厚生労働科学研究費補助金(再生医療実用化研究事業)
自家骨髄間葉系幹細胞により活性化された椎間板髄核細胞を用いた椎間板再生研究
における細胞、組織の安全性、品質確保に関する技術開発
分担研究報告書

分担研究課題：活性化椎間板髄核細胞の変性椎間板への移植術に関する研究

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研究要旨：
20 歳代の椎間板ヘルニあるいは椎間板症の患者で椎間固定術を予定する症例の内、その隣接椎間板に中等度の変性を持つ7症例に対して、活性化髄核細胞の移植術を施行した。椎間板組織からの髄核組織の分離、腸骨からの骨髄採取は安全かつ症例の個体差なく順調に施行された。骨髄間葉系幹細胞との共培養によって活性化された髄核細胞（約 90 万個）の隣接変性椎間板内への移植は局所麻酔下に安定した手技で実施することができた。骨髄液採取部や活性化髄核細胞移植部の椎間板部分に起因する新たな愁訴は認められず、安全な組織採取ならびに細胞移植治療が実施されたと考える。採取された椎間板組織、骨髄液の cell processing center への移送、細胞の分散から単層培養、共培養は計画された時系列に従って実施された。活性化が終了された髄核細胞の移植用キット（注射針セット）への注入、手術場への搬送も支障なく実施された。

A. 研究目的

自家骨髄間葉系幹細胞との細胞間接着を伴う共培養で活性化された椎間板髄核細胞の組織採取から移植までの過程を検証し、安全で確実な移植術が実施可能かについて検討する。

B. 研究方法

椎体間固定時に採取された椎間板髄核組織の状態と、腸骨より採取された骨髄液の状態を術中に評価し、本細胞移植療法への細胞提供の可否を決定する。Cell processing center における細胞処理過程が計画通りに時系列を守って実施されることを検証し、最終製品として得られた活性化髄核細胞を、細胞判定委員会の議に従って受け取り、移植術が基準通りに実施されることを検証する。

C. 研究結果ならびに D. 考察

Cell processing center に移送する段階での椎間板髄核組織ならびに骨髄液は、組織の質、量ともに研究計画の基準を満たしていた。細胞の処理過程における観察では、細胞の活性化は極めて良好であり、その手技において感染が生じているというデーターは一切認められなかった。最終製品として得られた活性化髄核細胞の変性椎間板移植キットへの移行も、安全に実施された。麻酔科専門医の観察のもと、局所麻酔下で実施された活性化髄核細胞の移植手術は、平均 35 分間で実施され、術中の予想外の問題点当該移植部の疼痛、下肢神経障害の発生などの合併症は一切認められなかった。

E. 結論

組織採取から 7 日後の活性化髄核細胞移植術実施における手術室内ならびに cell processing center 内での工程は、研究計画に準拠し安全に実施された。

G. 研究発表　なし

H. 知的財産権の出願・登録状況　なし

厚生労働科学研究費補助金(再生医療実用化研究事業)
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分担研究報告書

分担研究課題：適応患者選択ならびに細胞の均一化に関わる外部評価に関する研究

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研究要旨：
2009 年以降、東海大学医学部附属病院における活性化髄核細胞移植症例の外部評価を行った。臨床研究参加者（患者）の資料、すなわち年齢、性別、既往歴、単純 X 線画像と MRI の画像所見は、症例ごとに東海大学における適応決定前の段階で送付された。これまで計 7 症例について評価を行い、20 才代の腰椎椎間板ヘルニアなどの症例で後方椎体間固定術を行う椎間があり、これに隣接した椎間が椎間板変性を有するが不安定性などがみられないなどの基準、すなわち活性化髄核細胞移植部位として適当であるかについて検討を行った。提示された 7 症例は全て本臨床研究の適応基準に合致していることを評価決定し、書面にて報告を行った。なお、移植された活性化椎間板髄核細胞の細胞処理の均一化については、東海大学において予定されている全 10 例の臨床研究が終了した時点で、全例のデーターを比較検討し、外部評価者としての見解を報告する。

A. 研究目的

椎間板再生医療プロジェクトの中で、骨髄間葉系幹細胞による椎間板髄核細胞の活性化を行い、安全性と有効性を科学的に検証する。

A. 研究方法

東海大学医学部整形外科学では椎間板の再生に関する基礎的研究を行い、骨髄間葉系幹細胞と髄核細胞を細胞間接着を伴うシステムで共培養する形の細胞培養系を開発した。活性化髄核細胞を 7 日後に椎体間固定術を施行した部分の隣接椎間で、中等度の変性を持ち、年齢や臨床症状、および画像上の基準に合致する症例に移植するプロジェクトである。

外部評価者としての研究方法は、ヒト幹細胞臨床研究に関する審査委員会で承認された適応基準に患者が合致していることを事前に検討し、また細胞処理が的確に実施されているかについての評価を実施することである。

B. 研究結果

症例は全例、20 代の男性で、問題となる既往歴はなかった。術前の画像上、活性化髄核細胞の移植先となる椎間固定術施行の隣接椎間板は MRI の Pfirrmann 分類で grade III であり、単純 X 線画像上、前方後方へのすべり、椎間の後方開大、動態不安定が認められず、ヒト幹細胞臨床研究に関する審査委員会で承認された適応基準に合致していた。

なお、活性化椎間板髄核の細胞処理の均一化については、全 10 例の臨床研究が終了した時点で、データーを比較検討し、外部評価者としての評価を行う。

C. 結論

東海大学において選択された 7 症例はすべ適応基準に合致しており、外部評価者として適応可との判定を行った。

G. 研究発表　なし

H. 知的財産権の出願・登録状況　なし

III.　研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Watanabe T, Sakai D, Yamamoto Y, Iwashina T, Serigano K, Tamura F, Mochida J	Human nucleus pulposus cells significantly enhanced biological properties in a coculture system with direct cell-to cell contact with autologous mesenchymal stem cells	J Orthop Res	28	623-630	2010
Hiyama A, Gajghate S, Sakai D, Mochida J, Shapiro IM, Risbud MV	Activation of TonEBP by calcium controls {beta} 1,3-glucuronosyltrans-ferase-I expression, a key regulator of glycosaminoglycan synthesis in cells of the intervertebral disc	J Biol Chem	284	9824-9834	2009
Sakai D, Nakai T, Mochida J, Alini M, Grad S	Differential phenotype of intervertebral disc cells: microarray and immunohistochemical analysis of canine nucleus pulposus and anulus fibrosus	Spine	34	1448-1456	2009
Rutges J, Creemers LB, Dhert W, Milz S, Sakai D, Mochida J, Alini M, Grad S	Variations in gene and protein expression in human nucleus pulposus in comparison with annulus fibrosus and cartilage cells: potential associations with aging and	Osteoarthritis Cartilage	18	416-423	2009
酒井大輔 持田譲治	細胞レベルからの椎間板再生　細胞移植療法のその先に	日本腰痛学会雑誌	15	95-98	2009

IV.　研究成果の刊行物・別刷

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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Received 11 March 2009; accepted 25 September 2009
Published online 1 December 2009 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/jor.21036

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.

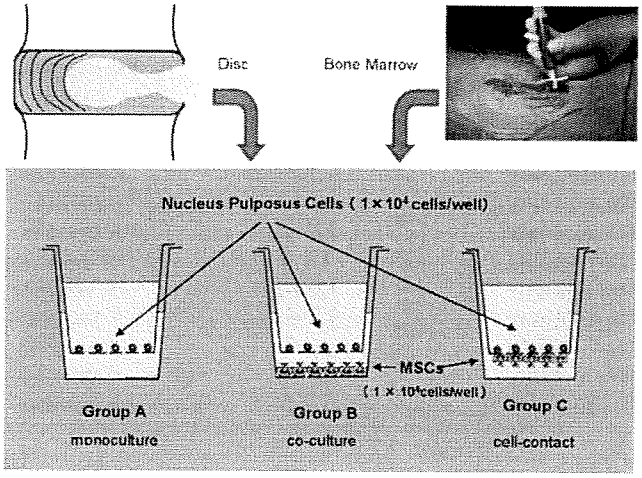

Figure 1. Study design: During surgery, the lumbar disc was removed, and 50 ml of autologous bone marrow was taken from the same patient. Nucleus pulposus (NP) cells and mesenchymal stem cells (MSCs) were isolated by enzymatic digestion and gradient centrifugation, respectively, and utilized for the 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-

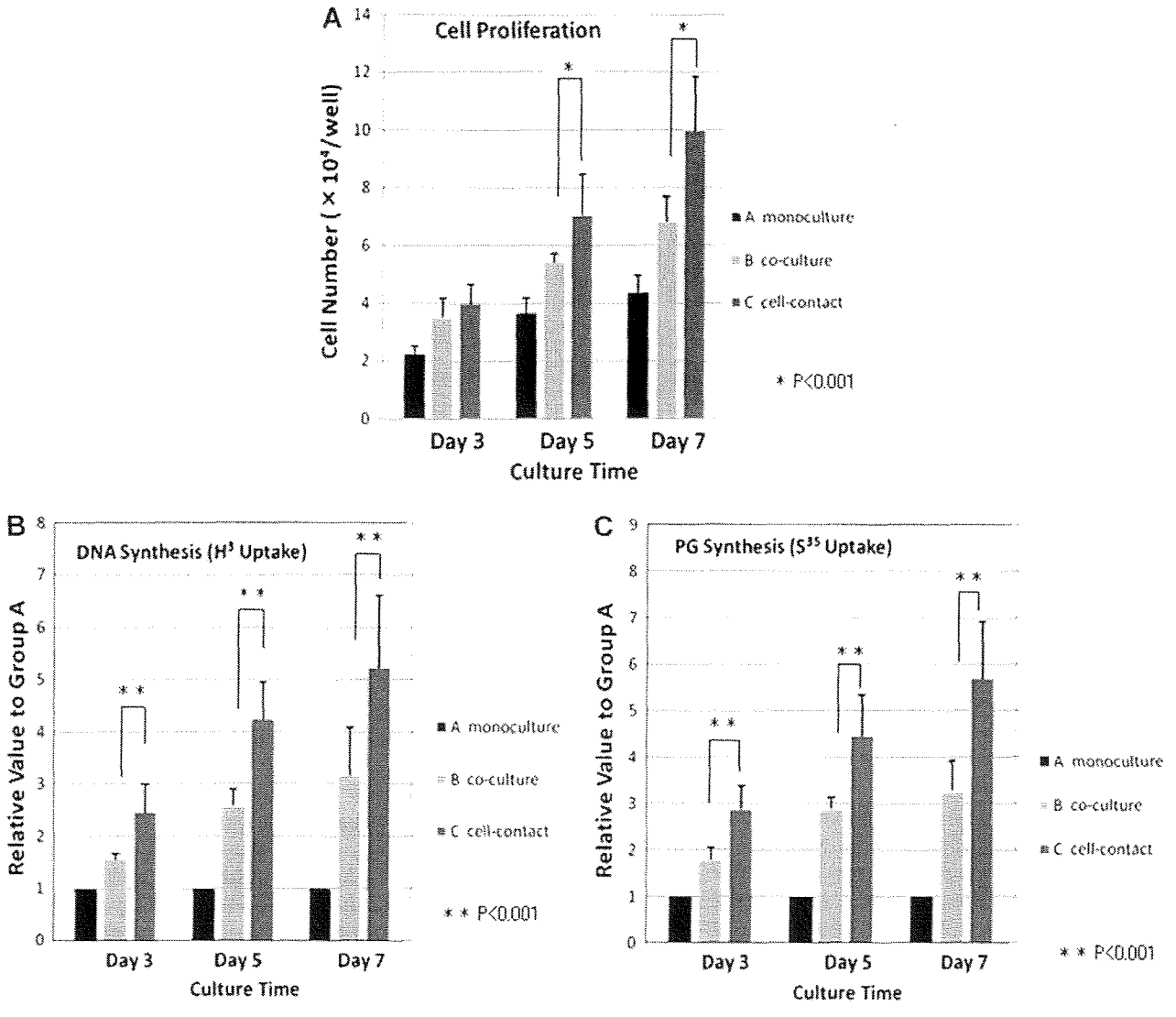

Figure 2. Nucleus pulposus (NP) cells were seeded onto culture inserts and divided into three groups: Group A, NP cell monoculture; Group B, standard coculture of NP cells and mesenchymal stem cells (MSCs); Group C, NP cells and MSCs cocultured with direct cell-to-cell contact. After 3, 5, and 7 days 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

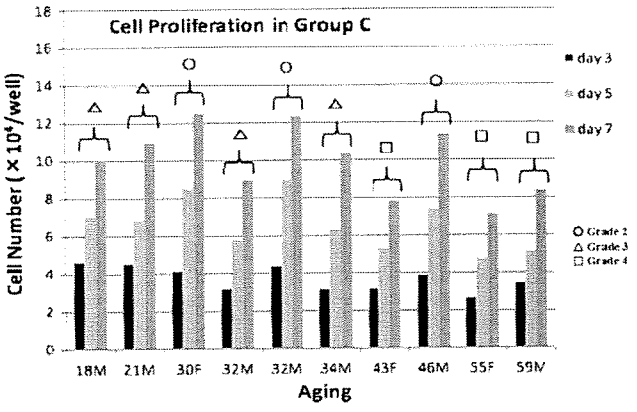

Figure 3. Cell proliferation is dependent on disc grading in Group C (Table 1). This result showed no significant differences among the 10 cases at 3 days of coculture dependent on the effects of age, the MRI disc grade. However, at 5 and 7 days of coculture, NP cells from the lower-graded discs (less-degenerated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

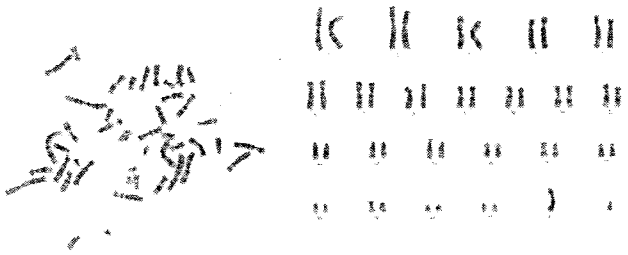

Figure 4. Microscopic examination of chromosomes (G-banding). Nucleus pulposus (NP) cells primarily cultured in monolayer for 4 days and then cocultured for 3 days with autologous mesenchymal stem cells (MSCs) with direct cell-to-cell contact were investigated for chromosomal abnormalities. Chromosomes 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

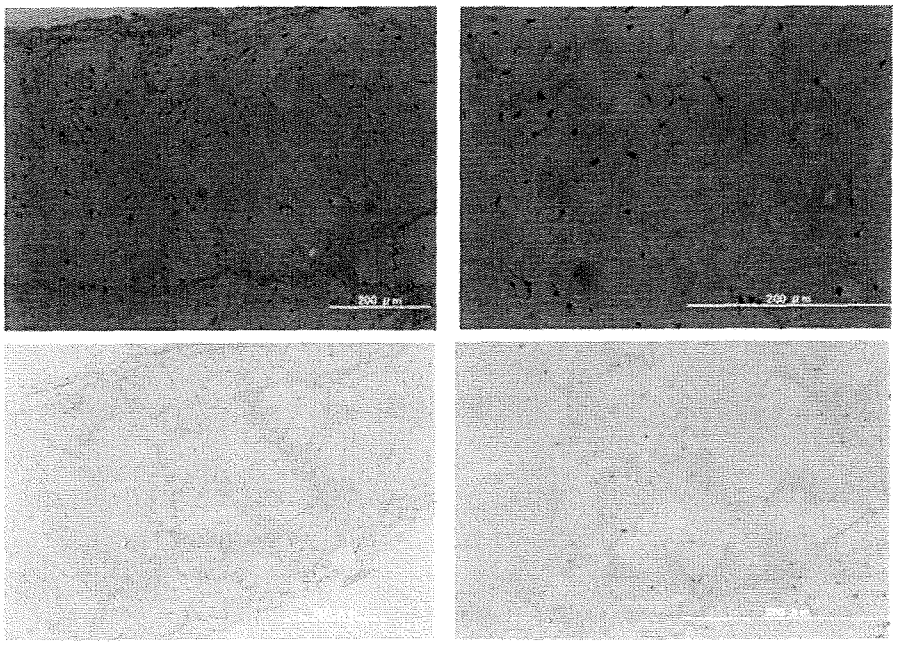

Figure 5. Histological examination of frozen sections. Nucleus pulposus (NP) cells from 10 clinical cases were primarily cultured in monolayer for 4 days and then cocultured for 3 days with autologous mesenchymal stem cells (MSCs) with direct cell-to-cell contact. Cocultured cells were suspended in a 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.

ACKNOWLEDGMENTS

We thank Satoshi Arakawa for supporting our studies by scanning chromosomal abnormalities using optical and fluorescent microscopes, and Ms. Kanae Tohyama for technical assistance. This work was supported in part by a Grant-in-Aid for Scientific Research, and a Grant of The Science Frontier Program, from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and a grant from AO Spine International to D. S.

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.

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 15, pp. 9824–9834, April 10, 2009
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Activation of TonEBP by Calcium Controls β1,3-Glucuronosyltransferase-I Expression, a Key Regulator of Glycosaminoglycan Synthesis in Cells of the Intervertebral Disc*

Received for publication, September 11, 2008, and in revised form, January 7, 2009 Published, JBC Papers in Press, January 15, 2009, DOI 10.1074/jbc.M807081200

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The goal of this investigation was to study the expression and regulation of β1,3-Glucuronosyltransferase-I (GlcAT-I), a key enzyme regulating GAG synthesis in cells of the intervertebral disc. There was a robust expression of GlcAT-I in the nucleus pulposus *in vivo*. Treatment with the calcium ionophore ionomycin resulted in increased GlcAT-I expression, whereas GlcAT-I promoter constructs lacking TonE site or a mutant TonE were unresponsive to the ionophore. Experiments using TonEBP and DN-TonEBP constructs showed that TonEBP positively regulated GlcAT-I promoter activity. ChIP analysis confirmed binding of TonEBP to the promoter. We further validated the role of TonEBP in controlling GlcAT-I expression using mouse embryo fibroblasts from TonEBP null mice. GlcAT-I promoter activity in null cells was significantly lower than the wild type cells. In contrast to wild type cells, treatment with ionomycin failed to increase GlcAT-I promoter activity in null cells. We then investigated if calcineurin (Cn)-NFAT signaling played a regulatory role in GlcAT-I expression. Inhibition of Cn following ionomycin treatment did not block GlcAT-I and *tauT*, a TonEBP-responsive reporter activity. GlcAT-I promoter activity was suppressed by co-expression of Cn, NFAT2, NFAT3, and NFAT4. Moreover, following ionomycin treatment, fibroblasts from CnAα and CnAβ null mice exhibited robust induction in GlcAT-I promoter activity compared with wild type cells. Results of these studies demonstrate that calcium regulates GlcAT-I expression in cells of the nucleus pulposus through a signaling network comprising both activator and suppressor molecules. The results suggest that by controlling both GAG and aggrecan synthesis, disc cells can autoregulate their osmotic environment and accommodate mechanical loading.

The intervertebral disc is a specialized structure that permits rotation as well as flexure and extension of the human spine. It consists of an outer ligament, the annulus fibrosus, that encloses a gel-like tissue, the nucleus pulposus. Although sparse, cells in the nucleus pulposus secrete a complex extracellular matrix that contains fibrillar collagens and the proteoglycan aggrecan. Glycosoaminoglycan (GAG)[2] components of the aggrecan molecule provide a robust hydrodynamic system that serves to accommodate applied biomechanical forces (1, 2). Surprisingly, although the importance of aggrecan secretion and function has been discussed by many investigators, mechanisms of control of GAG synthesis are poorly understood.

In the nucleus pulposus, the principle GAG is chondroitin sulfate. Structurally, this molecule is a heteropolysaccharide containing repeating units of *N*-acetylgalactosamine linked to glucuronic acid. In its fully sulfated form, the molecule exhibits a high charge density, and when hydrated, it assumes a linear configuration. Bound to the aggrecan core protein and associated with hyaluronic acid, the chondroitin sulfate chains form a giant polydispersed supramolecular structure. The high osmotic pressure of the aggregate contains the forces applied to the spine (3).

In an earlier study, we showed that nucleus pulposus cells responded to changes in osmotic pressure by up-regulating the transcription factor, TonEBP (tonicity enhancer-binding protein) (4). When expressed, this protein modulated aggrecan expression; whether it can regulate chondroitin chain synthesis has not been determined. It is noteworthy that other workers have shown that galactose-β1,3-glucuronysltransferase- 1 (GlcAT-I) activity was required for GAG chain synthesis (5); hence, there is the possibility that this enzyme serves as the rate-limiting step in GAG synthesis for chondrocytes and possibly other cell types (6, 7). Related to this point, it is now known that IL-1β suppressed GAG biosynthesis by down-regulating GlcAT-I expression and activity (8). A second factor regulating aggrecan synthesis is the intracellular calcium concentration (9, 10). In smooth muscle and Sertoli cells, calcium channel blockers have been shown to alter proteoglycan synthesis (11, 12). Furthermore, using *p*-nitrophenyl-β-D-xyloside as an exogenous primer for chain initiation, it was demon-

* This work was supported, in whole or in part, by National Institutes of Health Grants AR050087 and AR055655. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[2] The abbreviations used are: GAG, glycosoaminoglycan; GlcAT-I, β1,3-glucuronosyltransferase-I; NFAT, nuclear factor of activated T cells; Cn, calcineurin; BAPTA, 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid acetoxymethyl ester; PMA, phorbol 12-myristate 13-acetate; DN, dominant negative; CA, constitutively active; MEF, mouse embryo fibroblast; PBS, phosphate-buffered saline; ChIP, chromatin immunoprecipitation; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; ERK, extracellular signal-regulated kinase.

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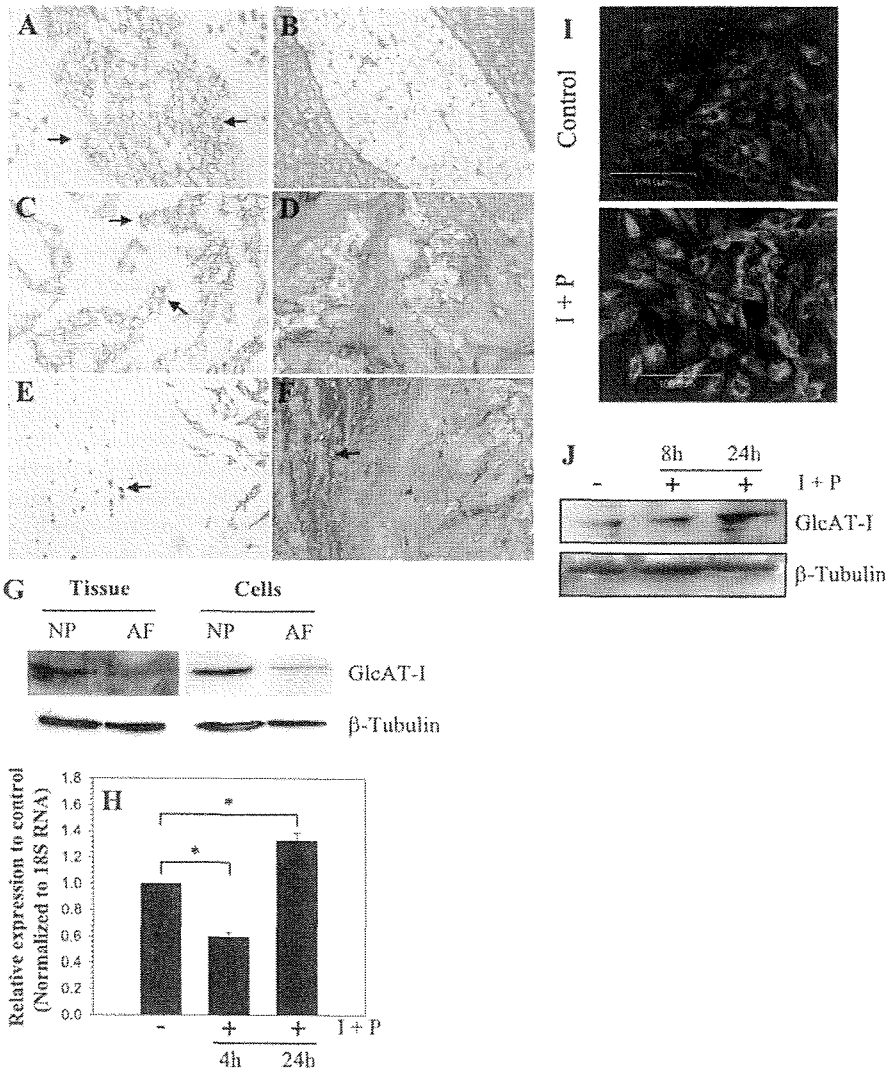

FIGURE 1. **Saggital and coronal sections of disc tissue from neonatal (*A*) and mature (*C* and *E*) rat spines stained with an antibody against GlcAT-I or stained with hematoxylin and eosin and Alcian blue (*B*, *D*, and *F*).** Note that nucleus pulposus cells in the neonatal (*A*) as well as skeletally mature disc cells (*C* and *E*) express GlcAT-I protein; much of the staining is localized to the cytosol and plasma membrane (*A* and *C*; *arrows*). Furthermore, inner annulus fibrosus cells localized in amorphous Alcian blue-positive matrix (*F*; *arrow*) express GlcAT-I protein (*E*; *arrow*). Isotype and secondary antibody controls were negative (not shown). Magnification was ×20. *G*, Western blot analysis of GlcAT-I expression by nucleus pulposus (*NP*) and annulus fibrosus (*AF*) tissue and cultured cells. Note, the expression of the 43-kDa GlcAT-I band in tissue extracts. The native nucleus pulposus tissue and cells in culture expressed higher GlcAT-I protein levels than the annulus tissue and cells. *H*, real time reverse transcription-PCR analysis of GlcAT-I expression by cells treated with ionomycin (1 μM) and PMA (100 ng) (*I* + *P*) for 4–24 h. There was a time-dependent change in mRNA expression following treatment. At 4 h, GlcAT-I expression was suppressed; at 24 h, there was increased expression of the gene. *I*, immunofluorescent analysis of nucleus pulposus cells treated with ionomycin and PMA. Cells showed increased GlcAT-I expression 24 h after the treatment. *J*, Western blot of nucleus pulposus cells treated with ionomycin and PMA. Note the increased GlcAT-I expression 24 h after treatment.

strated that GAG synthesis was inhibited by calcium blocking agents (12). Since these agents affected both chondroitin sulfate and heparin sulfate, the results suggested that calcium ions controlled a common early step in the GAG biosynthetic pathway. In a recent study, calcium was shown to control GlcAT-I expression through Sp1 transcription factor (13).

The major objective of the investigation was to examine the regulation of GlcAT-I expression by nucleus pulposus cells of the intervertebral disc. We show for the first time that TonEBP regulates GlcAT-I expression and that regulation is dependent on intracellular calcium ions. We also demonstrate that calcium-dependent calcineurin (Cn)-NFAT signaling serves as a negative regulator of GlcAT-I expression in these cells. From this perspective, by controlling GAG as well as aggrecan synthesis, TonEBP permits nucleus pulposus cells to autoregulate the osmotic environment of the disc.

EXPERIMENTAL PROCEDURES

Plasmids and Reagents—Rabbit polyclonal TonEBP antibody was a kind gift from Dr. H. Moo Kwon (University of Maryland). TonEBP/NFAT5 wild type and null MEFs (originally from Dr. Steffan N. Ho), were provided by Dr. Feng Chen (Washington University, St. Louis, MO). Primary kidney medullary fibroblasts derived from CnAα null, CnAβ null, and CnA wild type mice were provided by Dr. Jennifer Gooch (Emory University). Plasmids were kindly provided by Dr. Takashi Ito (Osaka University, Japan) (*tauT* (taurine transporter) wild type (WT) and mutant (MT) reporter) (14) and Dr. Ben C. Ko (University of Hong Kong) (FLAG-DN-TonEBP, FLAG-TonEBP, and FLAG-CMV2) (15). DN-TonEBP contains amino acids 157–581 of human TonEBP (from clone KIAA0827). Dr. Gerald Crabtree (Stanford University) provided catalytic subunit (CnA) and regulatory subunit (CnB). Dr. Jeffery Molkentin (Cincinnati Children's Hospital Medical Center) supplied NFAT4, pECE, and Dr. Gerald Thiel (University of Saarland Medical Center, Germany) supplied DN-Sp1. Plasmids for NFAT1 (catalogue number 11100) and constitutively active NFAT2 (CA-NFAT2) (catalog number 11102) with key serine residues changed to alanine to prevent phosphorylation developed by Dr. Anjana Rao (16), plasmid for NFAT3 (catalog number 10961) developed by Dr. Toren Finkel (17), and plasmid for 3×NFAT-Luc (catalog number 17870) developed by Dr. Gerald Crabtree (18) were obtained from Addgene. 3×NFAT-Luc contains three NFAT binding sites upstream of the minimal interleukin-2 promoter and used to measure calcineurin-dependent NFAT activation. As an internal transfection control, vector pRL-TK (Promega)

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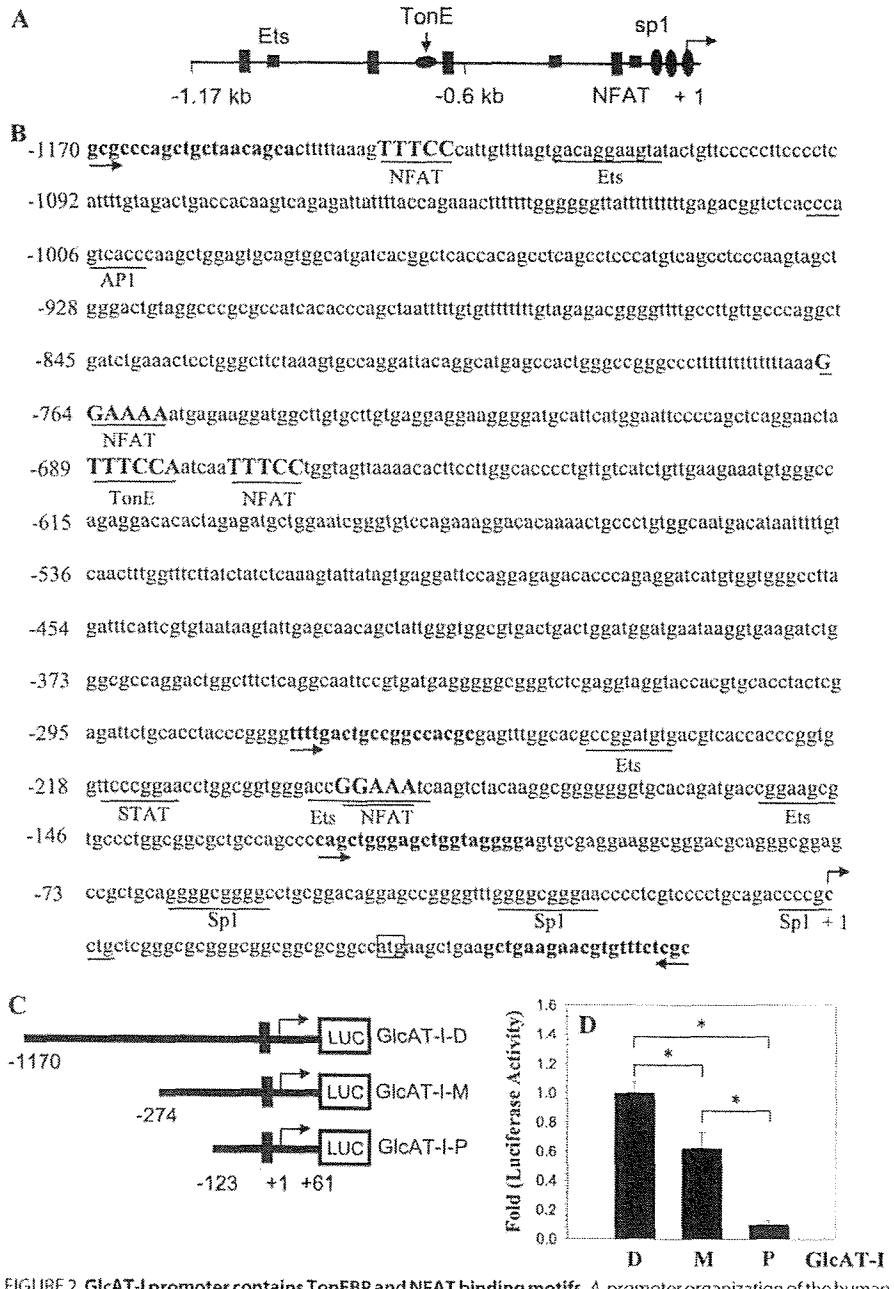

FIGURE 2. **GlcAT-I promoter contains TonEBP and NFAT binding motifs.** *A*, promoter organization of the human GlcAT-I gene. The transcription start site is marked as *+1*. The TonE site is shown as a *flattened circle*, Sp1s are indicted as *ovals*, and the NFAT binding motifs are shown as *rectangles*. *B*, DNA sequence of the promoter region of the human GlcAT-I gene. TonE (TTTCCA) and NFAT (TTTCC or GGAAA) consensus sequences are marked in *boldface type* and *underlined*. The *arrows* indicate the starting location of the primers used to generate promoter constructs. The transcription start site is marked as *+1*; *ATG* marks the translation start site. Sp1 sites are *underlined* and lie within first 100 bases. *C*, schematic diagram showing a map of successive PCR-generated 5′ deletion constructs of the human GlcAT-I promoter. The GlcAT-I-D construct consists of a 1,231-bp fragment containing 1,170 bp of the upstream GlcAT-I promoter sequence linked to 61 bp of exon 1 (*i.e.* −1170/+61), whereas GlcAT-I-M and GlcAT-I-P constructs contain a 335-bp fragment (−274/+61) and a 184-bp fragment (−123/+61), respectively. *D*, basal activities of GlcAT-I promoter constructs relative to full-length construct GlcAT-I-D in nucleus pulposus cells. Cells showed maximal luciferase activity for the GlcAT-I-D construct, whereas the shortest construct, GlcAT-I-P, showed minimal activity. Values shown are mean ± S.D. of three independent experiments. *, $p < 0.05$.

containing the *Renilla reniformis* luciferase gene was used. The amount of transfected plasmid, the pretransfection period after seeding, and the post-transfection period before harvesting have been optimized for rat nucleus pulposus cells using pSV β-galactosidase plasmid (Promega) (19).

Immunohistological Studies—Freshly isolated discs were immediately fixed in 4% paraformaldehyde in PBS and then embedded in paraffin. Transverse and coronal sections, 6–8 μm in thickness, were deparaffinized in xylene, rehydrated through graded ethanol, and stained with Alcian blue, eosin, and hematoxylin. For localizing GlcAT-I, sections were incubated with the anti-GlcAT-I antibody (Novus) in 2% bovine serum albumin in PBS at a dilution of 1:100 at 4 °C overnight. After thoroughly washing the sections, the bound primary antibody was incubated with biotinylated universal secondary antibody, at a dilution of 1:20 (Vector Laboratories) for 10 min at room temperature. Sections were incubated with a streptavidin-peroxidase complex for 5 min and washed with PBS, and color was developed using 3′,3-diaminobenzidine (Vecta Stain Universal Quick Kit; Vector Laboratories).

Isolation of Nucleus Pulposus Cells and Treatments of Cells—Rat nucleus pulposus cells were isolated using a method reported earlier by Risbud *et al.* (19). Nucleus pulposus cells and MEFs were maintained in Dulbecco's modified Eagle's medium and 10% fetal bovine serum supplemented with antibiotics. In some experiments, cells were treated with 1 μM ionomycin and PMA (100 ng/ml) with or without FK505 (10 ng/ml) and cyclosporine A (1 μg/ml) or BAPTA-AM (10 μM) or bisanthracycline (WP631; 50–100 nM).

Real Time RT-PCR Analysis—Following treatment, total RNA was extracted from nucleus pulposus cells using RNAeasy minicolumns (Qiagen). Before elution from the column, RNA was treated with RNase-free DNase I. 100 ng of total RNA was used as template for real time PCR analysis. Reactions were set up in microcapillary tubes using 1 μl of RNA with 9 μl of a LightCycler FastStart DNA Master SYBR Green I mix (Roche Applied Science) to which gene-specific forward and reverse

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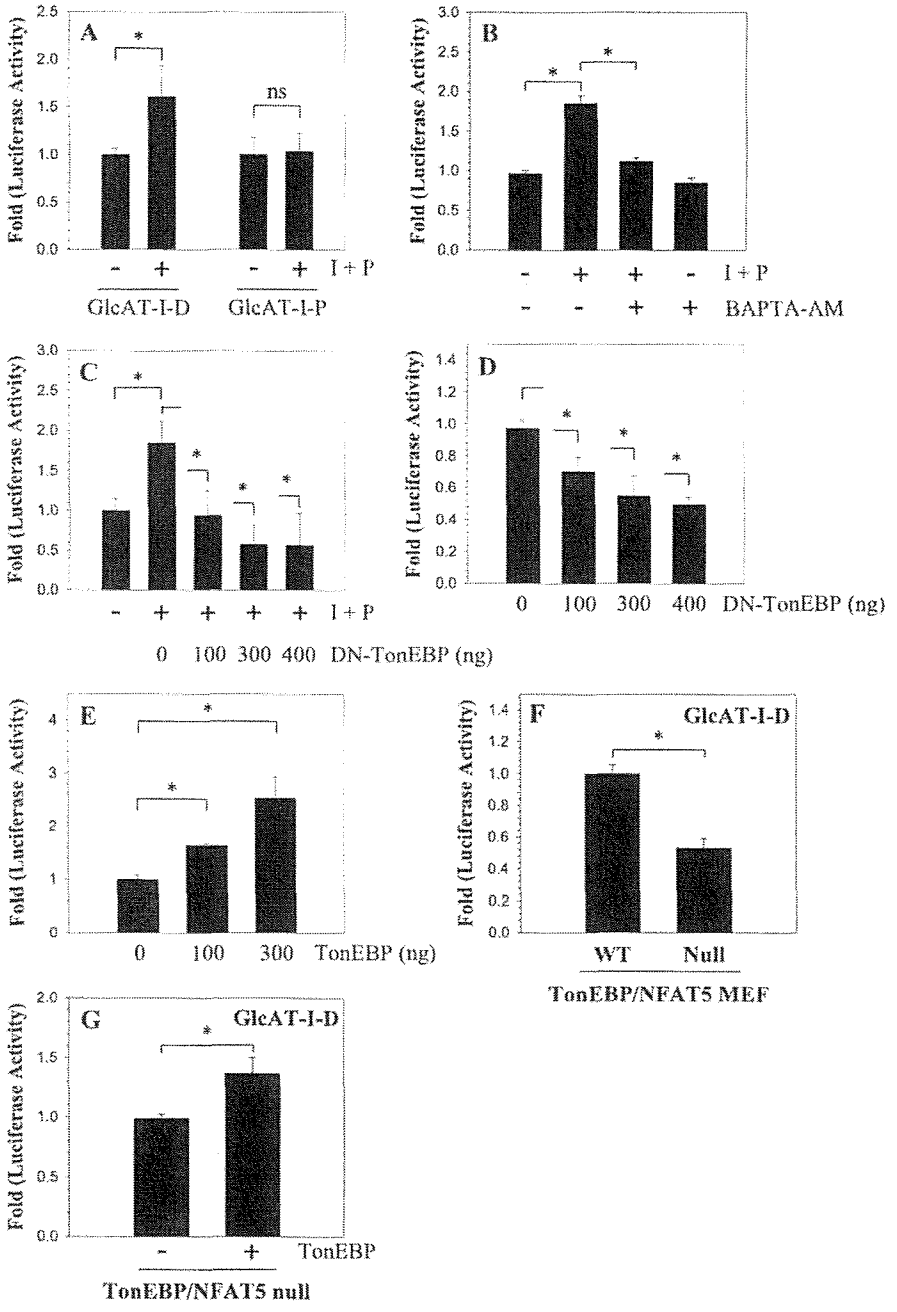

FIGURE 3. **Calcium regulates GlcAT-I promoter activity through TonEBP.** *A*, GlcAT-I-D or GlcAT-I-P reporter activity measured following ionomycin and PMA (*I* + *P*) treatment. Treatment resulted in induction in GlcAT-I-D but not GlcAT-I-P reporter activity. *B*, effect of BAPTA-AM (10 μM) on GlcAT-I-D reporter activity. The calcium chelator completely blocks ionomycin-mediated activity but not basal activity of the GlcAT-I-D reporter in nucleus pulposus cells. *C* and *D*, effect of ionomycin treatment on cells transfected with GlcAT-I-D reporter construct along with DN-TonEBP or empty backbone FLAG- CMV2. Note that the expression of DN-TonEBP resulted in a complete suppression of ionomycin-mediated induction in GlcAT-I promoter activity. In addition, when TonEBP function was blocked, there was suppression of basal GlcAT-I-D activity. *E*, effect of TonEBP on GlcAT-I promoter activity. When pTonEBP was co-expressed, there was a dose-dependent increase in GlcAT-I reporter activity. *F*, GlcAT- I promoter activity of TonEBP/NFAT5 null and wild type cells. Null cells evidenced decreased basal activity of reporter compared with wild type cells. *G*, effect of co-expression of TonEBP and GlcAT-D in null cells. When co-expressed, TonEBP increased GlcAT-I-D reporter activity. Values shown are mean ± S.D. of three independent experiments performed in triplicate. *, $p < 0.05$.

PCR primers were added (GlcAT-I (NCBI number NM_001128184), forward (5′-atgcccagtttgatgctactg-cac-3′) and reverse (5′-tgttcctcct-gcttcatcttcggt-3′)). Each set of samples included a template-free control. PCRs were performed in a LightCycler (Roche Applied Science) according to the manufacturer's instructions. All the primers used were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA).

Immunofluorescence Microscopy—Cells were plated in flat bottom 96-well plates (5 × 103/well) and treated with ionomycin for 6–24 h. After incubation, cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 in PBS for 10 min, blocked with PBS containing 5% fetal bovine serum, and incubated with antibodies against GlcAT-I (1:200) (Novus), TonEBP (1:200) (Calbiochem), NFAT-2 (1:200; Abcam), NFAT-3 (1:100; Cell Signaling), or NFAT-4 (1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 4 °C overnight. As a negative control, cells were reacted with isotype IgG under similar conditions. After washing, the cells were incubated with Alexa Fluor-488-conjugated anti-mouse secondary antibody (Invitrogen) at a dilution of 1:50 and 10 μM propidium iodide for 1 h at room temperature. Cells were imaged using a laser-scanning confocal microscope (Olympus Fluoview).

Nuclear Protein Extraction and Western Blotting—Cells were placed on ice immediately following treatment and washed with ice-cold Hanks' balanced saline solution. Nuclear and cytosolic proteins were prepared using the CellLytic NuCLEAR extraction kit (Sigma). All of the wash buffers and final resuspension buffer included 1× protease inhibitor mixture (Pierce), NaF (5 mM), and Na_3VO_4 (200 μM). Nuclear or total cell proteins were resolved on 8–12% SDS-polyacrylamide gels and transferred by electroblotting to nitrocellulose membranes (Bio-Rad). The membranes

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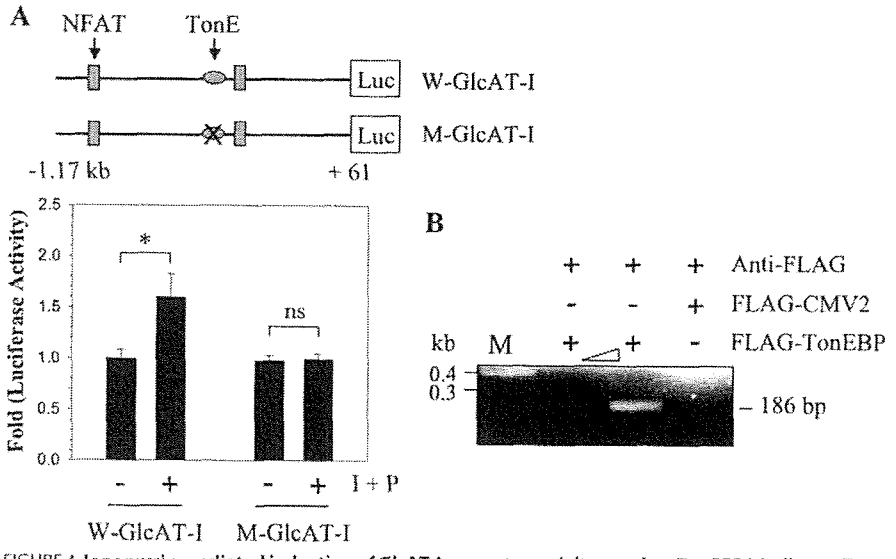

FIGURE 4. **Ionomycin mediated induction of GlcAT-I promoter activity requires TonEBP binding to TonE.** Effect of a 4-bp mutation introduced into the TonE motif of the GlcAT-I-D reporter plasmid. Nucleus pulposus cells were transfected with wild type GlcAT-I-D (*W-GlcAT-I*) or mutant GlcAT-I-D (*M-GlcAT-I*) reporter plasmids, and the induction of the luciferase activity was determined following ionomycin treatment. Treatment caused an induction of wild type reporter activity, whereas the mutant reporter failed to increase activity. Values shown are mean ± S.D. of three independent experiments. *, $p < 0.05$. *B*, interaction of TonEBP with the GlcAT-I promoter measured using a chromatin immunoprecipitation assay. COS7 cells were transfected with GlcAT-I-D along with either FLAG-TonEBP or FLAG-CMV2 empty vector. PCR amplification was performed using primer pairs that encompass TonE sequences of the GlcAT-I promoter. The use of anti-FLAG antibody resulted in generation of a PCR amplicon containing TonE only when FLAG-TonEBP was present. The addition of increasing amounts of FLAG-TonEBP vector evidenced enhanced binding to TonE. Pull-down using anti-FLAG antibody did not result in the formation of a PCR product when cells received empty FLAG-CMV2 vector.

were blocked with 5% nonfat dry milk in TBST (50 mM Tris, pH 7.6, 150 mM NaCl, 0.1% Tween 20) and incubated overnight at 4 °C in 3% nonfat dry milk in TBST with the anti-GlcAT-I (1:500; Novus) or anti-TonEBP antibody (1:3,000; from Dr. Kwon). Immunolabeling was detected using the ECL reagent (Amersham Biosciences).

Generation of GlcAT-I Reporter and Deletion Constructs—PCR amplification using genomic DNA of a 1,231-bp fragment containing 1,170 bp of the upstream promoter sequence linked to 61 bp of exon 1 (*i.e.* −1170 to +61) of the human GlcAT-I gene was performed using the following primers (forward, 5′ CTAGCTAGCGCGCCCAGCTGCTAACAGCA-3′ (NheI site underlined); reverse, 5′-CCCAAGCTTGCGAGAAACACGT-TCTTCAGC-3′ (HindIII site underlined)) with the addition of GC buffer 1 and LA Taq polymerase (Takara Mirus Bio). Similarly, to generate successive 5′ deletions, promoter fragments of 335 bp (−274 to +61) and 184 bp (−123 to +61) were PCR-amplified using specific forward primers and the same reverse primer. The resulting DNA fragments were subcloned into pCR2.1 TA vector (Invitrogen), isolated by restriction digestion with NheI and HindIII, and ligated into the luciferase basic expression vector, pGL3 (Promega). The identity of each GlcAT-I promoter sequence was confirmed by sequencing.

Site-directed Mutagenesis of TonE—GlcAT-I-D reporter plasmid was used to mutate the TonE site (TTTCCA to TAAAAA). Mutants were generated using the QuikChange II XL site-directed mutagenesis kit (Stratagene), using forward and reverse primer pair containing the desired mutation, following the manufacturer's instructions. The mutation was verified by sequencing.

Chromatin Immunoprecipitation (ChIP) Assay—COS7 cells were transfected with GlcAT-I-D along with either FLAG-TonEBP (increasing amounts) or FLAG-CMV2 vector and cultured for 48 h. ChIP analysis was performed as described before (20). Cross-linked and fragmented lysates were immunoprecipitated with monoclonal anti-FLAG M2 antibody (Sigma). PCR analysis to identify the coprecipitated TonE fragment in GlcAT-I promoter was performed using the following primer sequences: forward, 5′-GGAAAAATGAGAAG-GATGGCTTG-3′; reverse, 5′-TGGA-CACCCGATTCCAGCATCTCTA-3′.

Transfections and Dual Luciferase Assay—Cells were transferred to 24-well plates at a density of 6×10^4 cells/well 1 day before transfection. To investigate the effect of TonEBP overexpression on GlcAT-I promoter activity, cells were cotransfected with 100–500 ng of pTonEBP or DN-TonEBP or backbone vector pcDNA3.1 with 250 ng of GlcAT-I reporter and 250 ng of pRL-TK plasmid. In some experiments, cells were transfected with 500 ng of GlcAT-I reporter plasmids with 500 ng of pRL-TK plasmid. Lipofectamine 2000 (Invitrogen) was used as a transfection reagent. For each transfection, plasmids were premixed with the transfection reagent. For measuring the effect of ionomycin on GlcAT-I reporter activity, 24 h after transfection, the cells in some wells were ionomycin-treated with or without inhibitors FK506 and CsA or BAPTA-AM. The next day, the cells were harvested, and a Dual-Luciferase™ reporter assay system (Promega) was used for sequential measurements of firefly and *Renilla* luciferase activities. Transfection efficiency for rat nucleus pulposus cells was about 50–60%, whereas for fibroblasts, it was close to 90%. Quantification of luciferase activities and calculation of relative ratios were carried out using a luminometer (TD-20/20; Turner Designs, CA). At least three independent transfections were performed, and all analyses were carried out in triplicate.

Statistical Analysis—All measurements were performed in triplicate; data are presented as mean ± S.D. Differences between groups were analyzed by Student's *t* test. *, $p < 0.05$.

RESULTS

Saggital sections of the neonatal rat (Fig. 1, *A* and *B*) and skeletally mature rat discs (Fig. 1, *C–F*) were stained with an antibody to GlcAT-I (Fig. 1, *A*, *C*, and *E*) or counterstained with hematoxylin and eosin and Alcian blue for morphology assessment (Fig. 1, *B*, *D*, and *F*). GlcAT-I is expressed by cells of the nucleus pulposus, annulus fibrosus, and cartilaginous end plate

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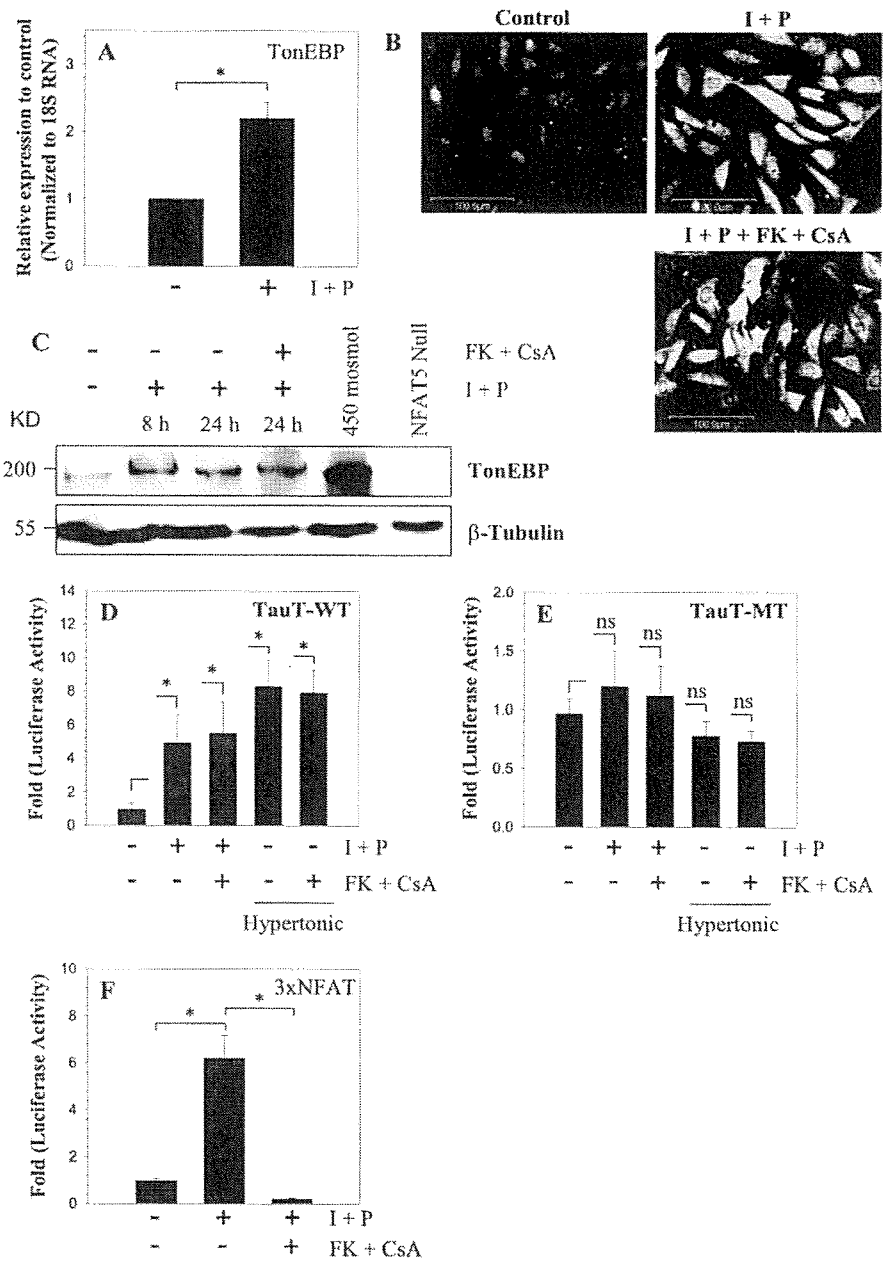

FIGURE 5. **Effect of calcium ions on TonEBP expression.** *A*, nucleus pulposus cells were treated with the calcium ionophore ionomycin (*I*; 1 μM), along with PMA (*P*; 100 ng), and TonEBP expression was measured. Ionomycin treatment resulted in significant increase in TonEBP mRNA expression. *B* and *C*, immunofluorescence and Western blot analysis of cells as treated in *A*. note the increase in TonEBP protein after ionomycin treatment. The addition of Cn inhibitors FK506 (10 ng/ml) and cyclosporine A (*CsA*; 1 μg/ml) did not suppress synthesis of TonEBP protein. When nucleus pulposus cells were cultured under hyperosmotic conditions (450 mosmol/kg), there was high induction in TonEBP, whereas TonEBP protein was undetectable in TonEBP null cells. *D* and *E*, reporter activity of nucleus pulposus cells transfected with wild type (*D*) or mutant (*E*) *tauT* reporter and treated with ionomycin and PMA with or without FK506 and cyclosporine. Note that treatment with ionomycin increased the activity of the WT but not the TonE-MT *tauT* reporter. FK506 and CsA did not inhibit ionomycin-mediated increase in *tauT*-WT promoter activity. As expected under hyperosmotic conditions, there is a robust induction in activity of *tauT*-WT but not *tauT*-MT reporter. *F*, induction of the activity of 3×NFAT reporter in NP cells following ionomycin and PMA treatment. The reporter is highly induced, whereas the addition of FK506 and cyclosporine completely blocks activation, indicating a requirement for Cn in this process. Values shown are mean ± S.D. of three independent experiments performed in triplicate. *, $p < 0.05$.

in rat disc (Fig. 1, *A*, *C*, and *E*). In all cases, staining is localized to the cytosol (Fig. 1, *A* and *C*). Expression of GlcAT-I in native disc tissues and cultured cells was studied using Western blot analysis. Fig. 1*G* indicates that nucleus pulposus tissue expresses a prominent 43-kDa GlcAT-I band. Moreover, the expression level of GlcAT-I in nucleus pulposus tissue is higher than in the annulus fibrosus (Fig. 1*G*). Similar to native tissue, cultured rat disc cells exhibit a similar pattern of expression (Fig. 1*G*). To explore the premise that intracellular calcium regulated GlcAT-I expression, nucleus pulposus cells were treated with ionomycin, a calcium ionophore, along with PMA, and expression of GlcAT-I was analyzed. Fig. 1*H* shows that treatment with ionomycin for 24 h results in increased GlcAT-I mRNA levels in nucleus pulposus cells. In addition, we studied the expression of GlcAT-I in nucleus pulposus cells using immunofluorescence microscopy and Western blot analysis. Ionomycin treatment results in increased GlcAT-I protein expression (Fig. 1, *I* and *J*); the increase is pronounced 24 h after treatment (Fig. 1*J*).

To investigate the regulation of expression, we analyzed the 1.17-kb promoter sequence of human GlcAT-I and measured the activity of different size promoter fragments. Analysis revealed that the GlcAT-I promoter contains four NFAT (GGGAA or TTTCC) as well as a conserved TonE (TTTCCA) motif at −684 bp (Fig. 2, *A* and *B*). To analyze promoter function, we generated luciferase reporter constructs containing a −1170/+61 bp (pGlcAT-I-D), a −274/+61 bp (pGlcAT-I-M), and a −123/+61 bp (pGlcAT-I-P) fragment of the human GlcAT-I promoter (Fig. 2*C*). We measured the basal activity of all three fragments in nucleus pulposus cells. Fig. 2*D* shows that the −1170/+61 bp fragment has maximal basal activity, whereas the −123/+61 bp fragment exhibits the least activity.

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