Dexamethasone enhances SOX9 expression in chondrocytes

I Sekiya, P Koopman¹, K Tsuji, S Mertin², V Harley², Y Yamada³,
K Shinomiya⁴, A Nifuji and M Noda

Department of Molecular Pharmacology, Tokyo Medical and Dental University, Tokyo, Japan

¹Center for Molecular and Cellular Biology, University of Queensland, Brisbane, Australia

²Howard Florey Institute of Experimental Physiology and Medicine, University of Melbourne, Parkville, Victoria, Australia

³Laboratory of Developmental Biology, National Institutes of Dental and Craniofacial Research, Bethesda, Maryland, USA

⁴Department of Orthopaedic Surgery, Tokyo Medical and Dental University, Tokyo, Japan

(Requests for offprints should be addressed to M Noda, Department of Molecular Pharmacology, Medical Research Institute, Tokyo Medical and Dental University, 2-3-10 Kanda-Surugadai, Chiyoda-ku, Tokyo, 101, Japan; Email: noda.mph@mri.tmd.ac.jp)

Abstract

SOX9 is a transcription factor that activates type II procollagen (*Col2a1*) gene expression during chondrocyte differentiation. Glucocorticoids are also known to promote chondrocyte differentiation via unknown molecular mechanisms. We therefore investigated the effects of a synthetic glucocorticoid, dexamethasone (DEX), on *Sox9* gene expression in chondrocytes prepared from rib cartilage of newborn mice. *Sox9* mRNA was expressed at high levels in these chondrocytes. Treatment with DEX enhanced *Sox9* mRNA expression within 24 h and this effect was observed at least up to 48 h. The effect of DEX was dose dependent, starting at 0.1 nM and maximal at 10 nM. The half life of *Sox9* mRNA was approximately

45 min in the presence or absence of DEX. Western blot analysis revealed that DEX also enhanced the levels of SOX9 protein expression. Treatment with DEX enhanced *Col2a1* mRNA expression in these chondrocytes and furthermore, DEX enhanced the activity of *Col2*-CAT (chloramphenicol acetyltransferase) construct containing a 1.6 kb intron fragment where chondrocyte-specific *Sry/Sox*-consensus sequence is located. The enhancing effect of DEX was specific to SOX9, as DEX did not alter the levels of *Sox6* mRNA expression. These data suggest that DEX promotes chondrocyte differentiation through enhancement of SOX9.

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Introduction

Sox9 is a member of the family of *Sox* (*Sry*-type HMG box) genes which were first identified on the basis of a region with a high homology to *Sry* (Sex-determining region Y) (Gubbay *et al.* 1990, Wright *et al.* 1993). This region encodes a 79 amino acid motif known as the HMG box, which is responsible for sequence-specific DNA binding. Several HMG box proteins are known to act as transcription factors (Giese *et al.* 1992, Harley *et al.* 1994) and some of the *Sox* genes have been shown to be expressed in a tissue-specific manner during development (reviewed by Wegner 1999). *Sox9* is expressed predominantly in mesenchymal condensations throughout the embryo in the regions where deposition of cartilage matrix is taking place, suggesting a role in skeletal formation (Wright *et al.* 1995, Ng *et al.* 1997, Zhao *et al.* 1997). In addition, mutations in human *SOX9* result in campomelic dysplasia, characterized by skeletal malformation and XY sex reversal (Foster *et al.* 1994, Wagner *et al.* 1994). In mouse chimeras, *Sox9*^{−/−} cells are excluded from all cartilaginous tissues during embryonic development but

are present in juxtaposed mesenchyme that does not express chondrocyte-specific markers such as procollagen type II, IX, XI and aggrecan (Bi *et al.* 1999). SOX9 regulates expression of the gene encoding type II procollagen (*Col2a1*), the major matrix protein characteristic of chondrocytes. SOX9 protein binds to the specific sequences in the first intron of *Col2a1* gene and activates *Col2a1* gene expression *in vitro* (Lefebvre *et al.* 1997) and *in vivo* (Bell *et al.* 1997). In addition, we showed that SOX9 enhanced aggrecan gene promoter/enhancer activity in a cartilage-derived cell line (Sekiya *et al.* 2000). These observations indicate that *Sox9* plays a key role in chondrogenesis and skeletogenesis.

Glucocorticoids have been demonstrated to promote differentiation of chondrocytes and to maintain the integrity of the cartilaginous matrix in isolated primary cell populations (Jones & Addison 1975, Kato & Gospodarowicz 1985, Takano *et al.* 1985, Horton *et al.* 1987, Quarto *et al.* 1992) as well as in chondrogenic cell lines (Calcagno *et al.* 1970, Grigoriadis *et al.* 1988, 1989). In addition, glucocorticoids suppress cartilage phenotype expression *in vitro* and *in vivo* depending on culture

conditions (Tessler & Salmon 1975, Silbermann & Maor 1985).

In this study we examined whether the effects of glucocorticoids on chondrocyte differentiation might be mediated by modulation of *Sox9* gene expression in chondrocytes.

Materials and Methods

Cell culture

Our experiments were conducted in accordance with the guidelines of the animal care committee of Tokyo Medical and Dental University. Chondrocytes were prepared from rib cartilage of 0- to 7-day-old ICR mice (Lefebvre *et al.* 1994). Rib cages were incubated in 680 units/ml collagenase (Sigma, St Louis, MO, USA) in DMEM (Gibco BRL, Rockville, NY, USA) for 30 min at 37 °C, rinsed with PBS and then incubated in 680 units/ml collagenase in DMEM at 37 °C in a CO₂ incubator for 3 h. Undigested bony parts were discarded, and primary chondrocytes were plated at 10⁵ cells/cm² in tissue culture plastic dishes and subjected to experiments within several days. Standard culture medium consisted of DMEM supplemented with antibiotics (100 units/ml penicillin G sodium, 100 µg/ml streptomycin sulfate and 0.25 µg/ml amphotericin B) and 10% FBS (Gibco BRL). Dexamethasone (DEX) (Sigma) was dissolved in 95% ethanol and DEX solutions (10⁻², 10⁻³, 10⁻⁴, 10⁻⁵ and 10⁻⁶ M) were prepared. All cultures including control received an equivalent amount of ethanol at 0.0095%.

Northern blotting

We used a 1.6 kb EcoRI–BglII fragment of the mouse *Sox9* cDNA (Wright *et al.* 1995), a 0.4 kb EcoRI–Hind III fragment of the mouse *Col2a1* cDNA (Metsaranta *et al.* 1991), and a 0.5 kb XhoI–BamHI fragment of the mouse *Sox6* cDNA as a probe (Connor *et al.* 1995). A 1.2 kb EcoRI fragment of the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) probe was used as a control.

Total cellular RNA was prepared according to the acid guanidium thiocyanate–phenol–chloroform method (Chomczynski & Sacchi 1987). Aliquots of 10 µg of the total RNA per lane were electrophoresed in 1.0% agarose gels containing 0.66 M formaldehyde and transferred to nylon filters (Hybond-N, Amersham Pharmacia Biotech, Piscataway, USA) by electroblotting. Filters were incubated at 42 °C for 1 h in a hybridization buffer (50% formamide, 250 mM sodium phosphate pH 7.2, 25 mM sodium chloride, 0.5% sodium dodecylsulfate (SDS), 0.2 mg/ml herring sperm DNA, 10% polyethylglycol MW 6000, 10 × Denhardt's solution (0.4% Ficoll type 400, 0.4% polyvinyl-pyrrolidone, 0.4% BSA fraction V)). Each cDNA was labeled using the BcaBEST random

primer labeling kit (Takara Shuzo Co., Ltd, Tokyo, Japan) and α-³²P-dCTP (NEN Life Science Products, Boston, MA, USA). Hybridization was performed at 42 °C for 24 h in a fresh hybridization buffer containing 1 × 10⁶ c.p.m./ml of the labeled probe.

Filters were washed in 2 × SSC, 0.5% SDS for 20 min at room temperature and in 0.2 × SSC, 0.5% SDS for 20 min at 65 °C. Filters were exposed to X-ray film using intensifying screens at –80 °C. The bands in the northern blot autoradiographs were quantified by densitometry and each value was normalized against that of the *GAPDH* band in the corresponding lane. The normalized values obtained were used to calculate fold induction.

Western blotting

Twenty-four hours after DEX treatment, approximately 6 million chondrocytes per dish were scraped in a lysis buffer (150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% Triton × 100, 1 × protease inhibitor Complete (Boehringer Mannheim, Indianapolis, IN, USA), 20 mM Tris pH 8.0) (Murakami *et al.* 2000a,b). Approximately 2.6 mg protein per dish was obtained and 40 µg protein per lane was used for Western blotting. The supernatants were used for Western blotting. Extracts were fractionated on 10% SDS-polyacrylamide gel and transferred to Immobilon-P membrane (Millipore, Molsheim, France). The filters were blocked in 5% skim milk in Tris-buffered saline overnight and incubated with a 1/5000 dilution of SOX9 antibody and then incubated with a 1/4000 dilution of horseradish peroxidase-conjugated goat anti-rabbit antibody. The SOX9 protein was detected by using ECL kit (Amersham Pharmacia Biotech). SOX9 antibody was produced in a rabbit which was injected with a chemically synthesized peptide coupled to KLH (keyhole limpet hemocyanin). This peptide corresponds to the amino acid sequence 486 to 509 at the C-terminus of human SOX9 protein. Hemagglutinin (HA)-tagged full length SOX9 protein was used as a positive control and luciferase protein was used as a negative control. For the production of full-length human SOX9, PBS–HA–SOX9 plasmid was constructed by PCR amplification using SOX9 cDNA as a template (Mertin *et al.* 1999). SOX9 protein (509 amino acids) was produced using the TNT T3-coupled rabbit reticulocyte lysate system (Promega, Madison, WI, USA).

Chloramphenicol acetyltransferase (CAT) assay

CAT assay was performed as described previously (Gorman *et al.* 1982). Primary chondrocytes were plated in six-well cluster plates (35 mm well diameter) at 6–7 × 10⁴ cells/cm². One day later, these cells were transfected with pCII4–C CAT (*Col2*–CAT) (Horton *et al.* 1987) reporter construct in the absence or presence of 100 nM DEX. Transfection was performed by using FuGENE6

(Boehringer Mannheim). The cells were harvested 60 h after transfection. Cell extracts were prepared and used for the analysis of CAT activity. Protein concentrations in the cell lysates were determined according to the Coomassie Brilliant Blue G method (Spector 1978). Equivalent amounts of the cellular proteins were incubated for 2 h at 37 °C in a reaction buffer (0.25 M Tris-HCl, pH 7.5, 40 mM acetyl CoA (Sigma), [^{14} C]chloramphenicol (Amersham Corp.)). The levels of acetylation were estimated by TLC followed by autoradiography of the TLC plates. Quantitation of the acetylation levels was performed by using a Bioimaging Analyzer BAS 2000 system (Fuji Film Inc., Tokyo, Japan). The effect of *Sox9* promoter activity was estimated by measuring the conversion rate. Experiments were repeated two times in triplicate with independent preparations of cell extracts. To monitor transfection efficiency, CAT activity was normalized against luciferase (LUC) activity of pGL2-control LUC-construct cotransfected with the reporter plasmids and SOX9 expression plasmid in a part of the experiments. Such experiments gave similar results to those where CAT activity was normalized against total protein contents.

Statistical analysis

For Northern blot assay, relative *Sox9* expression levels were calculated as ratios of *Sox9* mRNA levels normalized against those of *GAPDH*. These ratios were compared between DEX-treated and control cells. Statistical evaluations were conducted by using one-sample *t*-test. *P* values less than 0.05 were considered to be statistically significant.

For CAT assay, statistical evaluations of the data were conducted by using unpaired Student's *t*-test. Data are presented as mean $\pm$ standard deviation (s.d.) *P* values less than 0.05 were considered to be statistically significant.

Results

DEX enhances *Sox9* mRNA expression

Sox9 mRNA is expressed at high levels as a 4.7 kb band in primary cultures of chondrocytes (Fig. 1). This expression was further enhanced in the presence of DEX. The enhancement was observed within 24 h and lasted at least up to 48 h (Fig. 1). DEX enhanced *Sox9* mRNA levels in a dose-dependent manner from 0.1 nM, with a maximum at 10 nM (Fig. 2a,b).

To examine if DEX affects the stability of *Sox9* mRNA, primary cultures of chondrocytes were first incubated for 48 h in the absence (–) or presence (+) of 100 nM DEX, after which DRB, an inhibitor of RNA polymerase II, was added to the cultures and the stability of *Sox9* mRNA was examined. *Sox9* mRNA half life was

Figure 1 Time course of DEX on *Sox9* mRNA expression in chondrocytes. Primary cultures of chondrocytes were treated for the indicated hours in the absence (–) or presence (+) of 100 nM DEX. Total RNA was extracted and subjected to Northern blot analysis as described under Materials and Methods. Experiments were repeated three times, and representative data are shown. The enhancing effect of DEX on *Sox9* mRNA levels normalized against those of *GAPDH* at the time point of 48 h was statistically significant ($P < 0.05$).

Sox9 / *GAPDH* relative scale

Figure 2 Dose effect of DEX on *Sox9* mRNA expression in chondrocytes. Primary cultures of chondrocytes were treated with the indicated doses of DEX for 48 h. Total RNA was extracted and subjected to Northern blot analysis as described under Materials and Methods. (a) Dose dependence of DEX effects on *Sox9* mRNA expression. (b) Quantification of the data shown in (a). Data are expressed as fold induction normalized against *GAPDH* mRNA expression. The figure indicates the data of one of two independent experiments with similar results.

approximately 45 min in both the presence and absence of DEX (Fig. 3a,b), indicating that DEX does not affect the stability of *Sox9* mRNA.

Figure 3 Stability of Sox9 mRNA enhanced by DEX treatment. (a) After primary cultures of chondrocytes were treated for 48 h in the absence (–) or presence (+) of 100 nM DEX; DRB (25 µg/ml) was added to inhibit RNA synthesis (time 0). Total RNA was extracted at 0, 1, 2 or 3 h thereafter, and subjected to Northern blot analysis as described under Materials and Methods. (b) Quantitative data indicated by fold induction normalized against GAPDH mRNA expression. Experiments were repeated twice, and representative data are shown.

DEX enhances SOX9 protein expression

In order to determine whether the enhancing effect of DEX on Sox9 mRNA levels is translated into protein levels, Western blot analysis was conducted. Primary cultures of chondrocytes expressed SOX9 protein, which was detected as a 69 kDa band. DEX enhanced the levels of SOX9 protein expression by about 50% (Fig. 4), indicating that the effect of DEX on Sox9 mRNA levels is translated into increased protein levels.

DEX enhances transcription of the Col2a1 gene

As DEX enhanced SOX9 expression in primary cultures of chondrocytes and SOX9 is known to enhance Col2a1 gene expression (Bell *et al.* 1997, Lefebvre *et al.* 1997), we

examined whether DEX enhances transcriptional activity of Col2a1 in primary cultures of chondrocytes prepared from rib cartilage of newborn mouse (Lefebvre *et al.* 1994). DEX at 10 and 100 nM has previously been reported to enhance endogenous Col2a1 mRNA expression in these cells within 48 h (Kato & Gospodarowicz 1985) (Fig. 5a). We then examined whether DEX enhanced transcriptional activity of a Col2a1 gene fragment containing chondrocyte-specific Sry/Sox-consensus sequences. For this purpose, we used pCII4-C CAT (Col2-CAT) reporter construct that harbors a 1.6 kb intron fragment of murine Col2a1 gene (Horton *et al.* 1987). DEX at 100 nM enhanced transcriptional activity of this CAT construct by about 50% (Fig. 5b). DEX did not alter the activity of pSV2-CAT, indicating the specificity of its effect on the Col2a1 gene fragment (Fig. 5b).

Figure 4 DEX effect on SOX9 protein expression. Primary cultures of chondrocytes were treated for 24 h in the absence (–) or presence (+) of 100 nM DEX. Cell extract was prepared and was subjected to Western blot analysis under Materials and Methods. HA-tagged full length SOX9 protein that migrated as a 69 kDa band was used as a positive control and luciferase protein was used as a negative control. Experiments were repeated twice, and representative data are shown.

Figure 5 DEX enhancement of transcriptional activity of *Col2a1* gene in chondrocytes. (a) Primary cultures of chondrocytes were treated with the indicated doses of DEX for 48 h. Total RNA was extracted and subjected to Northern blot analysis as described under Materials and Methods. Experiments were repeated three times, and representative data are shown. (b) DEX effect on the transcriptional activity of the pCII4-C CAT (*Col2*–CAT) reporter construct containing chondrocyte-specific *Sry*/Sox-consensus sequence within the enhancer region of *Col2a1* gene was examined. Primary cultures of chondrocytes were transfected with 1 µg of reporter plasmid and were cultured in the absence or presence of 100 nM DEX for 60 h. pSV2–CAT plasmid was used as a positive control. CAT activity was determined as described in Materials and Methods. Experiments were repeated twice in triplicate with independent preparations of cell extracts. The representative data are shown. Data are expressed as means ± S.D. (n=3).

DEX does not enhance *Sox6* mRNA expression

Another SOX protein, SOX6, is co-expressed with SOX9 during chondrogenesis and cooperates with SOX9 in

Figure 6 DEX effect of *Sox6* mRNA expression. Primary cultures of chondrocytes were treated with 100 nM DEX for 48 h. Total RNA was extracted and subjected to Northern blot analysis as described in Materials and Methods. Experiments were repeated three times, and representative data are shown. The difference between *Sox6* mRNA levels normalized against those of *GAPDH* in DEX-treated cultures and those in control cells was not statistically significant ($P=0.20$).

activating *Col2a1* (Lefebvre & Crombrughe 1998). To test whether enhancement by DEX is specific to *Sox9*, we examined the effect of DEX on *Sox6* expression in rib primary chondrocytes. In Northern blots, *Sox6* mRNA expression was observed as an 8 kb band for these cells. DEX at 100 nM did not alter the levels of *Sox6* mRNA expression (Fig. 6). These results indicate that the effect of DEX is specific to SOX9.

Discussion

Chondrocytes produce a unique extracellular matrix composed of proteins such as type II collagen, aggrecan and link protein. Although DEX has been shown to enhance expression of these genes (Calcagno *et al.* 1970, Jones & Addison 1975, Kato & Gospodarowicz 1985, Takano *et al.* 1985, Horton *et al.* 1987, Grigoriadis *et al.* 1988, 1989, Quarto *et al.* 1992), the molecular mechanism(s) by which DEX exerts its effects has not been elucidated. Our data indicate that DEX enhances expression of *Sox9*, which encodes a transcription factor known to regulate expression of genes encoding major chondrocyte-related extracellular matrix proteins, such as *Col2a1*, *aggrecan* and *CD-RAP* (Lefebvre *et al.* 1997).

The physiological relevance of the action of glucocorticoids on chondrocytes is well established. Kato & Gospodarowicz (1985) showed that glucocorticoids promote formation of cartilage-like tissues both morphologically and biochemically in cultures of isolated

chondrocytes. It was also reported by McCumbee and co-workers (McCumbee & Lebovitz 1980, McCumbee *et al.* 1980) that glucocorticoid together with growth hormone (GH) stimulated growth of Swarm rat chondrosarcoma implanted in hypophysectomized. In addition, Quarto *et al.* (1992) indicated that DEX supported chondrocyte viability. Our observation that DEX enhances *Sox9* expression in chondrocytes also shows that dexamethasone acts as a positive factor for the maintenance of chondrogenic cells. However, Kato & Gospodarowicz (1985) indicated that glucocorticoids are not required in cartilage explant system. Thus our observations on the DEX effect on SOX9 could be dependent on the cellular context that could vary according to the culture conditions such as monolayer cultures.

We showed that DEX enhanced *Sox9* mRNA expression in a dose-dependent manner. Its effect was first observed at 0.1 nM and maximal at 10 nM. Takano *et al.* (1985) reported a biphasic effect of hydrocortisone on the incorporation of ³⁵S-sulfate into glycosaminoglycan in confluent cultures of rabbit costal chondrocytes, where the enhancing effect was maximal at 100 nM and less at higher concentrations. Grigoriadis *et al.* (1989) also reported a dose-dependent DEX enhancement in the number of cartilage nodules formed in RCJ 3.1C5 cells, with a biphasic response with the peak at 10 nM. Although our data did not reveal biphasic action, these studies and ours are similar in that glucocorticoids enhance expression of the cartilage phenotype in a dose-dependent manner.

DEX enhanced *Sox9* mRNA and SOX9 protein expression within 24 h, and this DEX enhancement of *Sox9* mRNA expression was observed at least up to 48 h. Recently, Murakami *et al.* (2000a) reported that FGF2 enhanced *Sox9* mRNA expression in primary cultures of chondrocytes as early as 30 min after addition of FGF2 and that this increase lasted at least up to 24 h. It is still to be determined whether DEX enhances SOX9 expression directly or indirectly through the enhancement of intermediary factors in chondrocytes.

DEX enhanced the transcriptional activity of a *Col2*-CAT construct that contains a *Sry/Sox*-consensus sequences from the first intron fragment of the *Col2a1* gene. However, the 50% increase in *Col2*-CAT activity was not as high as the enhancement of endogenous *Col2a1* mRNA expression. This suggests the existence of additional pathways by which DEX enhances *Col2a1* gene expression, presumably through other sites outside the 1.6 kb first intron fragment.

In conclusion, we show here that SOX9 is a target of DEX regulation of chondrocyte differentiation. Whether glucocorticoid regulation of SOX9 plays a major role in regulating the chondrogenic cells as a master transcription factor requires further elucidation of downstream events following SOX9 expression.

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