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The Effect of Bone Morphogenetic Protein-2 on Rat Intervertebral Disc Cells *in Vitro*

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Study Design. An *in vitro* experiment to determine the molecular and cellular effect of recombinant human bone morphogenetic protein-2 on cultured rat intervertebral disc cells was performed.

Objectives. To determine the effect of recombinant human bone morphogenetic protein-2 on cell proliferation, production of sulfated-glycosaminoglycan, and the expression of genes specific for chondrocytes (Type II collagen, aggrecan, and Sox9) in cultured rat intervertebral disc cells.

Summary of Background Data. Intervertebral disc degeneration is associated with cellular and biochemical changes, which include decreased synthesis of cartilage specific gene products such as Type II collagen and aggrecan. Although bone morphogenetic protein-2 is known to induce chondrogenesis during new bone formation, the effects on intervertebral disc cells have not been characterized.

Method. Cells were isolated from the anulus fibrosus and transition zones of lumbar discs from Sprague-Dawley rats. The cells were grown in monolayer and treated with recombinant human bone morphogenetic protein-2 (0, 10, 100, 1000 ng/mL) in Dulbecco's Modified Eagle Medium/F-12 with 1% fetal bovine serum (day 0). On days 2, 4, and 7 after recombinant human bone morphogenetic protein-2 treatment, sulfated-glycosaminoglycan content in the media was quantified using 1,9-dimethylmethylene blue staining. The results were normalized according to culture duration and cell number. On day 7, mRNA was extracted for reverse transcriptase-polymerase chain reaction and real-time polymerase chain reaction to quantitate mRNAs of Type I collagen, Type II collagen, aggrecan, Sox9, osteocalcin, and glyceraldehyde phosphate dehydrogenase. Cell number was determined with a hemocytometer.

Results. Recombinant human bone morphogenetic protein-2 at 100 and 1000 ng/mL yielded a 17% and 42% increase in cell number on day 4, and a 59% and 79% on day 7, respectively. Recombinant human bone morphogenetic protein-2 at 10 ng/mL had no effect on cell number. Sulfated-glycosaminoglycan increase was greatest at day 7, increasing by 1.3-, 2.1-, and 3.6-fold with recombinant human bone morphogenetic protein-2 treatments of 10, 100, and 1000 ng/mL, respectively. Increases in mRNA levels of Type II collagen, aggrecan, Sox9, and osteocalcin were observed with recombinant human bone morphogenetic protein-2 concentrations of 100 and 1000 ng/mL on day 7 as determined by reverse transcriptase-polymerase chain reaction. No detectable increase in mRNA level of Type I collagen was observed with any levels of recombinant human bone morphogenetic protein-2. Real-time polymerase chain reaction showed the greatest effect at 1000 ng/mL recombinant human bone morphogenetic protein-2, leading to an 11.5-fold increase in aggrecan, a 4.6-fold increase in Type II collagen, a 5.3-fold increase in Sox9, and a 1.9-fold increase in osteocalcin mRNA above untreated controls at day 7.

Conclusion. The results of this study show that recombinant human bone morphogenetic protein-2 enhances disc matrix production and chondrocytic phenotype of intervertebral disc cells. Recombinant human bone morphogenetic protein-2 increases cell proliferation and sulfated-glycosaminoglycan (proteoglycan) synthesis. It increases mRNA of Type II collagen, aggrecan, and Sox9 genes (chondrocyte specific genes), and osteocalcin, but not Type I collagen or glyceraldehyde phosphate dehydrogenase. [Key words: intervertebral disc cell, bone morphogenetic protein, collagen, aggrecan, Sox9, osteocalcin, sulfated-glycosaminoglycan] **Spine 2003;28:1773–1780**

The pathogenesis of intervertebral disc degeneration is poorly understood, although it is known to be associated with a variety of cellular and biochemical changes. The most prominent of these changes include a decrease in cell density in the disc and a decrease in synthesis of cartilage specific gene products such as Type II collagen and proteoglycans.[1–3] As the disc degenerates, there is a change in the extracelluar matrix (ECM), and this is thought to lead to human disc disease.[4–6] Biologic therapy to enhance the production of Type II collagen and proteoglycans may be an effective method of retarding, or even reversing, the disc degeneration.

One approach to biologic treatment is to stimulate matrix synthesis by the disc cells using cytokines. Preliminary work with several candidate cytokines such as transforming growth factor-beta (TGF–β) and epidermal growth factor (EGF) have been performed *in vitro*.[7–9] These growth factors were found to stimulate proteoglycan synthesis rates and cellular proliferation in disc cells.[8–10] Bone morphogenetic protein-2 (BMP-2) is a member of the TGF–β family and holds promise as another stimulatory cytokine.

Bone morphogenetic protein-2 is an osteoinductive cytokine that can stimulate mesenchymal stem cell differentiation into a variety of cell types including chondrocytes and osteoblasts.[11,12] Bone morphogenetic pro-

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Table 1. Primer Sequences for Collagen Type I, Collagen Type II, Aggrecan, Sox9, Osteocalcin, and GAPDH

Primer	Sequence
Type I collagen (forward)	ATGTTCAGCTTTGTGGAC
Type I collagen (reverse)	GGATGCCATCTTGTCCAG
Type II collagen (forward)	CTCAAGTCGCTGAACAACC
Type II collagen (reverse)	CTATGTCCACACCAAATTCC
Aggrecan (forward)	AGGATGGCTTCCACCAGTGC
Aggrecan (reverse)	TGCGTAAAAGACCTCACCCTCC
Sox9 (forward)	ACTTCCGCGACGTGGACATC
Sox9 (reverse)	TGTAGGTGACCTGGCCGTG
Osteocalcin (forward)	CAGACACCATGAGGACCCTC
Osteocalcin (reverse)	GTCCATTGTTGAGGTAGCGC
GAPDH (forward)	ACCACAGTCCATGCCATCAC
GAPDH (reverse)	TCCACCACCCTGTTGCTGTA

GAPDH = glyceraldehyde phosphate dehydrogenase.

tein-2 is also known to induce chondrocyte differentiation and maintain articular chondrocyte phenotypes in long-term culture.[13–15] However, as far as we are aware, there are no published studies on the effects of BMP-2 on intervertebral disc cells.

We were particularly interested in the effect of BMP-2 on Type I and II collagens, aggrecan, Sox9, and osteocalcin. Type I and II collagens are fibrillar molecules that constitute the majority of the collagens in the disc.[16–18] Type II collagen is specific for articular cartilage and intervertebral disc matrix.[18,19] Aggrecan consists of a core protein to which sulfated-glycosaminoglycans (s-GAGs) are attached, and it is a major component of disc proteoglycans.[20,21] Sox9 upregulates both aggrecan and Type II collagen and is a transcriptional regulator that is specific to chondrocytes.[22–24] Osteocalcin is a marker of bone cells and is expressed by osteoblasts.[25,26]

We therefore decided to carry out an experiment using rat intervertebral disc cells to determine the effect of recombinant human BMP-2 (rhBMP-2) on cell proliferation, proteoglycan synthesis, the expression of chondrogenic genes (Type II collagen, aggrecan, and Sox9), an osteoblast marker gene (osteocalcin), and nonspecific genes (Type I collagen and glyceraldehyde phosphate dehydrogenase [GAPDH]).

■ Method

Disc Cell Isolation. Unless otherwise stated, all reagents were purchased from GibcoBRL (Grand Island, NY). Lumbar intervertebral discs from Sprague-Dawley rats (age 11 months and sexually mature) were harvested immediately after they were killed. Anulus fibrosus, including the transition zone between the anulus and the nucleus, was dissected out and placed in an incubator (5% CO_2 + 95% room air at 37 C) in Dulbecco's Modified Eagle Medium and Ham's F-12 medium (DMEM/F-12) with 10% fetal bovine serum (FBS) + 100 U/mL penicillin + 100 μg/mL streptomycin for 12 hours. To isolate the cells, the disc tissues in DMEM/F-12 media were digested with 0.2% pronase (Sigma Chemical, St. Louis, MO) for 1 hour, followed by 0.025% collagenase (Sigma Chemical) for 4 hours. After enzyme digestion, the suspension was filtered through 70 μm mesh (Falcon, Franklin Lakes, NJ). The filtered cells were then washed with the DMEM/F-12 media and a primary culture was started. About 150,000 cells were extracted from each rat disc.

Rat disc cells from anulus fibrosus and transition zone were used for this study. In our preliminary culture experiments, notochordal cells from the nucleus pulposus region of the disc were isolated, but they disappeared quickly without proliferation.[19,27,28] Notochordal cells are not present in humans beyond the age of 4 to 10 years and are probably not representative of normal adult human nucleus cells.[19,29] We elected not to include these notochordal cells in our experiments.

Cell Culture in Selected Concentration of Recombinant Human Bone Morphogenetic Protein-2. When the primary cell culture became confluent, the cells were trypsinized and subcultured into 6-well plates at 300,000 cells/well. Cells were cultured in DMEM/F-12 media with 10% FBS + 100 U/mL penicillin + 100 μg/mL streptomycin + 2 mmol/L L-glutamine + 50 μg/mL Vitamin C. When confluence in each well was over 80%, the media were replaced with media containing 1% FBS. Recombinant human bone morphogenetic protein-2 (Genetic Institute, Cambridge, MA) was added to the media to reach final concentrations of 10, 100, and 1000 ng/mL. Cultures without added rhBMP-2 acted as controls. On days 2 and 4, the culture media were changed with the same concentration of rhBMP-2 in each well. On day 7, the cells were harvested for RNA extraction.

To determine the effect of rhBMP-2 on disc cell proliferation, cells grown under the same conditions as those described above were prepared in triplicate, trypsinized, stained with tryphan blue, and counted on days 2, 4, and 7 using a hemocytometer. In all cases, more than 90% of the cells were viable based on tryphan blue dye exclusion. The ratio of cell number of rhBMP-2 treated samples (10, 100, 1000 ng/mL) to control (no rhBMP-2) was calculated.

All experiments were performed at least three times to insure consistency.

Proteoglycan Production Assay. Staining with 1,9-dimethylmethylene blue (DMMB) for s-GAG was used to measure proteoglycan production.[30,31] On days 2, 4, and 7 after the start of the experiment, the culture medium was collected. Culture medium was concentrated (20×) with Centricon YM-50 (Milipore Co., Bedford, MA) centrifugal filtration (5000*g* for 30 minutes). Twenty microliters (20 μL) of each concentrated medium was mixed with 200 μL of DMMB dye and optical density was determined with a 520 nm wavelength filter. A standard curve was made using serial dilutions of chondroitin sulfate. Total s-GAGs in the media were normalized according to counted cell number and duration of culture and described as the ratio of rhBMP-2-treated samples to control.

Reverse Transcription Polymerase Chain Reaction. A reverse transcription polymerase chain reaction (RT-PCR) technique was used to determine the mRNA level of Type I collagen, Type II collagen, aggrecan, Sox9, and osteocalcin. Glyceraldehyde phosphate dehydrogenase primer was used as an internal control. On day 7, total RNA was extracted by single-step method of the gonidium thiocyanate-phenol-chloroform technique.[32] Concentration of total RNA was determined with a spectrophotometer (DU-500; Beckman, Fullerton, CA) at 260 nm wavelength. Reverse transcription was carried out in 40 μL volume with 2 μg of total RNA: 30 U Avian Myeloblastosis virus reverse transcriptase (Promega,

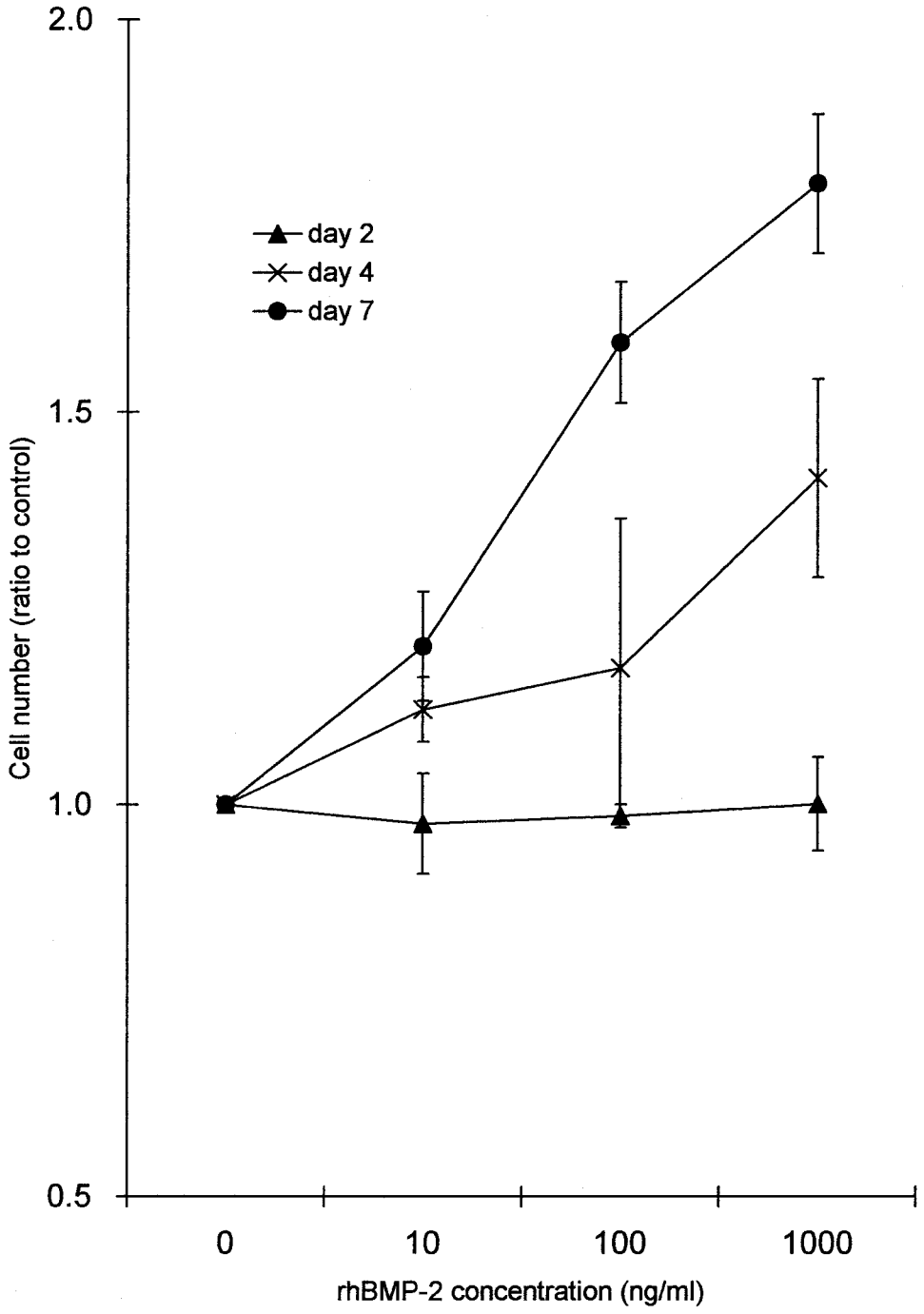

Figure 1. The effect of rhBMP-2 on disc cell proliferation. The number of viable cells was determined by staining with trypan blue and counting the cells on a clinical hemocytometer. The data are presented as a ratio of rh-BMP-2 treated samples (10, 100, 1000 ng/mL) to control (no rh-BMP-2). Statistically significant increases were seen with rh-BMP-2 concentrations of 100 and 1000 ng/mL at day 4 and day 7. Recombinant human BMP-2 at 10 ng/mL showed little effect on disc cell number.

Madison, WI); 5 mmol/L of $MgCl_2$; 60 U/μL of RNAsin (Promega, Madison, WI); 1 mmol/L of each deoxyadnosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP), deoxythymidine triphosphate (dTTP); and 1 μg oligo$(dT)_{15}$ primer for 45 minutes at 42 C.

One microgram of complementary deoxyribonucleic acid (cDNA) was used for each PCR amplification. Fifty microliters of reaction volume included 30 pmole of each forward and reverse primers; 1.25 U of Taq polymerase (Promega, Madison, WI); 0.2 mmol/L of each dATP, dCTP, dGTP and dTTP; and 1.5 mmol/L of $MgCl_2$. Thirty cycles of PCR were done for denaturation (95 C for 30 seconds), annealing (62 C for 30 seconds), and elongation (72 C for 45 seconds). The sequences of forward and reverse primers of Type I collagen, Type II collagen, aggrecan, Sox9, osteocalcin, and GAPDH are summarized in Table 1. Electrophoresis on 1.5% agarose gel was performed with 10 μL of each PCR product.

Quantitative Real-Time Polymerase Chain Reaction. To quantify mRNA levels of Type I collagen, Type II collagen, aggrecan, and Sox9, the real-time PCR method was used. Real-time PCR has been reported to be a rapid, reliable, and reproducible method for quantitative detection of specific mRNA.[33,34] The iCycler (Biorad, Hercules, CA) that detects SYBR Green fluorescent dye incorporated in double strand DNA was used. Fifty microliters of reaction volume included 2 μL of cDNA of RT-PCR, 35 pmole of each primer (collagen Type I, Type II, aggrecan, Sox9, osteocalcin, and GAPDH), and

25 μL of SYBR Green mater mix (2×; Biorad, Hercules, CA). Forty cycles of real-time PCR were done for denaturation (95 C for 30 seconds), annealing (62 C for 30 seconds), and elongation (72 C for 45 seconds). To confirm amplification specificity, the PCR products were subjected to a melting curve analysis. A positive standard curve of each primer was obtained by real-time PCR with serially diluted cDNA samples. Threshold cycles (Ct) of Type I collagen, Type II collagen, aggrecan, Sox9, and osteocalcin were standardized according to GAPDH. The amount of each mRNA in rhBMP-2 treated samples (10, 100, 1000 ng/mL) was compared to control samples and reported as a ratio of treated to control.

Statistical Analysis. The Student two-tailed *t* test was used. A *P* value less than 0.05 was used to define statistical significance.

■ Results

Cell Number and Morphology

The effect of rhBMP-2 on cell number during various stages of culture was determined. The cell number after culture for 2, 4, and 7 days is presented in Figure 1 as a ratio of the rhBMP-2 treated group to control (no rhBMP-2 added) group. Although no effect was seen at day 2, significant increases in cell numbers were seen at day 4, and the most pronounced effect was seen at day 7. There was an increase in cell number with increasing concentration of rhBMP-2. The addition of rhBMP-2 at concentrations of 100 and 1000 ng/mL increased disc cell number by 17% ($P = 0.413$) and 42% ($P = 0.030$) on day 4, and 59% ($P = 0.044$) and 79% ($P = 0.0008$) on day 7, respectively. Recombinant human BMP-2 at 10 ng/mL showed little effect on disc cell proliferation. Because there was essentially no cell death in the culture based on tryphan blue staining, the increase in cell number reflects cell proliferation.

Distinct and consistent differences in cell morphology were seen between the different concentrations of rhBMP-2. A representative phase contrast microscopic view after 7 days culture is shown in Figure 2. The control cells are more circular in shape and evenly scattered throughout the culture plate (Figure 2A). With the addition of 10 ng/mL of rhBMP-2, the cells tend to appear more polygonal or spindle shaped. The cells also attain a more organized spatial distribution on the culture plate, establishing a swirling configuration. With higher concentrations of rhBMP-2 (100 and 1000 ng/mL), the cells tend to organize into aggregates (Figure 2C and 2D).

Proteoglycan Production

Sulfated-glycosaminoglycan production as measured by the DMMB assay was used to estimate proteoglycan production. The total s-GAG present in the media was measured as a function of rhBMP-2 concentration and presented as a ratio to control in after normalization by cell number and culture duration (Figure 3). On day 2 and day 4, the s-GAG production reached a plateau at 100 ng/mL or rhBMP-2. However, on day 7, the s-GAG production increased significantly from 100 ng/mL to 1000 ng/mL of rhBMP-2, indicating that a plateau concentration was probably not reached at that time point. At day 7, the s-GAG production was 1.3, 2.1, and 3.6 times higher than control at 10, 100, and 1000 ng/mL of rhBMP-2, respectively. Statistically significant increases compared to control were seen at day 7 with 100 ng/mL ($P = 0.000006$) and 1000 ng/mL ($P = 0.00005$) of rhBMP-2.

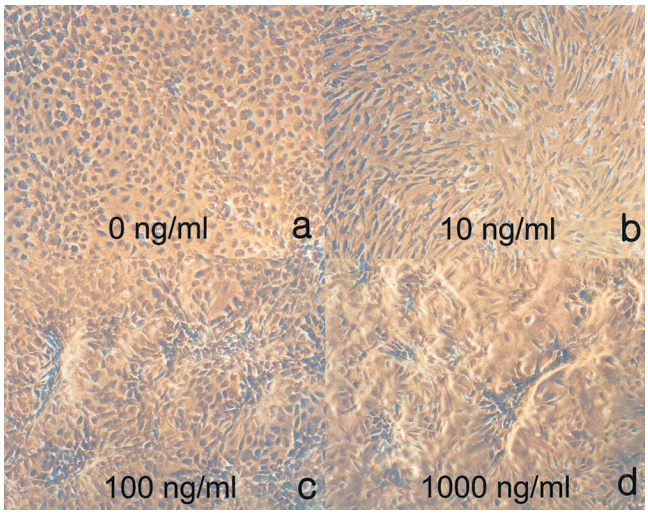

Figure 2. Phase contrast photomicrographs showing the change in disc cell morphology with different concentrations of rhBMP-2 (**A,** no rhBMP-2; **B,** 10 ng/mL; **C,** 100 ng/mL; **D,** 1000 ng/mL). These photographs were taken on day 7. In control and 10 ng/mL rhBMP-2 (A and B), disc cells were evenly scattered on the plate. In the 100 and 1000 ng/mL of rhBMP-2 (C and D), the cells tended to organize into aggregates. Original magnification of all photomicrographs ×40.

Quantitation of mRNA Levels

The gene expression profile of the disc cells in response to different concentrations of rhBMP-2 was determined by RT-PCR and real-time PCR methods. We assayed the mRNA levels of genes specific for chondrocytes (Type II collagen, aggrecan, and Sox9), an osteoblastic marker gene (osteocalcin), and nonspecific genes (Type I collagen and GAPDH). The RT-PCR shown in Figure 4 provides a qualitative assessment of the mRNA levels of Type I collagen, Type II collagen, aggrecan, Sox9, osteocalcin, and GAPDH obtained from the same total mRNA templates. Clean single amplicon bands were obtained. This experiment proved the quality of our PCR templates and primers. Type II collagen, aggrecan, and Sox9 showed strong dose-dependent increases. Osteocalcin increased slightly. In contrast, the Type I collagen did not display any noticeable change with rhBMP-2 treatment at any dose. The GAPDH bands were even in all of the lanes, indicating a relatively even load of total mRNA template.

Real-time PCR analysis was used to quantitate the changes in mRNA levels. Normalization was performed with internal control (GAPDH) and standard curve of each primer, and the results are shown in Figure 5 as a relative ratio of mRNA level of rhBMP-2 treated group

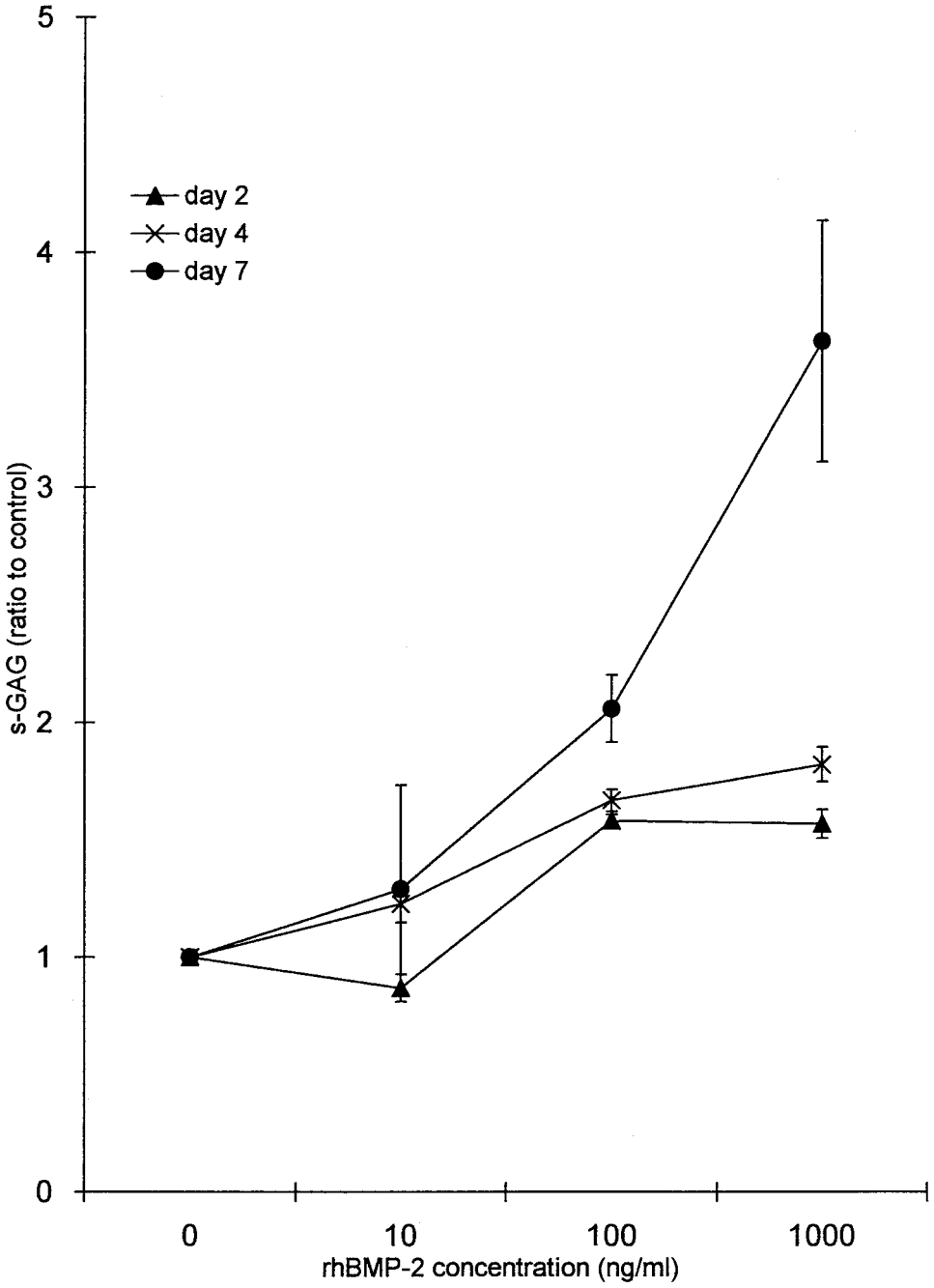

Figure 3. The effect of rhBMP-2 on the production of s-GAG from disc cells. The s-GAG content in the media was normalized by cell number and culture duration and described as a ratio of rhBMP-2 treated samples (10, 100, 1000 ng/mL) to control (no rhBMP-2). This result shows that s-GAG production increased with rhBMP-2 treatment. On day 7, statistically significant increases compared to control was seen with 100 ng/mL ($P = 0.000006$) and 1000 ng/mL ($P = 0.00005$) rhBMP-2.

to control. Type II collagen mRNA was significantly increased with rhBMP-2 treatment: by 2.02 ± 0.35 ($P = 0.007$) and 4.63 ± 2.05 ($P = 0.037$) times in 100 and 1000 ng/mL of rhBMP-2, respectively. Aggrecan mRNA was significantly increased with rhBMP-2: by 2.55 ± 0.63 ($P = 0.013$) and 11.59 ± 1.84 ($P = 0.0006$) times in 100 and 1000 ng/mL of rhBMP-2, respectively. Sox9 mRNA was also upregulated by rhBMP-2 treatment in a dose-dependent manner. Sox9 mRNA was increased by 2.55 ± 0.14 (100 ng/mL of rhBMP-2, $P = 0.0003$) and 5.31 ± 1.60 (1000 ng/mL of rhBMP-2, $P = 0.003$) times over controls. Osteocalcin mRNA was increased by 1.85 ± 0.49 (1000 ng/mL of rhBMP-2, $P = 0.04$). Type I collagen mRNA level was not changed by any concentration of rhBMP-2; even with 1000 ng/mL of rhBMP-2, there was no statistically significant increase ($P = 0.41$).

■ Discussion

In disc degeneration, there is a broad spectrum of physical and biologic changes that range from mild to severe.[1–3,5,35] One can speculate that the mild changes may not require treatment and that the severe changes might be beyond treatment. Between these extremes, there are moderate degenerative changes that may benefit from biologic treatment. One approach to biologic treatment is to use a cytokine to stimulate the existing disc cells. We chose to study BMP-2 because it is a cytokine known to be effective in stimulating ECM production in other cell

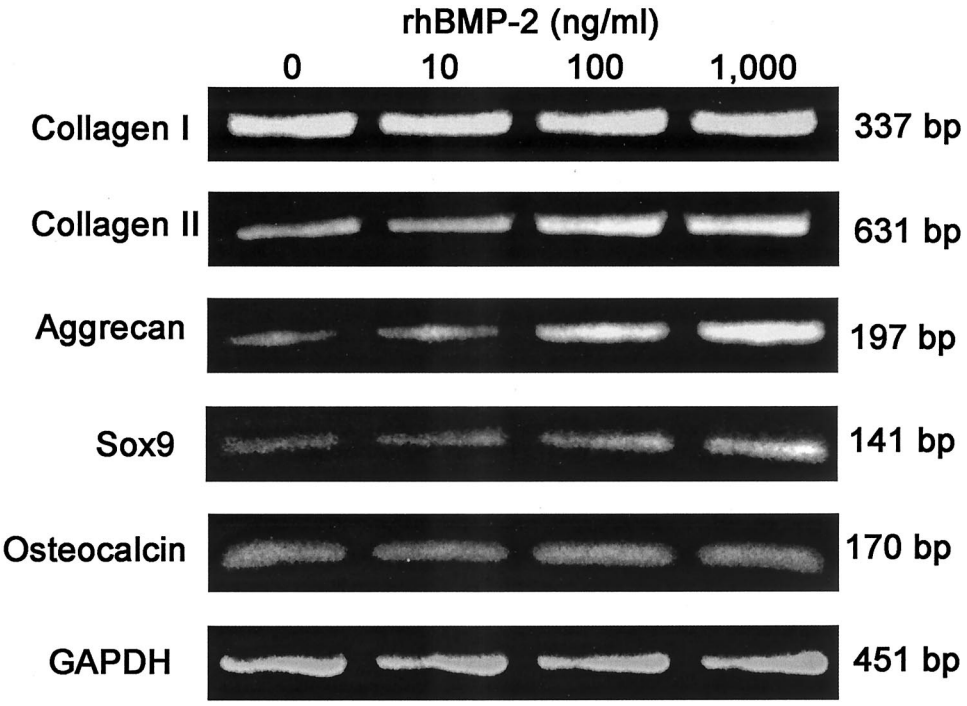

Figure 4. Reverse transcriptase-PCR results demonstrate expression extracellular matrix genes in intervertebral disc cells. Type II collagen, aggrecan, and Sox9 showed strong dose-dependent increases with rhBMP-2 treatment. Osteocalcin showed little increase at 100 and 1000 ng/mL of rhBMP. In contrast, Type I collagen did not show any noticeable change at any dose of rhBMP-2 treatment. The housekeeping gene, GAPDH, was used as a control.

types.[10,36] Furthermore, it is the only cytokine that is approved for use in spine surgery (for use with collagen sponge and cage) by the Federal Drug Administration. This indicates a relatively high degree of safety when applied in the lumbar disc space. The results of our study show that rhBMP-2 increases disc cell proliferation and promotes cellular aggregation. Recombinant human BMP-2 also increases s-GAG production and upregulates Type II collagen and aggrecan genes, which are important components of disc ECM.

Cell number increased with increasing concentration of rhBMP-2 in the media. The increase in cell number represents an increase in cell proliferation, because there was very little cell death during the culture period as seen on trypan blue staining. Although the morphogenic activity of BMP-2 is well established, the mitogenic activity of BMP-2 less is well documented.[12] Our results indicate that rhBMP-2 is a mitogen for intervertebral disc cells under the conditions of our experiment. We feel that further studies are necessary to make definitive statements about BMP-2 effect on human cells from degenerated discs.

Cell morphology and organization also changed with increased concentration of rhBMP-2. The cellular aggregates in our experiments cannot be explained by cell division in a confined space because the cell number only increased by about 60% over controls. The aggregate formation could be related to an increased differentiation state of the cells and an expression of more or different adhesion molecules. The formation of cellular aggregates has been noted during chondrogenesis in other cell culture experiments.[37–39]

Our results of s-GAG production represent an increase in synthesis rate per cell because total proteoglycan in the media was normalized by cell number and culture duration. The increase in s-GAG was greater with increased time of culture for the same concentration of rhBMP-2. Of note, increasing rhBMP-2 concentration from 100 ng/mL to 1000 ng/mL had little effect on s-GAG production at day 2 and day 4, indicating a plateau level after 100 ng/mL. However, the same change in rhBMP-2 concentrations produced a marked increase in s-GAG synthesis at day 7. The late peak of s-GAG synthesis suggests that it may be important to maintain effective concentrations for an extended period of time to alter disc cell metabolism.

The enhanced sensitivity and higher production of s-GAG at later time points after rhBMP-2 exposure reflects a change in cellular phenotype. Changes in one or more parts of the BMP-2 signaling pathway may explain this cellular phenotype change. One possibility is changes in SMADs,[12] which are part of the intracellular signaling pathway of BMP-2. Another possibility is a change in BMP-2 receptors. This latter explanation fits our data particularly well. It may be that there are low levels of BMP-2 receptors on the disc cells at day 2 and 4, and this leads to early saturation of receptors and an early plateau of s-GAG production. However, if rhBMP-2 can increase the expression of its own receptor on these cells, then the cells would become more sensitive to rhBMP-2 and would have a higher peak response.

Quantitative assay of mRNA levels after rhBMP-2 treatment showed a specific increase of Type II collagen and aggrecan gene expression. However, Type I collagen gene expression was not changed at any dose of rhBMP-2. This preferential stimulation of Type II collagen and aggrecan is consistent with the response of articular chondrocytes to rhBMP-2.[40]

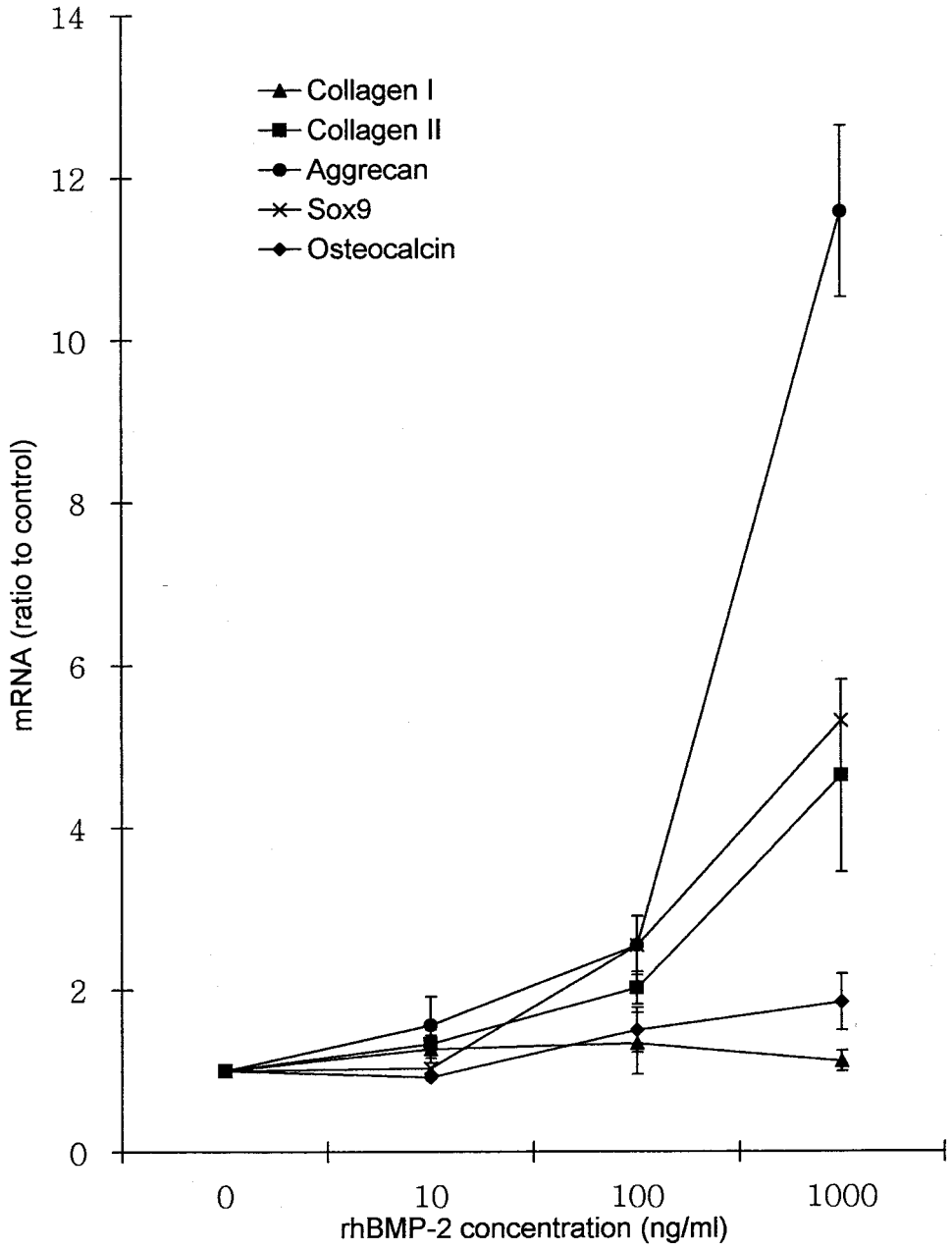

Figure 5. Quantitation of mRNA levels of Type I and Type II collagen, aggrecan, Sox9, osteocalcin, and GAPDH using real-time PCR at day 7. Normalization was performed with internal control (GAPDH) and a standard curve of each primer. Type II collagen, aggrecan, and Sox9 were significantly increased with rhBMP-2 concentrations of 100 and 1000 ng/mL. Osteocalcin increased slightly compared to the other chondrogenic genes. Type I collagen level did not change with rhBMP-2 treatment.

Sox9 is a known chondrogenic transcriptional regulator.[22,23,41–43] In our study, rhBMP-2 increased Sox9 gene expression in disc cells in a dose dependent manner. This correlated well with the dose-dependent increased production of s-GAG and upregulation of Type II collagen and aggrecan genes. These findings are consistent with the Sox9-mediated BMP-2 action in articular chondrocytes and further establish the similarity between in disc cells and articular chondrocytes.[22–24]

Because BMP-2 is associated with bone formation, we evaluated its effect using an osteoblastic marker, osteocalcin. Our experiments showed that osteocalcin mRNA is upregulated by 1.8-fold. This is a relatively lower increase than that found for chondrocyte specific genes (aggrecan, collagen Type II, and Sox9). We think that it is unlikely that intradiscal bone formation will develop with rhBMP-2 treatment *in vivo* because the intradiscal environment is avascular, which reduces oxygen tension and prevents ingrowth of mesenchymal tissue. There is a possibility that rhBMP-2 could induce a hypertrophic chondrocyte phenotype in disc cells; hypertrophic chondrocytes are associated with matrix calcification.[44,45] In the future, this possibility could be tested by evaluating collagen Type X gene expression.[44,45] Definitive studies with regards to whether rhBMP-2 causes bone formation and calcification in the disc will have to be determined with careful *in vivo* experiments.

■ Conclusion

The results of this study suggest that rhBMP-2 may be useful in enhancing disc matrix production and enhanc-

ing chondrocytic phenotype of intervertebral disc cells. Recombinant human BMP-2 increases cell number and s-GAG synthesis. It increases the mRNA of Type II collagen, aggrecan, Sox9, and osteocalcin genes, but not Type I collagen or GAPDH.

■ Key Points

- RhBMP-2 increases intervertebral disc cell proliferation and changes cellular morphology.
- RhBMP-2 increases sulfated-glycosaminoglycan synthesis.
- RhBMP-2 increases Type II collagen, aggrecan, Sox9, and osteocalcin gene expression, but not Type I collagen.

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