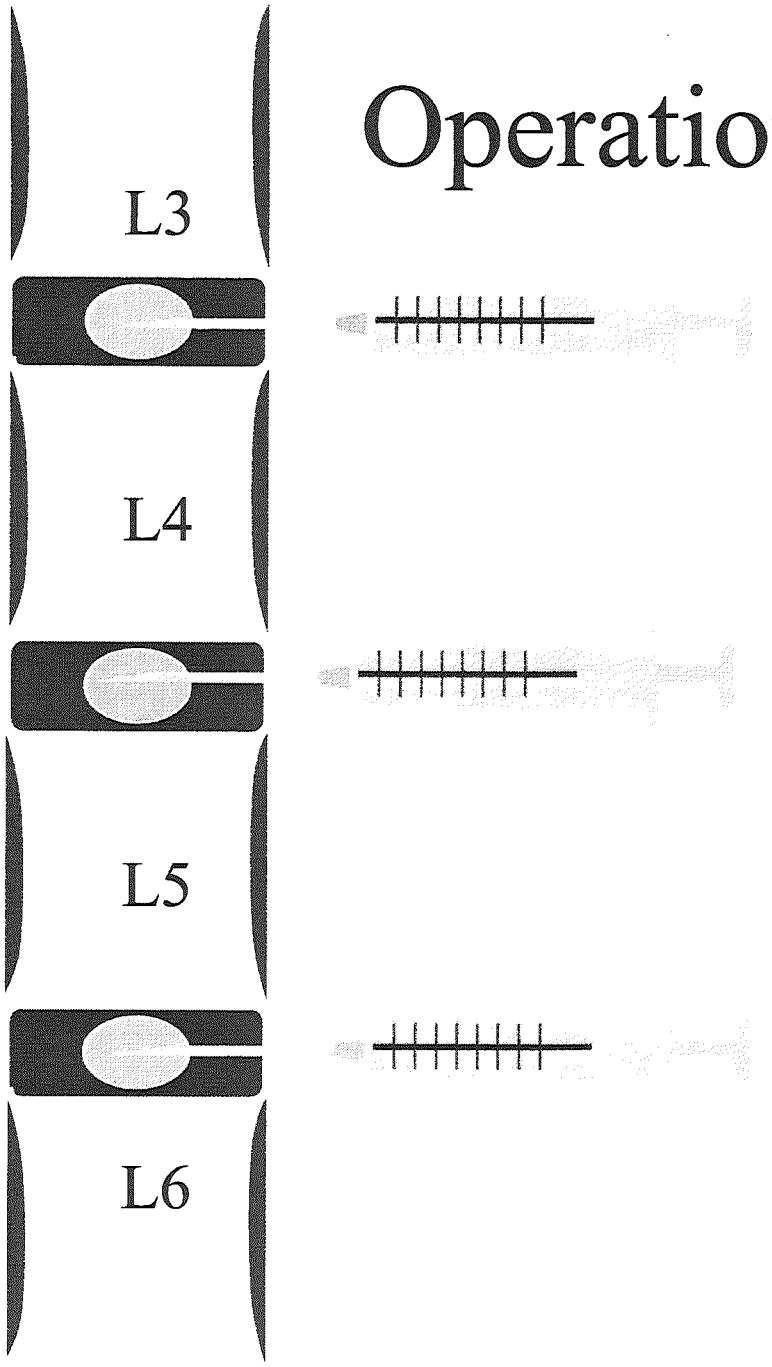
NP aspiration
(operation 1)

Cell transplantation therapy

(at 2 weeks after operation)

Tx group

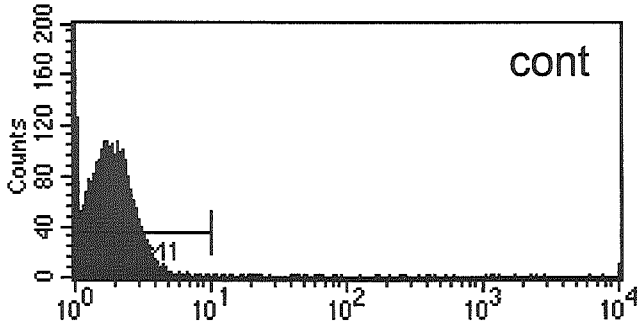
Operation 1: Disc degeneration induction

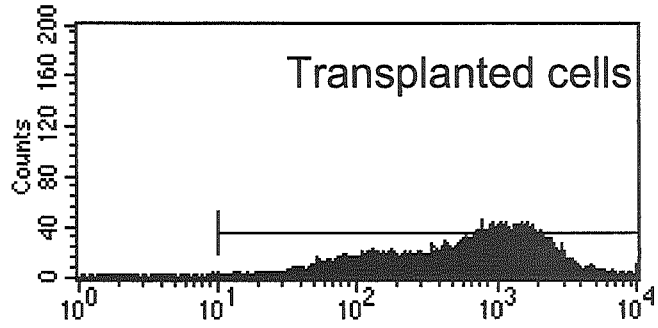


Techniques of aspiration

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

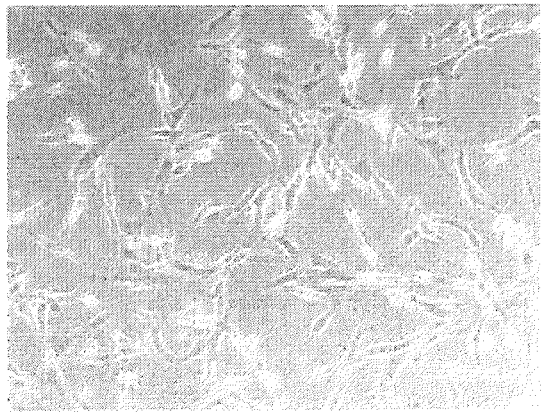
Labeling of transplanted cells with retrovirus vector expressing GFP

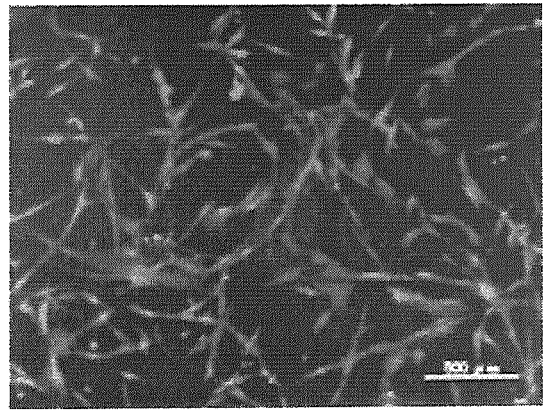




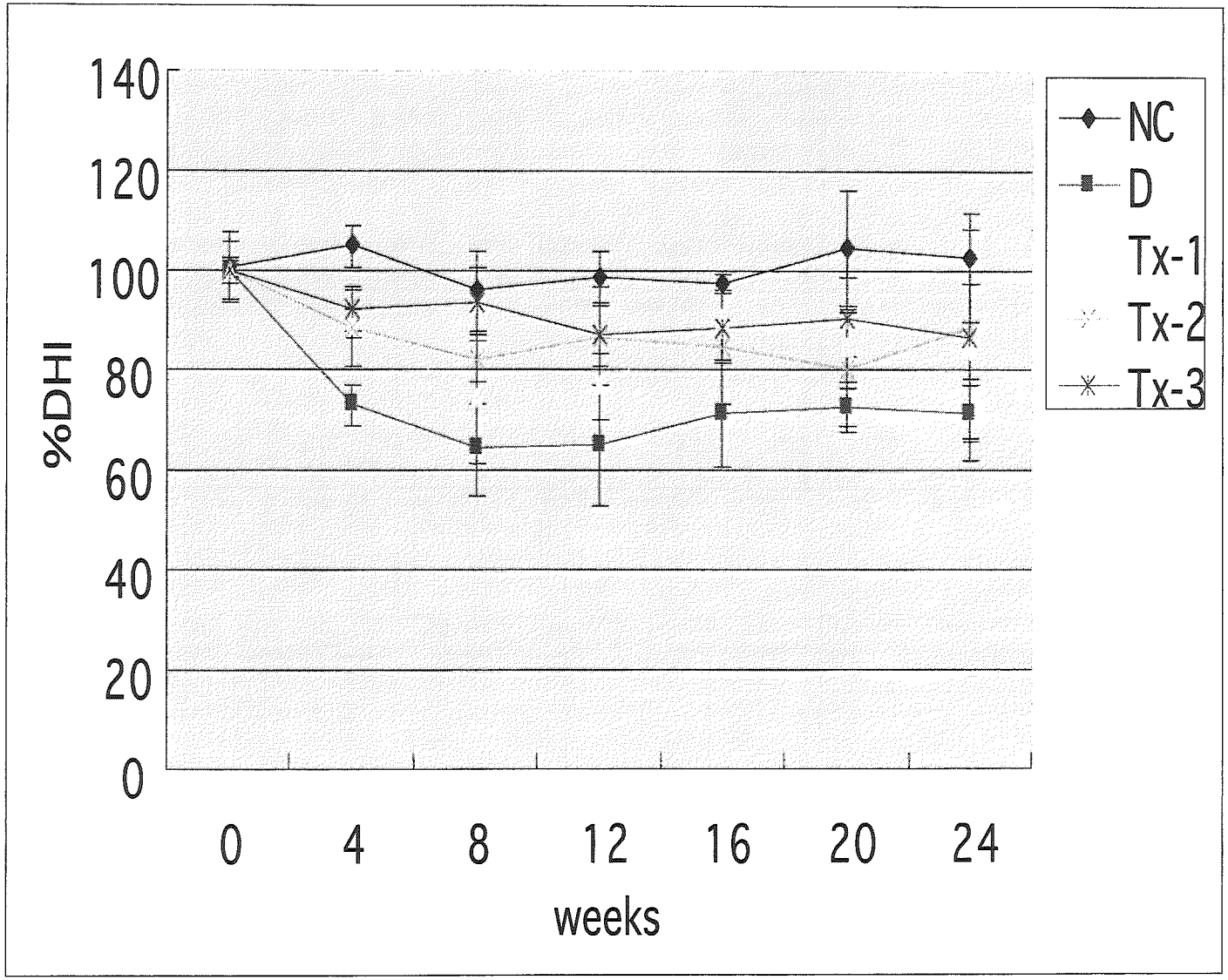
Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	SD	CV	Median	Peak	Peak Ch
All	1, 9910	10000	100.00	100.00	23.96	1.85	443.69	1851.96	1.76	780	1
M1	1, 10	9912	99.12	99.12	1.89	1.77	0.75	39.86	1.76	780	1

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



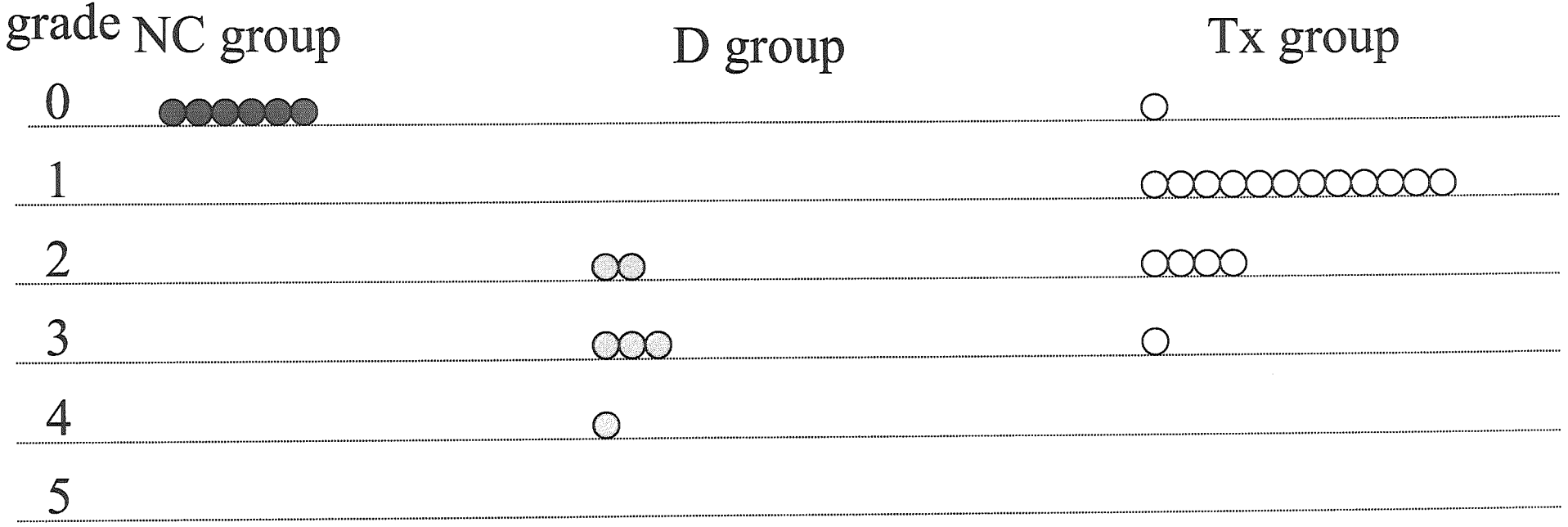


Radiographic assessment : %DHI



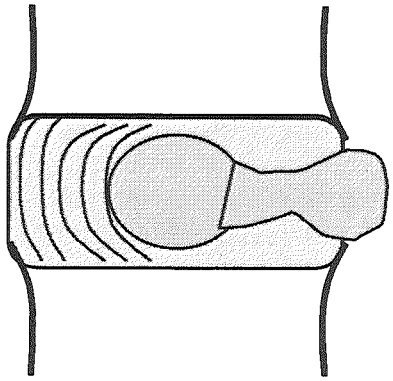
Degenerative changes in the anulus fibrosus

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

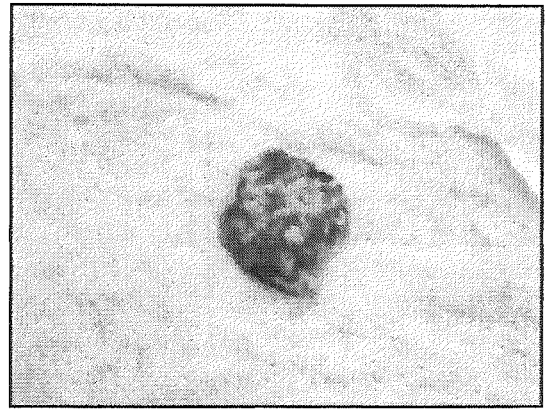


at 24weeks (22weeks after cell transplantation)

Materials and methods



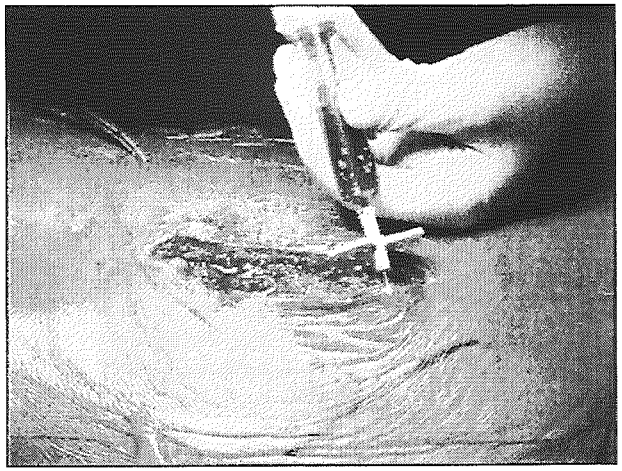
surgical specimens



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

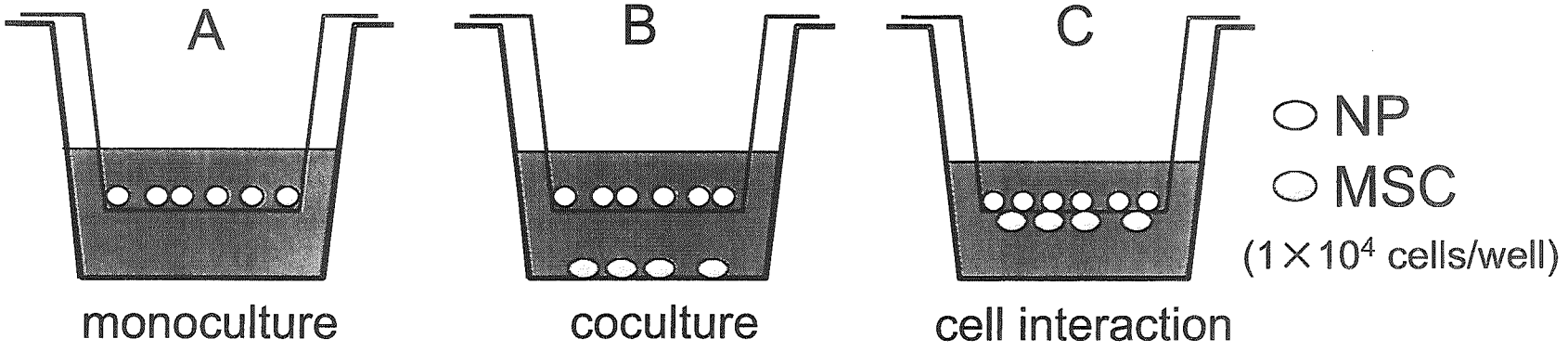
↓

CD45 negative plated cells were regarded as MSCs

↓

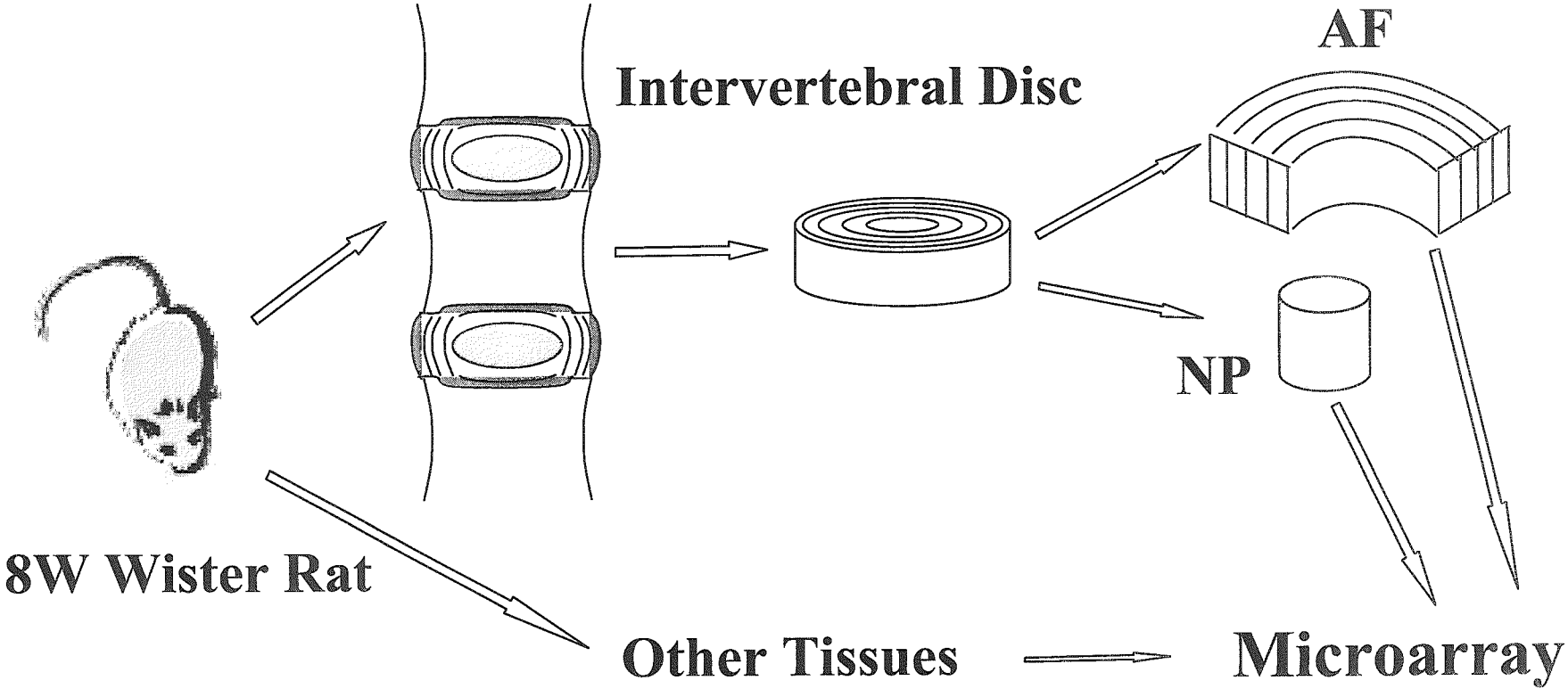
MSCs

Study design



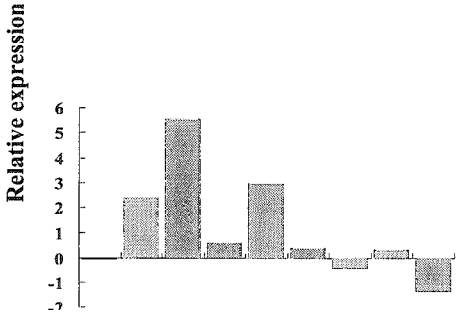
Medium : DMEM/F12　+10% autologous serum

Microarray Analysis

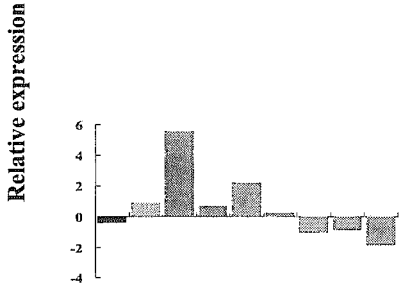


Identification of NP Specific Cell Surface Molecules

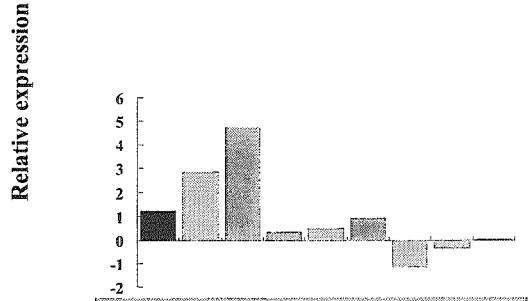
Na-K-Cl cotransporter (Nkcc1)



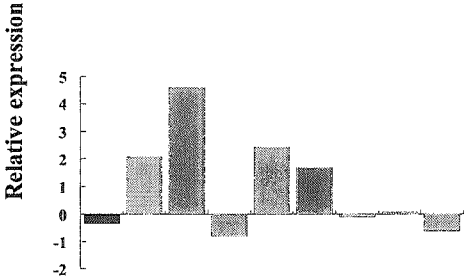
solute carrier family 12, member 2



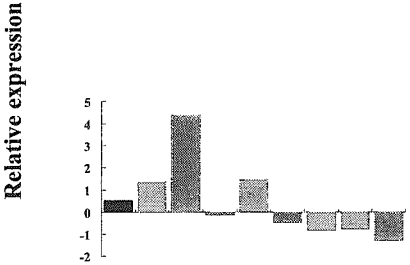
Neuropeptide Y5 receptor (Npy5r)



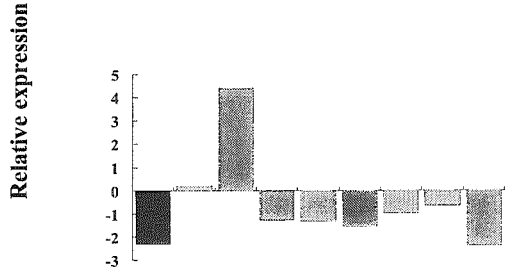
brain glucose-transporter protein

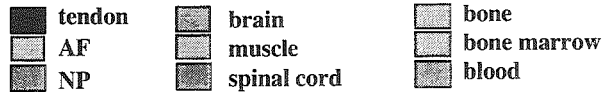


Glypican 3 (Gpc3)



CD24 antigen (CD24)





SPINE Volume 29, Number 14, pp 1508–1514

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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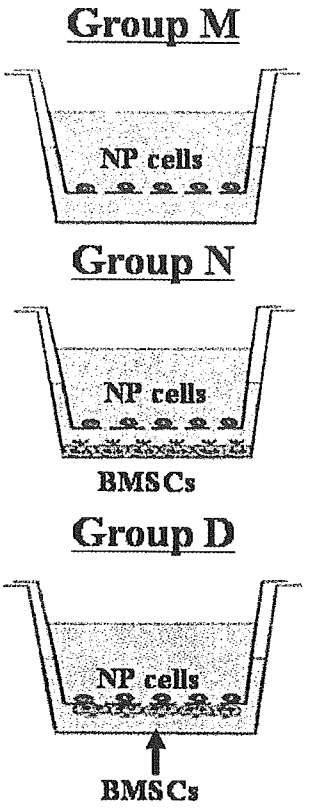

Figure 1. Diagrams of several culture systems used in the study. Group M: NP cells cultured monolayer (top). Group N: NP cells cultured in conventional coculture system with BMSCs having no cell-to-cell contact (middle). Group D: NP cells cultured in coculture system with BMSCs having direct cell-to-cell contact (bottom).

using a novel coculture system having direct cell-to-cell contact with autologous bone marrow-derived stromal cells (BMSCs) *in vitro* or not.

Materials and Methods

The current study was conducted under the protocol approved by the Animal Experimentation Committee of Tokai University School of Medicine.

Cell Isolation and Culture. To isolate BMSCs, bone marrow aspirates were taken from the iliac crest of 20 New Zealand white rabbits weighing an average of 1.5 kg while under inhalation anesthesia with 2.5% isoflurane (Abbot Laboratories, North Chicago, IL). Mononucleated cells were isolated with a density gradient (Nycoprep™; Axis-Shield, Oslo, Norway) centrifugation. These cells were then cultured with 25 cm^2 culture flask in Dullbecco Modified Eagle Medium (DMEM, Gibco, Grand Island, NY) with 20% fetal bovine serum and penicillin (100 μg/mL), streptomycin (250 ng/mL), and amphotericin B (85 μg/mL) at 37 C in a humid atmosphere containing 5% CO_2. After 24 hours, nonadherent cells were discarded, and adherent cells were thoroughly incubated for 7 days. Next, the same rabbits were killed while under general anesthesia by intravenous injection of pentobarbital sodium (120 mg/kg) (Abbot Laboratories). Thoracolumbar spines including T5 to L7 levels were removed under aseptic conditions. Each disc was cut transversely, and NP was separated from the disc with a spatula. Obtained NP tissue was digested in a mixture of 0.4% pronase (Kakenkagaku, Tokyo, Japan) and 0.0125% collagenase P (Boehringer Mannheim, Mannheim, Germany) for 30 minutes. The digested cells were washed 3 times with DMEM, being collected by centrifugation at 1500 rpm. Six-well culture plate (Becton Dickinson, Franklin Lakes, NJ) and culture insert (Becton Dickinson) were used for coculture. The culture insert we used in the study consists of a polyethylene terephthalate track-etched membrane with 0.4 μm pores at the bottom, which prevent an exchange of cellular component. Diagrams of the culture systems used in this study are displayed in Figure 1. Nucleus pulposus cell cultured monolayer (Group M, Figure 1, top), conventional coculture system with no cell-to-cell contact with BMSCs (Group N, Figure 1, middle), and a new coculture system having direct cell-to-cell contact with BMSCs (Group D, Figure 1, bottom) were designed. The 1×10^4 NP cells and BMSCs were seeded in each group, and 7 sets of each group were made. In Group D, BMSCs were cultured on the reverse side of the membrane of the insert in DMEM with 10% fetal bovine serum. After 2 hours, culture insert was set in culture plate, and then NP cells were seeded on the front side of the membrane of the insert and cocultured in DMEM with 10% fetal bovine serum and penicillin (100 μg/mL), streptomycin (250 ng/mL), and amphotericin B (85 μg/mL) at 37 C in a humid atmosphere containing 5% CO_2. On day 4 of coculture, all groups were evaluated, because NP cells in Group D became confluent. The medium was exchanged once on the second day.

Evaluations. Evidence of direct cell-to-cell contact was evaluated in Group D. Cell proliferation, deoxyribonucleic acid (DNA) synthesis, proteoglycan (PG) synthesis, and cytokine expression analysis were evaluated for every groups.

Evidence of Direct Cell-to-Cell Contact. Specimens from Group D were processed for scanning electron microscopic (SEM) evaluation. Specimens were soaked in 0.1 mol/L phosphate buffer and fixed in 2.5% glutaraldehyde for 2 hours. Next, samples were fixed in 1% osmium solution for 1 hour and dehydrated in ascending concentrations of ethanol (50%, 70%, 80%, 90%, 95%, and 100%). Specimens were spatter-coated with gold and affixed to an adhesive interface for observation by SEM (JSM-840, Tokyo, Japan).

Measurement of Cell Proliferation. Cell proliferation was determined by WST-8 assay using Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technology, Gaithersburg, MD).[11] A 96-well plate containing NP cells from each of the 3 groups was inoculated with 10 μL of prepackaged CCK-8 solution. They were incubated at 37 C in a humid atmosphere containing 5% CO_2 for 2 hours, and absorbance at 450 nm of the supernatant was measured spectrophotometrically. Cell counts were determined with a calibration curve.

Measurement of DNA Synthesis. The DNA synthesis was examined by uptake of [^{3}H]-thymidine.[12] Cultures were labeled with 74 kBq of [^{3}H]-thymidine per well for 2 hours. Cells were washed twice with phosphate buffered saline (PBS), and 2 mL of 10% trichloroacetic acid (TCA) was added to each well. Cultures were centrifuged (3000 rpm for 10 minutes), and supernatant (TCA) was removed. This procedure was repeated 5 times, and TCA-insoluble material was collected and dried

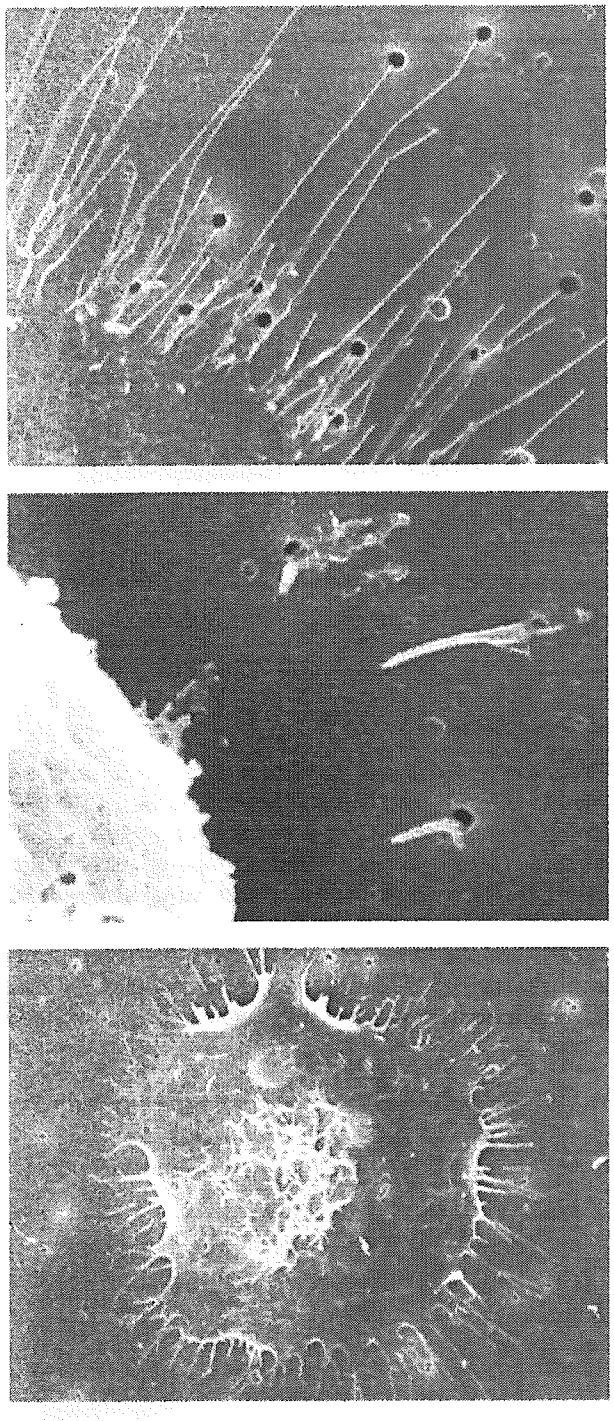

Figure 2. Electron microscopic view of the novel coculture system in Group D. Bone marrow-derived stromal cells on the reverse side of the membrane were adhering NP cells of the front side by extending cytoplasmic processus *via* pores of membrane (top). On the front side of the membrane, the cytoplasmic processus of BMSCs were extending toward NP cells through pores of membrane, and NP cells showed the longer processes and the faster wide spreading on the membrane (middle, bottom).

with 70% ethanol. The dried material was treated overnight with 1 mL of solvent (Solvable™; Packard, Meriden, CT) at 45 C and 10 mL of liquid scintillation cocktail (Atomlight™; Packard) was added for counting of emissions (Beckman LS4800, Fullerton, CA). Radioactivity, or disintegrations per minute (DPM), of these results was divided by number of cells counted by using the cell counting kit. The radioactivity of each sample was expressed as DPM per cell.

Measurement of Proteoglycan Synthesis. The incorporation of [^{35}S]-sulfate was used to measure PG synthesis. At the indicated times, cultures were labeled with 370 kBq of [^{35}S]-sulfate per well for 6 hours. Subsequent PBS washes, TCA treatment, drying, and scintillation counting were carried out with the same procedure as for [^{3}H]-thymidine uptake.

Protein Array Assay of Cytokine Expression. Ray Bio™ Cytokine Array (Ray Biotech, Norcross, GA) was used to evaluate cytokine expression of supernatants in every groups. To prepare media from 3 groups, cultures were performed in serum-free DMEM/F12 (DMEM: nutrient mixture Ham F-12; Gibco, Grand Island, NY). Cells were cultured for 5 days and supernatants were collected. Membranes immobilized with capture antibodies were blocked with 5% bovine serum albumin (BSA)/tris buffered saline (TBS) [0.01 M Tris HCl (pH 7.6), 0.15 M NaCl] for 1 hour. Membranes were then incubated with 1 mL of each media for 2 hours at room temperature. After extensive washing with TBS 0.1%/Tween 20 (3 times, 5 minutes each) and TBS (2 times, 5 minutes each) to remove unbound cytokines, membranes were incubated individually or collectively with biotin-conjugated streptavidin (2.5 pg/mL) for 1 hour at room temperature. Unbound materials were washed using TBS 0.01%/Tween 20 and TBS. Signals then were detected by an enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech, Aylesbury, UK). The obtained expressions on tagged image file format (TIFF) images were quantified using CS analyzer 2.0 (Atto, Tokyo, Japan). Determining value of positive control as 1000, each value for transforming growth factor-beta (TGF-β), insulin-like growth factor-1 (IGF-1), epidermal growth factor (EGF), and platelet-derived growth factor (PDGF) were quantified and evaluated.

Statistical Analysis. Statistical significance was determined based on $P < 0.01$. Comparison of group means between different cell cultures was determined using the Wilcoxon signed ranks test.

Results

Scanning Electron Microscopic Findings

In Group D, on day 4 of coculture, the BMSCs on the reverse side of the membrane were seen to adhere NP cells on the front by extending cytoplasmic processus through the 0.4 μm pores of membrane. Nucleus pulposus cells cultured on the front side of the membrane in Group D demonstrated longer processes and faster, wide spreading compared to NP cells cultured in other 2 groups (Figure 2).

Cell Proliferation

Mean NP cell proliferation measured by WST-8 assay resulted in 3.97×10^4 cells in Group M, 21.7×10^4 cells in Group N, and 39.5×10^4 cells in Group D. Cell proliferation in Group N was approximately 5 times higher than that of Group M. Furthermore, cell prolifer-